Recent Advances in Biofertilizers and Biofungicides (PGPR) for Sustainable Agriculture

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CHAPTER ONE

PRIVATE SECTOR PERSPECTIVE: GLOBAL SUCCESS OF PRATHISTA INDUSTRIES ORGANIC AGRI-INPUTS FOR SUSTAINABLE AGRICULTURE

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Abstract

The adoption of organic farming alone will bring about sustainability in agriculture. The indiscriminate use of chemical fertilizers over the years has resulted in the accumulation of toxic chemical substances in the soil, depletion of organic carbon content, micro flora and fauna, and damage to soil fertility with deleterious effects on crop productivity. The application of certified organic agri-inputs revives soil health and brings about ecological balances, reducing pollution and increasing the quality and quantity of crop yield. The availability of such organic inputs is a limiting factor for the farming community.

In an effort to overcome this limitation, Prathista Industries of Hyderabad, India has developed microbial-derived organic agri-inputs for all plant macro and micro nutrients. National and international experiments have proved the efficacy of these products in increasing the yield and quality of different crops. The present chapter reviews the results of experiments on yield improvement attained in various crops under diverse agro-climatic zones. Organic agriculture is a holistic production management system which promotes and enhances agro ecosystem health, including biodiversity, biological cycles and soil biological activity. Organic farming is necessary in view of economic, environmental and social concerns. The green revolution increased crop yields to attain self-sufficiency in food production by using high input external techniques.

The continuous use of inorganic fertilizers has had a negative impact on soil health resulting in a reduction in organic carbon content, nutrient uptake and expression of micro nutrient deficiencies, in addition to soil erosion and environmental pollution. The adoption of organic farming combats the ill effects of soil, water and the environment. In this direction, Prathista Industries manufactures microbial-derived organic agri inputs for all plant macro and micro nutrients developed through continuous research and development. The products are eco-friendly, bioavailable with a shelf life of 3–5 years, and do not contaminate the groundwater. These products are certified as organic by the Indian Organic Certification Agency (INDOCERT) in India, and the Organic Materials Review Institute (OMRI) in the USA. Field-level demonstrations of these products on various food crops (rice, corn, soyabean etc.), commercial crops (sugarcane and cotton), vegetables (cabbage, tomato, onion, cauliflower, aubergine and onion), horticultural crops (banana, mango, citrus, rose etc.), and spices (cardamom and cloves) were conducted in various parts of India and abroad (the USA, the Philippines, Uganda, Turkey and Kenya). The present chapter reviews the bio-efficacy results of Prathista organic inputs on different crops conducted at various locations.

Rice

Large-scale demonstrations on farmers' fields were conducted in different states of India, adopting the protocol of Prathista products (125 kgs of Aishwarya + 75 kgs each of Bio Potash and Bio Phos as basal, 12.5 kgs of bio zinc twenty days after planting [DAP], and foliar application of 625 mL each of Organic NPK +Suryamin and Bio Potash at 40 and 60 DAP, respectively/ha), which has convincingly indicated 20%–30% increased yield over the recommended dose of chemical fertilizers (80 N + 40 P_2O_5 + 40 K_2O/ha). The increase in the yield was attributed to the greater number of tillers, more grains per panicle, a greater number of panicles per plant, and the test weight of the grain. Trials conducted at Agricultural Research Institutions, Andhra Pradesh, on the rice-pulse cropping system utilizing Prathista products have indicated a greater uptake of plant nutrients from the soil, an increase in organic carbon content, and the enhancement of the enzymatic activity of Ureases, Phosphotases and Dehydrogenases, which ultimately resulted in a significant increase in the yield of rice as well as the succeeding pulse crop (*Phaseolus aures*).

Field trials on rice as per food and product agreement FPA protocols using a randomized complete block design (RCBD) with six treatments were conducted at Tiaong Quezyn in the Philippines to evaluate the

effectiveness of Bio Phos on the growth and yield of lowland rice (RC 18). Treatments include control, recommended rate of conventional fertilizer (90–60–60 Kg/ha), recommended rate of Bio Phos (2 mL/L), and different combinations of chemical fertilizer and Bio Phos. Straw and grain yields were significantly affected by the different treatments. The recommended rate of conventional fertilizer and recommended rate of Bio Phos increased the straw yield significantly over the control, but improvement due to Bio Phos was nevertheless comparable only with the improvement due to half the recommended rate of chemical fertilizer. The grain yield performance of Bio Phos was significantly better than the performance of control, but comparable with that of half the recommended rate of chemical fertilizer. This indicates a positive interaction between recommended rate of Bio Phos and half the recommended rate of chemical fertilizer. Based on the experiment results, Bio Phos has been recommended for FPA registration in the Philippines.

A similar study was conducted in various parts of Uganda by the Department of Crop Science, Makerere University, using the following products: New Suryamin, Megacal, Bio Zinc, Bio Phos, Bio Potash and Push (biopesticide). These products were also subjected to pathological and quality analyses, together with their field efficacy on various crops including rice. The results indicated that the products were organic and free from disease-causing plant pathogens. Field efficacy results emphasized the increased rice yields by 20%–22% compared to conventional fertilizers.

Studies conducted at Kansas State University on the effect of Suryamin, Bio Phos, Bio Potash and Bio Zinc on wheat, soyabean, and sorghum indicated the positive impulse of these products in increasing grain quality and yield. Application of New Suryamin increased the total nitrogen and protein content by 11% compared to control. Application of Suryamin, Bio Phos, Bio Potash and their combination on Soyabean improved the photosynthesis rate, transpiration rate leaf chlorophyll content, and photochemical efficiency. Due to the improved physiological traits, the grain yield was increased by 12%–20% and the protein content increased to 5% more than control.

Research studies conducted by the Department of Plant and Soil Sciences at Mississippi State University, USA, revealed an increase in corn yield and quality with the application of Bio Zinc and Bio Potash (8%), Suryamin (5%) and Bio Phos (15%) over control (1100 Kg/ha). The highest percentage of grain protein (9.5%) was observed with the application of Suryamin and Bio Phos compared to control (8.2%).

Sugarcane

The on-farm semi-commercial and commercial demonstrations conducted in sugarcane by Kakira Sugars, Uganda, using Prathista products indicated significantly higher cane and sugar yields compared to the recommended dose of chemical fertilizers. Further, this study has concluded that an integrated approach of adopting 50% of inorganic fertilizer recommendation (50 Kg N + 80 Kg P_2O_5 + 50 Kg K_2O) along with the recommended Prathista products (125 Kg Bio Phos + 50 Kg Bio Potash + 50 Kg New Suryamin) is the better strategy to realize higher yields with a low production cost. Field demonstrations conducted in collaboration with the World Health Organization (WHO) utilizing Prathista products at the Regional Agricultural Research Station, Anakapalle, India, indicated that the Prathista protocol received (Suryamin + Bio Phos + Bio Potash each 115 Kg/ha as basal and sprayings of Organic NPK @ 650 mL/ha at 45, 60, and 90 DAP) recorded the highest cane and sugar yields. Increases of 23% of cane yield and 33% sugar yield were recorded over farming practice. The same treatment, when compared with RDF (112 Kg N + 100 Kg P_2O_5 + 120 Kg K_2O), also recorded 7.6% and 11.7% increases in cane and sugar yields, respectively. Fertigation demonstrations with liquid organic formulations of Prathista indicated that 100% Prathista organic soluble nutrients contribute to higher cane yields, a greater number of millable canes, high quality juice and jaggery, and a higher B:C ratio compared to the recommended dose of chemical fertilizers. Higher populations of beneficial microbes like *Trichoderma* spp. (8.8×10^4 cfu/g) and *Pseudomonas flourescens* (6.3×10^6 cfu/g) were recorded in plots that received 100% Prathista organic fertilizers compared to control (RDF- 3.2×10^4 cfu/g, and 1.9×10^6 cfu/g, respectively). Field demonstrations of over thirty locations conducted by the Prudential Sugars Corporation (India) indicated an average additional yield of 25–30 t/ha of sugarcane with Prathista organic products compared to chemical fertilizers.

Vegetables

The Prathista Organic fertilizers New Suryamin, Aishwarya, Wonder, Miracle, Bio Phos, Bio Zinc and Jado were studied for their efficacy by the University of the Philippines both during the wet season (July to November) at the Central Experiment station, and at Barangay Sambat, Laguna during the dry season (March to May). The results show that organic formulations are effective in increasing Pechay crop yields over the control. A further improvement in yields was obtained by combining

Prathista Organic formulations with half the recommended rate of inorganic fertilizer. New Suryamin in combination with half the recommended rate of inorganic fertilizers resulted in the highest crop yield (11 t/ha) followed by Wonder (10.2 t/ha), Bio Zinc (10.2 t/ha), Safe (10.2 t/ha), Miracle (9.3 t/ha), Jado (8.4 t/ha), and Bio Phos (8.3 t/ha) in comparison to the recommended dose of conventional fertilizer (6–7 t/ha). Based on these results, the products were recommended for FPA registration in the Philippines.

Demonstrations of Prathista organic products conducted on various vegetable crops by officials of the Department of Agriculture, Sikkim (declared as an organic state in India) showed a significant increase in yield over control (cow dung 10 t/ha). An average additional yield of 8 q/ha in tomato was recorded followed by 5 q/ha and 4 q/ha in cabbage and cauliflower, respectively, using Prathista protocol (Aishwarya 75 Kg + Bio Phos 50 Kg + Bio Potash 50 Kg as basal and sprayings of Pushkal / New Suryamin + Megacal 4 mL/L at 35 DAP).

PART I.

PGPR AND OTHER MICROBIALS: GROWTH PROMOTION AND BIOLOGICAL CONTROL IN CROPS

CHAPTER TWO

CONTROL OF PLANT DISEASES BY THE ENDOPHYTIC RHIZOBACTERIAL STRAIN *PSEUDOMONAS AERUGINOSA* 23_{1-1}

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Abstract

The term endophyte refers to microorganisms colonising the interior of plants which do not have pathogenic effects on their hosts. Various endophytes have been found to play important roles in plant vitality and suppression of plant diseases as the action of endophytic microorganisms has been demonstrated in several pathosystems. Endophytic bacteria are able to enter the host plant and become systemically disseminated, actively colonising the xylem and occasionally the intracellular spaces. This colonisation represents an ecological niche, similar to that occupied by plant pathogens. Thus, endophytic bacteria can act as biological control agents against several plant pathogens. In our previous studies, the endophytic and antagonistic rhizobacterial strain *Pseudomonas aeruginosa* 23_{1-1}, isolated from the rhizosphere of a watermelon plant grown in the Mekong Delta of Vietnam, showed significant control of two important fungal diseases of watermelon, i.e. gummy stem blight caused by *Didymella bryoniae*, and vascular wilt caused by *Fusarium oxysporum* f.sp. *niveum* under greenhouse and field conditions. In this study, *Ps.*

aeruginosa 23_{1-1} was tested against a range of pathogens under *in vitro* and greenhouse conditions. All pathogens were significantly inhibited under *in vitro* conditions. Furthermore, *Ps. aeruginosa* 23_{1-1} also significantly reduced sheath blight disease caused by *Rhizoctonia solani* and bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* in rice, fruit rot caused by *Phytophthora capsici* and anthracnose caused by *Colletotrichum lagenarium* in watermelon, as well as soft rot caused by *Erwinia carotovora* and damping off caused by *R. solani* in cabbage under greenhouse conditions. These results suggest that *Ps. aeruginosa* 23_{1-1} is a promising biological control agent for plant disease management.

Introduction

Rhizobacteria are important microorganisms which play many roles in promoting plant growth. They can directly promote plant growth by producing phytohormones, solubilise phosphate, or fix nitrogen. Indirectly, they also improve plant growth through inhibition of pathogen development (Lugtenberg & Kamilova 2009). Many rhizobacteria isolated from different plant species were able to reduce a range of diseases, including soilborne and airborne diseases. *Pseudomonas aeruginosa* has been found to be antagonistic to different plant pathogens and pests such as *Pythium* sp. and the root-knot nematode *Meloidogyne javanica* (Ali et al. 2002; Wahla et al. 2012). The endophytic bacterium *Pseudomonas aeruginosa* 23_{1-1} was isolated from the rhizosphere of a watermelon plant in the Mekong Delta of Vietnam, and this bacterium is able to systemically protect watermelon from gummy stem blight caused by *Didymella bryoniae* both under greenhouse and field conditions (Nga et al. 2010). The protection was found to involve antibiosis and to induce resistance. In addition, the bacteria were found living inside the watermelon plant where they can grow to a high population density when the pathogen is present (Nga et al. 2010). *Ps. aeruginosa* 23_{1-1} also shows antagonistic ability against *Fusarium oxysporum* f.sp. *niveum* in dual culture tests on potato dextrose agar (PDA) plates. When drenching bacterial suspensions into the soil, it was possible to reduce Fusarium wilt on watermelon under greenhouse and field conditions (Pham & Nguyen 2010; Nguyen 2010). In the present study we investigated whether the bacterium could inhibit other bacterial and fungal pathogens on rice, watermelon and cabbage, including *Xanthomonas oryzae* pv *oryzae* causing bacterial leaf blight and *Rhizoctonia solani* causing sheath blight in rice, *Phytophthora capsici* causing fruit rot, and *Colletotrichum lagenarium* causing anthracnose on watermelon and cucurbits plants, as well as *Erwinia carotovora* causing

soft rot, and *Rhizoctonia solani* causing damping off on cabbage *in vitro* and under greenhouse conditions.

Materials and Methods

Microorganisms and culture conditions

Pseudomonas aeruginosa $23_{1\text{-}1}$ was cultured on agar plates containing King's B medium for 48 h. Then, the bacterium was harvested by adding sterile distilled water and gently releasing the cells. The bacterial density was determined by measuring OD at 600 nm and then, based on a standard curve, the bacterial density in the suspension was determined. Bacterial suspensions of 10^7, 10^8 and 10^9 cfu/ml were obtained by dilution.

Xanthomonas oryzae pv *oryzae* and *Erwinia carotovora* were cultured agar plates containing King's B medium for 48 h. Harvest, determination of cell density and dilution to 10^9 cfu/ml took place as for *Pseudomonas aeruginosa* $23_{1\text{-}1}$.

Rhizoctonia solani was cultured on PDA plates for two d. *Colletotrichum lagenarium* was cultured on modified PDA plates (PDA to which was added 5 g peptone per litre) for two weeks for sporulation. *Phytophthora capsici* was cultured on potato carrot agar medium for 7 d. Sterile distilled water was added to the plates, just submerging the colonies in water for 7–10 d to produce sporangia. Harvesting of zoospores took place by adding water to the plates covered with colonies of sporangia and then incubating the culture plates in a refrigerator at 4°C for 30 min. Subsequently, the suspension was filtered through cheese cloth to remove debris and this suspension with zoospores was used.

The conidial density of *C. lagenarium* and a zoospore density of *Ph. Capsici* were determined by a haemocytometer, and the inoculum concentrations were diluted to 10^5 spores/ml of *C. lagenarium* and 5×10^4 zoospores/ml for *Ph. capsici*.

In vitro experiments

Dual culture tests for the antagonistic ability of *Ps. aeruginosa* $23_{1\text{-}1}$ were performed with *Xanthomonas oryzae* pv *oryzae* and *Erwinia carotovora.* All bacteria were cultured as before. A volume of 200 µl *Ps. aeruginosa* suspension was added to a test tube containing 10 mL melted King's B agar at 50°C. After thorough mixing, the medium was poured onto a plate and sterile dishes of blotter paper were dipped into bacterial suspensions and then placed on the surface of the plate (five dishes across the plate).

The plates were incubated for 2 d and inhibition zones were induced by *Ps. aeruginosa* $23_{1\text{-}1}$ against *X. oryzae* pv. *Oryzae*, and *E. carotovora* were recorded.

For dual culture tests for the antagonistic ability of *Ps. aeruginosa* $23_{1\text{-}1}$ against *R. solani*, *Ph. Capsici,* and *C. lagenarium*, *Ps. aeruginosa* $23_{1\text{-}1}$ was cultured as described above. *R. solani*, *Ph. Capsici* and *C. lagenarium* were cultured on PDA for 2–4 d. Subsequently, fungal culture plugs (5 mm in diameter) were placed in the middle of agar plates. Two dishes of sterile blotter paper (5 mm in diameter) dipped in a *Ps. aeruginosa* $23_{1\text{-}1}$ suspension were placed on opposite sides of the fungal agar plug. The plates were incubated at room temperature for several days and the inhibition zones between the bacterium and the three pathogens were recorded each day after inoculation.

Greenhouse experiments

All experiments were conducted with cv. Jasmine.

Control of sheath blight and bacterial leaf blight in rice under greenhouse conditions

***Sheath blight* (*Rhizoctonia solani*).** The experiment followed a completely randomised block design with 7 treatments and 4 replications. Three treatments with sprayings of *Ps. aeruginosa* $23_{1\text{-}1}$ at 10^7, 10^8, and 10^9 cfu/ml on entire plants at 2 d before inoculation with the pathogen; three treatments with sprayings of *Ps. aeruginosa* $23_{1\text{-}1}$ at 10^7, 10^8, and 10^9 cfu/ml on entire plants at 2 d after inoculation; and one control treatment with a spraying of distilled water. Soil from a rice field was well mixed and weighed at 1 Kg/pot, then rinsed and washed with water several times for one week. One pot (17.5 × 12 cm, containing 10 plants) served as one replication. Pathogen inoculation was conducted at 40 d after sowing following the method of Khoa et al. (2011) by placing a plug from a 3 d old *R. solani* PDA culture (5 mm in diameter) inside the sheath at 2 cm above the soil line. The plants were incubated in a growth chamber at 25°C and high humidity for 2 d. Subsequently, they were transferred to a greenhouse at ambient environmental conditions. At 2, 4 and 6 d after inoculation, the relative lesion height (Sharma et al. 1990) [RLH (%): (lesion length/plant height) × 100] and the percentage of infected plants = (the number of infected plants/total number of plants×100) were measured.

Bacterial leaf blight in rice (*Xanthomonas oryzae* pv oryzae)
The experimental design and bacterial treatments were as described above, except that applications of *Ps. aeruginosa* $23_{1\text{-}1}$ took place 1 d before and 1 d after inoculation. Inoculation with *X. oryzae* pv *oryzae* was conducted at 40 d after sowing following the method of Kauffman et al. (1973) by using a pair of scissors dipped in a *X. oryzae* pv *oryzae* suspension (10^9 cfu/ml) to cut the top of two fully mature leaves per plant in each pot. Then, the plants were incubated in the growth chamber at 25°C and high humidity for 24 h. Subsequently, plants were transferred to greenhouse conditions. Disease recordings were performed according to the method described by Kauffman et al. (1973) using a scale with 5 levels: (1): no or only a trace of disease; (3): lesions covering less than 25% of the leaf; (5): lesions covering 25%–50% of the leaf; (7): lesions covering more than 50% of the leaf; (9): lesion reaching down to the sheath or "kresek."

Control of anthracnose and fruit rot in watermelon under greenhouse conditions

Anthracnose disease on watermelon (*Colletotrichum lagenarium*)
The experimental design was as described above, but with 7 treatments and 12 replications. Three treatments with soil drenched with *Ps. aeruginosa* $23_{1\text{-}1}$ at 10^7, 10^8, and 10^9 cfu/ml with 5 m of bacterial suspension for each 7-day-old-seedling; three treatments with a spraying of *Ps. aeruginosa* $23_{1\text{-}1}$ at 10^7, 10^8, and 10^9 cfu/ml on the surface of the leaves at 1 d before and 2 d after inoculation with the pathogen, and one control treatment inoculated with the pathogen and sprayed with distilled water instead of *Ps. aeruginosa* $23_{1\text{-}1}$ at 1 d before and after pathogen inoculation. Each replication comprised one pot containing 4 plants. Inoculation with the pathogen was conducted at 15 d after sowing by spraying a *C. lagenarium* suspension (0^6 spores/ml) onto the surface of the first true leaf until run-off. Subsequently, the plants were incubated in the growth chamber at 25°C and high humidity for 1 d, after which time they were transferred to the greenhouse. Disease was recorded as a percentage of infected leaf area per plant each day when symptoms appeared at 5 d after inoculation until the plants died.

Fruit rot in watermelon (Phytophthora capsici)
The experimental design was as described above with 10 treatments and 6 replications. Three treatments with a spraying of *Ps. aeruginosa* $23_{1\text{-}1}$ at 10^7, 10^8, or 10^9 cfu/ml on the surface of watermelon fruits at 1 d before pathogen inoculation; three treatments with a spraying of *Ps. aeruginosa*

23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml on the surface of watermelon fruits at 1 d before and 1 d after pathogen inoculation, and three treatments sprayed with *Ps. aeruginosa* 23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml on the surface of watermelon fruits at 1 d after pathogen inoculation. Spraying with distilled water followed by inoculation with the pathogen served as a control. Each replication was with one young watermelon fruit. Pathogen inoculation was conducted by pipetting 15 µl of zoospore suspension (6×10^4 zoospores/ml) onto the middle of the fruit. Inoculated fruits were placed in nylon bags supplemented with a plug of moist cotton with the bags being placed at room temperature (25°C). The diameters of the fruit rot lesions were recorded when symptoms appeared (at 3 d after pathogen inoculation and until the entire fruit was rotten [5 d]).

Control of anthracnose and fruit rot in cabbage under greenhouse conditions

Damping off on cabbage (*Rhizoctonia solani*)
The experimental design was as described above with 7 treatments and 7 replications. Three treatments with soil drenching with *Ps. aeruginosa* 23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml before sowing, three treatments with soil drenching with *Ps. aeruginosa* 23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml at 5 d after sowing, and a control treatment with application of distilled water instead of *Ps. aeruginosa* 23_{1-1} followed by pathogen inoculation. A pot with 40 plants was a replication. Pathogen inoculation took place by adding a mixture of 50 g sterilised rice straw with a fully grown PDA plate of *R. solani* (3 d-old) into a pot containing 950 g sterile soil and mixing well. Cabbage seeds were treated with 50°C warm water for 30 min and then incubated at 30°C for 2 d for seed germination. A total of 40 germinated seeds were sown in each pot. The pots were placed in the greenhouse. Disease was recorded as a percentage of infected plants at 5, 9, and 13 d after pathogen inoculation.

Soft rot disease on cabbage *(Erwinia carotovora)*
The experimental design was as described above with 7 treatments and 10 replications. This comprised three treatments with a spraying of *Ps. aeruginosa* 23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml on the surface of cabbage leaves at 1 d before pathogen inoculation, three treatments with a spraying of *Ps. aeruginosa* 23_{1-1} at 10^7, 10^8, or 10^9 cfu/ml on the surface of cabbage leaves at 1 d after pathogen inoculation, and a control treatment inoculated with the pathogen and sprayed with distilled water 1 d before and 1 d after

inoculation. Each replication was one pot with 5 plants. Inoculation took place by using a pipette to inject 5 µl of bacterial suspension (10^9 cfu/ml) in the middle of the fourth true leaf of each plant. Inoculated plants were incubated in the growth chamber at 25°C in darkness for 24 h. After inoculation, the plants were placed in the growth chamber at 25°C with cycles of 12 h light and 12 h darkness. Disease recordings were performed using a scale modified from Ren et al. (2001), with nine levels: (1) No symptoms; (2) Lesions smaller than 5 mm; (3) Lesions 5–10 mm; (4) Lesions bigger than 10 mm but not reaching the petiole; (5) Lesions spreading on leaf and petiole; (6) The stem infected, uninoculated leaves not infected; (7) Stem and uninoculated leaves infected; (8) Plant going to die soon; (9) Plant dead.

Data analysis

The data were subjected to analysis of variance (ANOVA) using the MstatC software, and the means were compared using Duncan's multiple range test at a significance level of 5%.

Results and Discussion

Disease control by *Pseudomonas aeruginosa* 23_{1-1} in rice, watermelon and cabbage

Pseudomonas aeruginosa 23_{1-1} showed a high antagonistic ability against both fungal and bacterial pathogens on rice, watermelon and cabbage (see Table 2.1 below). *Ps. aeruginosa* 23_{1-1} expressed antagonistic ability against the two rice pathogens *Rhizoctonia solani* and *Xanthomonas oryzae* pv *oryzae* with inhibition zones of 5.6 and 9.0 mm at 2 DAI, respectively (see Table 2.1 and Fig. 2.1 below). *Ps. aeruginosa* 23_{1-1} also had an inhibiting effect against the watermelon pathogens *Ph. capsici* (inhibition zone 11.0 mm), and against *C. lagenarium* (inhibition zone 6.0 mm) (see Table 2.1 and Fig. 2.2 below).

For cabbage pathogens, *Ps. aeruginosa* 23_{1-1} likewise showed a high antagonistic ability to *R. solani* and *Erwinia carotovora* with inhibition zones of 11.0 and 3.0 mm (see Table 2.1 and Fig. 2.3 below).

These results indicate that *Ps. aeruginosa* 23_{1-1} possesses a wide spectrum of antagonistic activity with both bacterial and fungal pathogens from different plants.

In a previous study, *Ps. aeruginosa* 23_{1-1} also inhibited *Didymella bryoniae* causing gummy stem blight and *Fusarium oxysporum* f.sp.

niveum causing vascular wilt in watermelon under *in vitro*, greenhouse, and field conditions (Nga et al. 2010; Pham & Nguyen 2010; Nguyen 2010).

Table 2.1. **Inhibition zones induced by *Pseudomonas aeruginosa* 23_{1-1} against six pathogens of rice, watermelon and cabbage**

Pathogens	Inhibition zones (mm)
From rice	
Xanthomonas oryzae pv *oryzae* (2 dai)	5.6
Rhizoctonia solani (2 dai)	9.0
From watermelon	
Colletotrichum lagenarium (6 dai)	6.0
Phytophthora capsici (5 dai)	11.0
From cabbage	
Rhizoctonia solani (2 dai)	11.0
Erwinia carotovora (2 dai)	3.0

The dual culture tests were conducted with four to five replications.
Dai: days after inoculation.

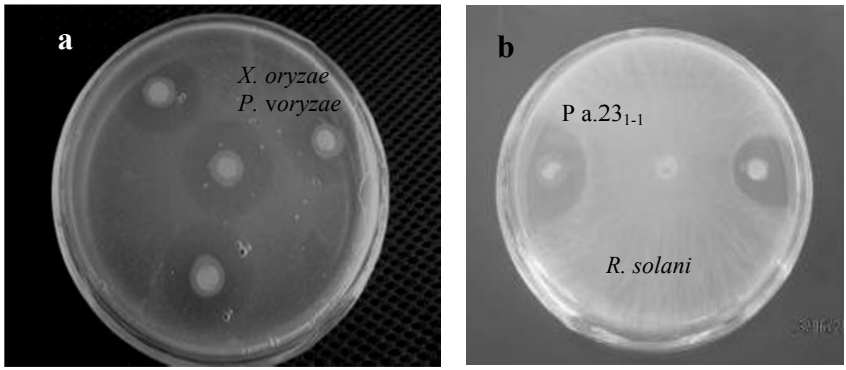

Fig. 2.1. Effect of *Ps*. *aeruginosa* 23_{1-1} against (a) *X. anthomonas oryzae* pv *oryzae* and (b) *R. hizoctonia solani* in dual culture tests at 2 d after inoculation

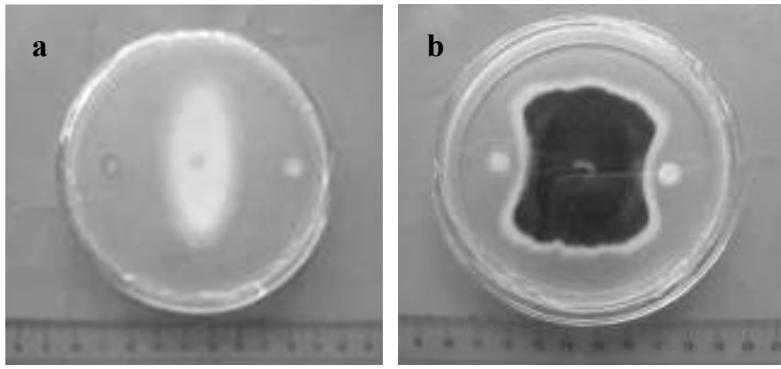

Fig. 2.2. Effect of *Ps. aeruginosa* $23_{1\text{-}1}$ against (a) *Ph. capsici* and (b) *C. lagenarium* after 5 and 6 d of inoculation

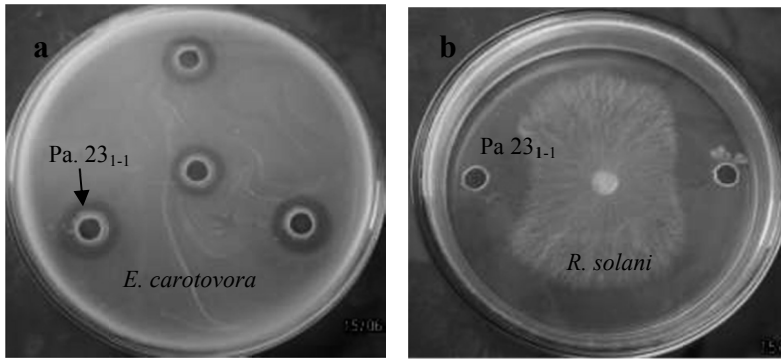

Fig. 2.3. Effect of *Ps. aeruginosa* $23_{1\text{-}1}$ against (a) *E. carotovora* and (b) *R. solani* in dual culture tests after 2 d of inoculation

Control of sheath blight and bacterial blight in rice under greenhouse conditions

Sheath blight caused by R. solani. Among six spray treatments with *Ps. aeruginosa* $23_{1\text{-}1}$ on rice for control of sheath blight, a concentration of 10^7 cfu/ml before pathogen inoculation resulted in a significant reduction in the percentage of diseased plants and relative lesion height at all-time points (see Table 2.2 and Fig. 2.4 below). The only other treatment which gave a significant reduction in relative lesion height was spraying with 10^8 cfu/ml before pathogen inoculation at 2 d. Thus, spraying with *Ps. aeruginosa* $23_{1\text{-}1}$ at a concentration of 10^7 or 10^8 cfu/ml before pathogen

inoculation could reduce infection by *Rhizoctonia solani* in rice.

Table 2.2. **Effects of bacterial treatments on the percentage of diseased plants and relative lesion height of sheath blight lesions caused by *R. solani* in rice under greenhouse conditions**

Treatment	% of diseased plants			Relative lesion height		
	Days after inoculation					
	2	4	6	2	4	6
Spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	52.5 b	83.2 a	83.2 a	1.1 c	3.6 b	4.5 b
Spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	75.7 a	95.8 a	95.8 a	1.8 bc	4.9 ab	6.7 a
Spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	72.7 a	92.7 a	97.2 a	2.3 abc	5.1 ab	6.0 ab
Spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	62.0 ab	87.5 a	92.5 a	2.1 abc	4.6 ab	5.7 ab
Spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	80.5 a	90.2 a	95.2 a	2.5 ab	5.5 a	7.1 a
Spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	73.1 a	93.8 a	93.8 a	2.2 abc	4.5 ab	6.2 ab
Control	81.4 a	94.7 a	94.7 a	3.3 a	6.1 a	7.1 a
Level of significance	*	NS	NS	*	*	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). NS: non-significant difference; *: significant at P < 0.05.*

Fig. 2.4. Sheath blight symptoms on cv. Jasmine after treatment with *Ps. aeruginosa* 23_{1-1} and water (control) under greenhouse conditions. (a) Spraying with *Ps. aeruginosa* 23_{1-1} at a concentration of 10^7 cfu/ml before pathogen inoculation; (b) Control sprayed with water

Bacterial blight caused by Xanthomonas oryzae *pv* oryzae. Six treatments with *Ps. aeruginosa* 23_{1-1} were tested for the control of bacterial leaf blight caused by *Xanthomonas oryzae* pv *oryzae* (see Table 2.3 and Fig. 2.5 below). Only two treatments reduced lesion length and disease level, i.e. the spray treatments with 10^9 cfu/ml before pathogen inoculation and the spray treatment with 10^8 cfu/ml after pathogen inoculation. Thus, the results indicate that spraying with *Ps. aeruginosa* 23_{1-1} can reduce bacterial leaf blight.

Table 2.3. Control of bacterial blight in rice by *Ps. aeruginosa* 23_{1-1} treatments under greenhouse conditions

Treatment	Average lesion length (cm)			Average disease level		
	Days after inoculation					
	10	12	14	10	12	14
Spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	6.4 ab	9.4 a	13.2 ab	3.3 ab	4.0 a	5.1 ab
Spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	5.6 b	9.7 a	13.1 ab	3.2 b	4.1 a	5.1 ab
Spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	5.3 b	8.3 a	12.2 b	3.1 b	3.8 a	4.6 b
Spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	5.4 b	9.1 a	12.8 ab	3.1 b	4.1 a	5.0 ab
Spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	5.2 b	8.3 a	12.2 b	3.2 b	3.9 a	4.9 ab
Spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	6.2 ab	9.4 a	13.7 ab	3.2 b	4.2 a	5.0 ab
Control	8.3 a	11.4 a	16.3 a	3.7 a	4.3 a	5.7 a
Level of significance	*	NS	*	*	NS	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). NS: non-significant difference; *: significant at $P < 0.05$.*

Control of anthracnose and fruit rot on watermelon under greenhouse conditions

***Anthracnose diseases on watermelon* (*Colletotrichum lagenarium*).** All six treatments with *Ps. aeruginosa* 23_{1-1} reduced anthracnose at 5 and 6 days after inoculation (dai) (See Table 2.4 and Fig. 2.6 below). Foliar spraying with 10^7 cfu/ml before and after pathogen inoculation gave the best disease-reducing effect.

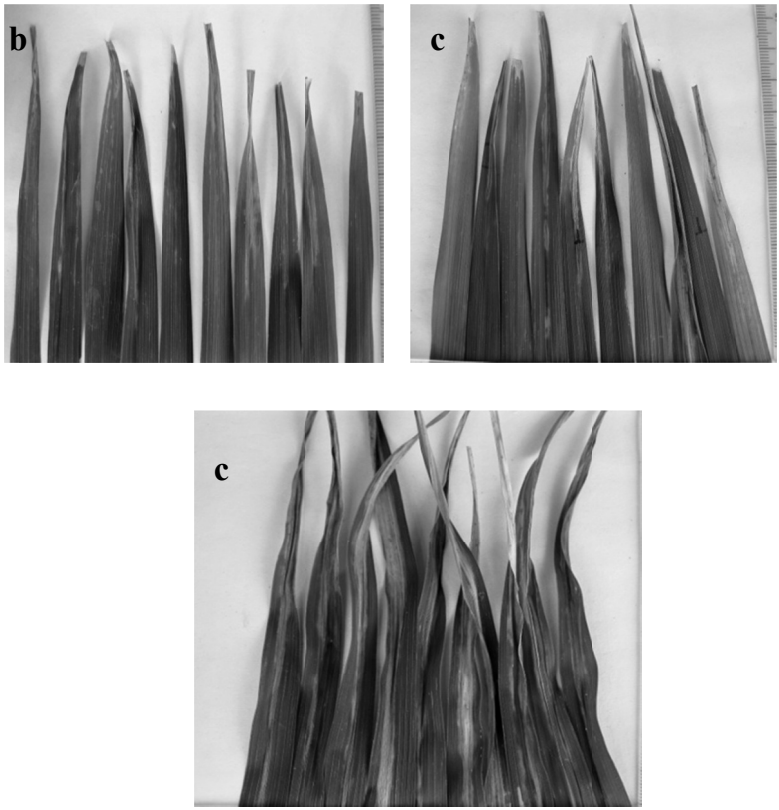

Fig. 2.5. Lesions of bacterial leaf blight caused by *X. oryzae* pv *oryzae* under greenhouse conditions of two bacterial treatments and controls. (a) spraying with a 10^9 cfu/ml suspension of *Ps. aeruginosa* $23_{1\text{-}1}$ before inoculation; (b) spraying with a 10^8 cfu/ml suspension of *Ps. aeruginosa* $23_{1\text{-}1}$ before inoculation; (c) control sprayed with water

Table 2.4. Percentage of infected leaf area with anthracnose caused by *Colletotrichum lagenarium* on watermelon under greenhouse conditions

Treatment	Percentage of infected leaf area			
	Days after inoculation			
	5	6	7	8
Soil drenching with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1}	1.5 bc	8.1 bc	50.5 a	73.9 a
Soil drenching with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1}	1.5 bc	9.5 b	55.1 a	74.8 a
Soil drenching with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1}	1.7 b	9.8 b	48.8 ab	64.6 ab
Foliar spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	1.3 bc	8.0 bc	57.3 a	75.1 a
Foliar spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	1.2 bc	8.5 bc	55.7 a	69.3 ab
Foliar spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	0.9c	6.9c	29.8b	52.7 b
Control	2.8 a	12.6 a	64.1 a	81.3 a
Level of significance	*	*	*	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). *: significant at $P < 0.05$.*

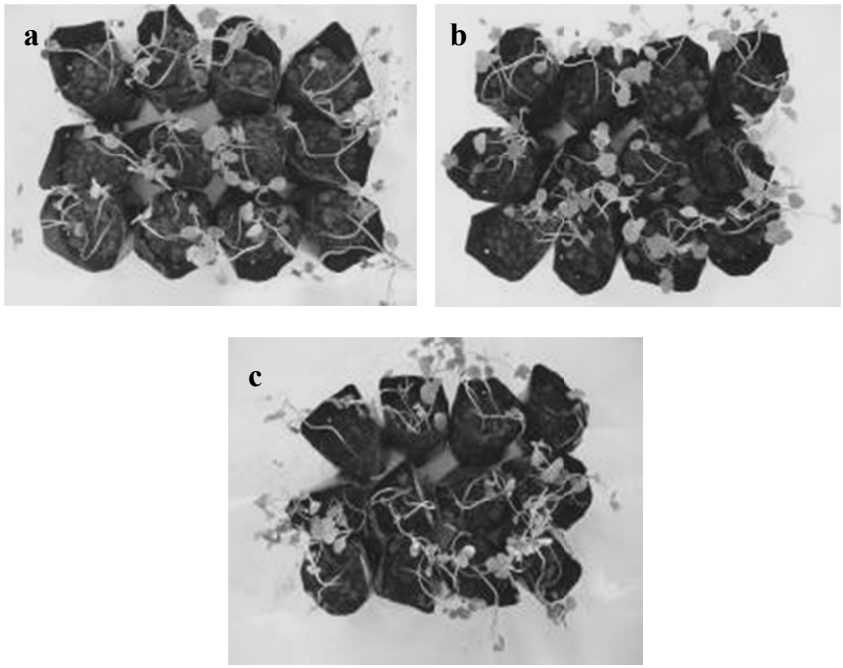

Fig. 2.6. Control of anthracnose on watermelon caused by *Colletotrichum lagenarium* at 8 d after inoculation. (a) Control; (b): Foliar spraying with *Ps. aeruginosa* $23_{1\text{-}1}$ (10^9 cfu/ml) before and after pathogen inoculation. (c) Soil drenching with *Ps. aeruginosa* $23_{1\text{-}1}$ (10^9 cfu/ml)

Fruit rot caused by Phytophthora capsici on watermelon. Out of nine treatments with *Ps. aeruginosa* $23_{1\text{-}1}$ for control of fruit rot, only six reduced fruit rot lesion size, i.e. fruit spraying (10^7, 10^8, and 10^9 cfu/ml) before pathogen inoculation, and fruit spraying (10^7, 10^8, and 10^9 cfu/ml) before and after pathogen inoculation (see Table 2.5 and Fig. 2.7 below).

Table 2.5. Fruit rot lesions caused by *P. capsici* on watermelon after treatment with *Ps. aeruginosa* 23_{1-1} under greenhouse conditions

Treatments	Fruit rot lesion diameter (cm)		
	Days after inoculation		
	3	4	5
Fruit spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	0.9bc	2.9bc	4.3cd
Fruit spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	1.2b	4.8 ab	6.7 abc
Fruit spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	0.4bc	2.4cd	3.0d
Fruit spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	0.0c	3.0bc	4.8bcd
Fruit spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	0.0c	0.8d	2.4d
Fruit spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before and after pathogen inoculation	0.0c	3.0bc	4.9bcd
Fruit spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	3.5 a	4.9 ab	6.4 abc
Fruit spraying with 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	3.8 a	5.4 a	6.7 abc
Fruit spraying with 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	4.3 a	5.9 a	7.5 ab
Control	3.9 a	6.1 a	8.2 a
Level of significance	*	*	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). *: significant at $P < 0.05$.*

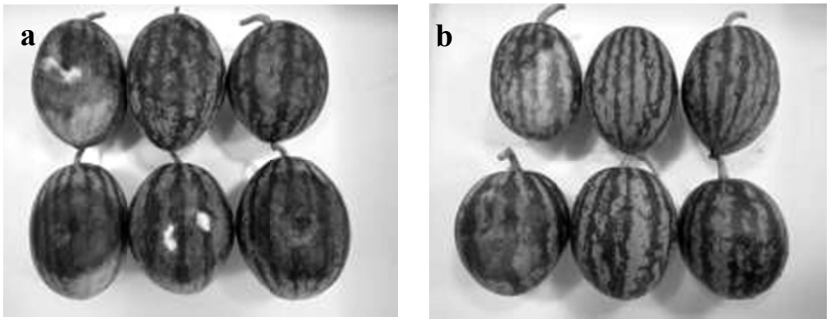

Fig. 2.7. Fruit rot symptoms caused by *Phytophthora capsici* after treatment with *Ps. aeruginosa* $23_{1\text{-}1}$. (a) Control sprayed with water; (b) Spraying with 10^8 cfu/ml bacteria before and after pathogen inoculation

Control of bacterial blight and damping off in cabbage under greenhouse conditions

Damping off in cabbage (*Rhizoctonia solani*). Of six treatments, only four were able to reduce the percentage of infected plants with damping off at 13 dai (see Table 2.6 and Fig. 2.8 below). These treatments were soil drenching with 10^7, 10^8 or 10^9 cfu/ml before pathogen inoculation, and soil drenching with 10^8 cfu/ml after inoculation. Soil drenching with 10^7 cfu/ml before inoculation showed the best disease-reducing effect.

Table 2.6. Percentage of infected plants with damping off symptoms causing by *R. solani* on cabbage after treatment with *Ps. aeruginosa* 23_{1-1} under greenhouse conditions

Treatments	Percentage of infected plants		
	Days after sowing		
	5	9	13
Soil drenching of 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	6.4b	31.1c	47.7d
Soil drenching of 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	11.5 ab	43.9 abc	60.6cd
Soil drenching of 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation	12.5 ab	55.3 ab	72.3 bc
Soil drenching of 10^7 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	26.0 a	50.8 abc	90.5 ab
Soil drenching of 10^8 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	20.8 ab	36.8bc	67.5cd
Soil drenching of 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} after pathogen inoculation	22.0ab	63.2a	80.6abc
Control drenched with water	20.3ab	63.7a	100.0 a
Level of significance	*	*	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). *: significant at P < 0.05.*

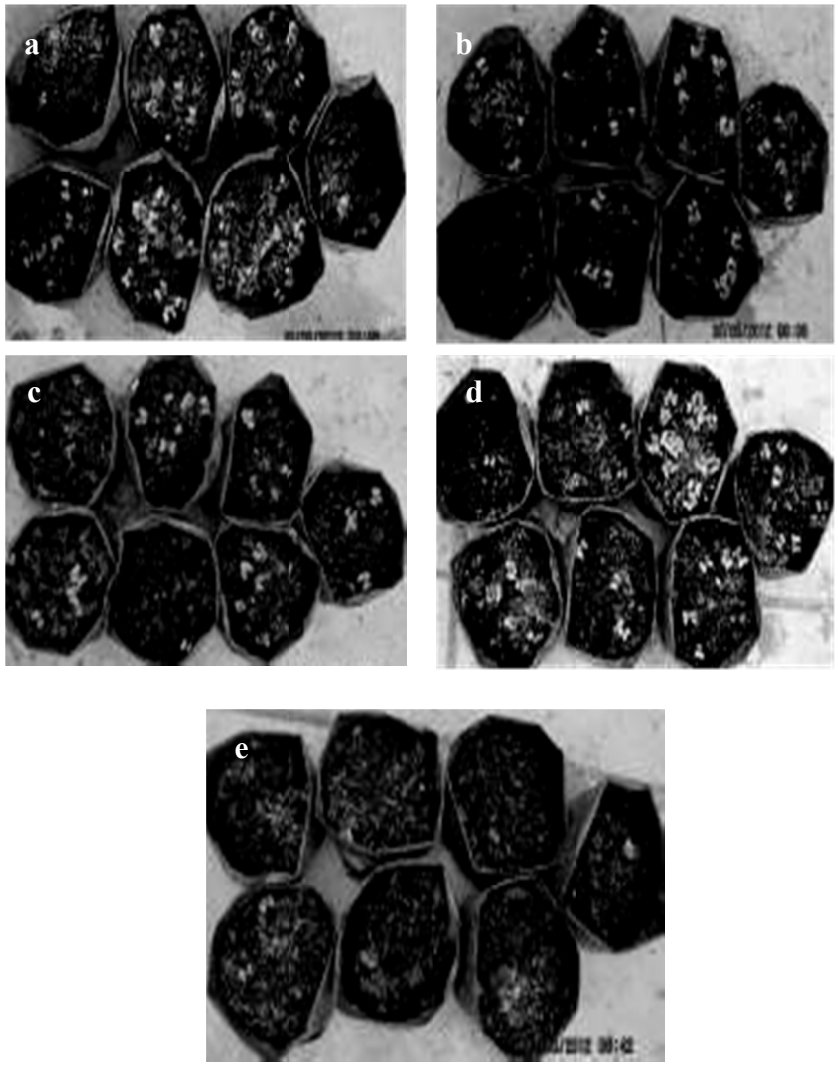

Fig. 2.8. Control of damping off symptoms caused by *R. solani* under greenhouse conditions after treatment with *Ps. aeruginosa* $23_{1\text{-}1}$. (a) Soil drenching with 10^7 cfu/ml before sowing. (b) Soil drenching with 10^8 cfu/ml before sowing. (c) Soil with 10^9 cfu/ml before sowing. (d) Soil drenching with 10^8 cfu/ml after sowing. (e) Control drenched with water

Control of soft rot in cabbage under greenhouse conditions

All six treatments, i.e. foliar spraying with *Ps. aeruginosa* 23_{1-1} (10^7, 10^8 and 10^9 cfu/ml) before and after pathogen inoculation were able to reduce soft rot symptoms on cabbage under greenhouse conditions (see Table 2.7 and Fig. 2.9 below). Foliar spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation and with 10^8 cfu/ml after pathogen inoculation gave the best disease reductions.

Table 2.7. Disease levels of soft rot on cabbage caused by *E. carotovora* after treatment with *Ps. aeruginosa* 23_{1-1} under greenhouse conditions

Treatment	Disease level		
	Days after inoculation		
	1	1.5	2
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^7 cfu/ml before pathogen inoculation	3.7b	4.9 bcd	7.4bc
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^8 cfu/ml before pathogen inoculation	3.5b	5.7 b	7.4bc
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^9 cfu/ml before pathogen inoculation	3.3b	4.8 bcd	6.4d
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^7 cfu/ml after pathogen inoculation	3.6b	4.6cd	7.1bcd
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^8 cfu/ml after pathogen inoculation	3.6b	4.5d	6.8cd
Foliar spraying with *Ps. aeruginosa* 23_{1-1} 10^9 cfu/ml after pathogen inoculation	3.7b	5.5 bc	7.9b
Control	4.6a	7.2 a	8.8 a
Level of significance	*	*	*

*Note: Means within a column followed by the same letter are not significantly different based on Duncan's multiple range test least significant difference (α= 0.05). *: significant at $P < 0.05$.*

Fig. 2.9. Control of soft rot caused by *E. carotovora* in cabbage by *Ps. aeruginosa* 23_{1-1} treatment under greenhouse conditions at 48 h after inoculation. (a) Control sprayed with water. (b) Foliar spraying with 10^9 cfu/ml *Ps. aeruginosa* 23_{1-1} before pathogen inoculation

In conclusion, *Ps. aeruginosa* 23_{1-1} was able to control several phytopathogenic bacteria and fungi in various crops. Thus, control was obtained for bacterial leaf blight (*X. oryzae* pv *oryzae*) and sheath blight (*Rhizoctonia solani*) in rice, anthracnose (*Colletotrichum lagenarium*), fruit rot (*Phytophthora capsici*) in watermelon, and damping off (*R. solani*) and soft rot (*Erwinia carotovora*) in cabbage under greenhouse conditions. This bacterium was isolated from the rhizosphere of a watermelon plant and was also able to protect watermelon against gummy stem blight (*Didymella bryoniae*) and Fusarium wilt under greenhouse and field conditions (Nga et al. 2010; Pham & Nguyen 2010; Nguyen 2010). These results indicate that *Ps. aeruginosa* 23_{1-1} is a promising rhizobacterium for controlling plant diseases. Similarly, *Ps. aeruginosa* SD12 was found to possess an antagonistic ability against many phytopathogenic fungi (Dharni et al. 2012). In addition, Aravind et al. (2012) revealed that *Ps. aeruginosa* could also control *Phytophthora* root rot on pepper.

Conclusion

Ps. aeruginosa $23_{1\text{-}1}$ is, through its broad-spectrum antagonistic activity, a promising rhizobacterium for controlling plant diseases against a range of phytopathogenic bacteria and fungi under *in vitro* as well as greenhouse conditions.

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CHAPTER THREE

POTENTIAL OF *BACILLUS SUBTILIS* AGAINST POWDER MILDEW OF GARDEN PEA

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Abstract

Powdery mildew caused by *Erysiphepisi* Syd. is one of the major threats to garden pea production worldwide. It causes significant yield losses and reduces pod quality. To minimize the fungicide inputs yet sustain the productivity of the crop, the potential of *Bacillus subtilis* isolated from infected garden pea leaves was further evaluated under greenhouse and field conditions. Egg yolk + canola oil (EYCO) at the rate of 1 egg yolk + 60 ml canola oil / 60 L water was the most effective as reflected by the area under the disease progress curve (AUDPC). Although significantly lower in efficacy than EYCO, *B. subtilis* (PM 2011-001) at the rate of 10^6 cfu/ml, Silicon (3.2 g/ 16 L water), Virtuoso (the commercial preparation of *B. subtilis*) applied at 720 mL/16 L water and *Verticillium* sp. (10^6 spores/ml) significantly suppressed powdery mildew infections comparable to the standard chemical, Sulfur, following the recommended rate (53 g/16 L water). On the other hand, baking soda (sodium bicarbonate) at the rate of 80 g/16 L water and *B. subtilis* (PCN-2011-003) applied at 10^7 cfu/ml were comparable with the untreated control. No significant differences, however, were noted in pod yield. The effectiveness of combining *B. subtilis* with selected organic-based products against the disease was also determined under greenhouse conditions. Based on AUDPC values, EYCO alone was the most effective followed by EYCO + garlic extract. However, *B. subtilis* + EYCO and *B. subtilis* + baking soda were comparable to EYCO + garlic. On the other hand, *B. subtilis* + pepper was comparable to the untreated control. Interestingly, plants applied with EYCO + garlic, *B. subtilis* + EYCO and *B. subtilis* + baking soda showed bigger leaves and more robust and longer

stems compared with those treated with EYCO alone. Numerically, plants applied with *B. subtilis* + EYCO gave the highest pod yield followed by EYCO + garlic and *B. subtilis*+ baking soda. Among the treated plants, EYCO alone gave the lowest pod yield. Follow-up studies under field conditions are in progress to further validate these findings.

Introduction

Garden peas (*Pisumsativum* L.) are one of the most important legume vegetables in Benguet and Mt. Province, grown for their edible pods and seeds. Like other legumes, garden peas are high in protein and carbohydrates, and are also a profitable crop (Kudan 2008). Aside from their nutritional contributions and economic value to farmers, peas also help in increasing soil nitrogen by having a symbiotic relationship with nitrogen-fixing bacteria.

One of the most important constraints to garden pea production is powdery mildew caused by *Erysiphe. pisi* Syd. (Anonymous 1979). An obligate parasite, its development depends on the photosynthetic status of the host; this pathogen cannot develop on photosynthetically inactive tissues (Caver & Jones 1988). The fungus is unique in that its haustoria penetrates only to epidermal cells devoid of chloroplasts; its successful development depends on the photosynthetic activity of the underlying cells. The pathogen causes up to 50% losses and reduces pod quality (Nisar et al. 2006; Singh 1998; Dixon 1987). Air currents spread the fungus locally and over long distances, whereas rain controls the disease by washing off the spores and making them burst instead of germinating. At present, there are no varieties/breeding lines that are resistant to the disease in the locality; hence, fungicide application is the only means of control. Unfortunately, the intensive use of chemicals against powdery mildew often results in the development of resistance, as happened in the case of most of the groups of chemicals applied (Mcgrath 2001). The use of biological control agents is one possible alternative to the use of chemicals that has been proposed and evaluated in numerous pathosystems, with different degrees of success. One of them is the use of *Bacillus subtilis*, which has been widely tested and is registered for use in several countries (Copping 2004). The inhibitory effect of *B. subtilis* on plant pathogenic fungi has been frequently reported in laboratory, greenhouse and field studies (Asaki & Shoda 1996; Cavaglieri et al. 2005; Lee et al. 2008). *B. subtilis* is able to synthesize more than sixty different types of antibiotics, mainly in polypeptides, many of which possess antifungal effects and belong to the iturin family (Phae et al. 1991).

Besides the antifungal effects, some compounds produced by *B. subtilis* may also act as plant growth promoters (Compant et al. 2005).

The potential of indigenous *B. subtilis* isolated from powdery- infected leaves has been observed in previous greenhouse trials. This investigation was carried out to further evaluate the effectiveness of *B. subtilis* against *E. pisi* under greenhouse and field conditions.

Materials and Methods

Isolation and identification of bacterial antagonist (*Bacillus subtilis*)

Bacillus subtilis was isolated from the powdery mildew infected leaves collected from the greenhouse at the Balili Experiment Station, and was later purified and brought to the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines, for identification.

Field experiment

A field experiment was carried out at the Benguet State University Experiment Station, the Philippines, to confirm the efficacy of *B. subtilis* and other organic-based products against the powdery mildew pathogen. Thirty-six plots measuring 1 × 5 m were thoroughly prepared and divided into 4 blocks representing the replications. The area was planted with garden pea seeds var. Chinese. Three biocontrol agents and three organic-based products (see Table 3.1 below) were evaluated. Plants treated with Virtuoso (a commercial preparation of *B. subtilis*) and those sprayed with Sulphur, including untreated plants, were provided as controls. The selection of treatments was based on the results of previous greenhouse experiments. The first treatment application was done five weeks after sowing, and thereafter weekly. Disease assessment was done weekly before treatment application using the powdery mildew rating, as shown in Fig. 3.1., on the area under the disease progress curve (AUDPC was calculated using the formula of Campbell & Madden [1990]). On the other hand, fresh pods were harvested weekly, being weighed and classified either as marketable or non-marketable yields.

Table 3.1. Treatments and rate of application

Treatments	Rate of Application
T_1 Untreated	--
T_2 *Bacillus subtilis* (isolate 1	10^6 cfu/ml
T_3 *Bacillus subtilis* (isolate 2	10^7 cfu/ml
T_4 Silicon (sodium metasilicate)	3.2 g/16 L
T_5 Baking soda (sodium bicarbonate)	80 g/16 L
T_6 *Verticillium sp.*	10^6 spores/ml
T_7 Egg yolk + canola oil (EYCO)	1 yolk and 60 mL canola oil in 20 L water
T_8 Virtuoso (commercial product of *B. subtilis*)	720 mL/16 L
T_9 Standard chemical (Sulphur)	52 /16 L

1. No visible symptoms

2. 1%–15% infection

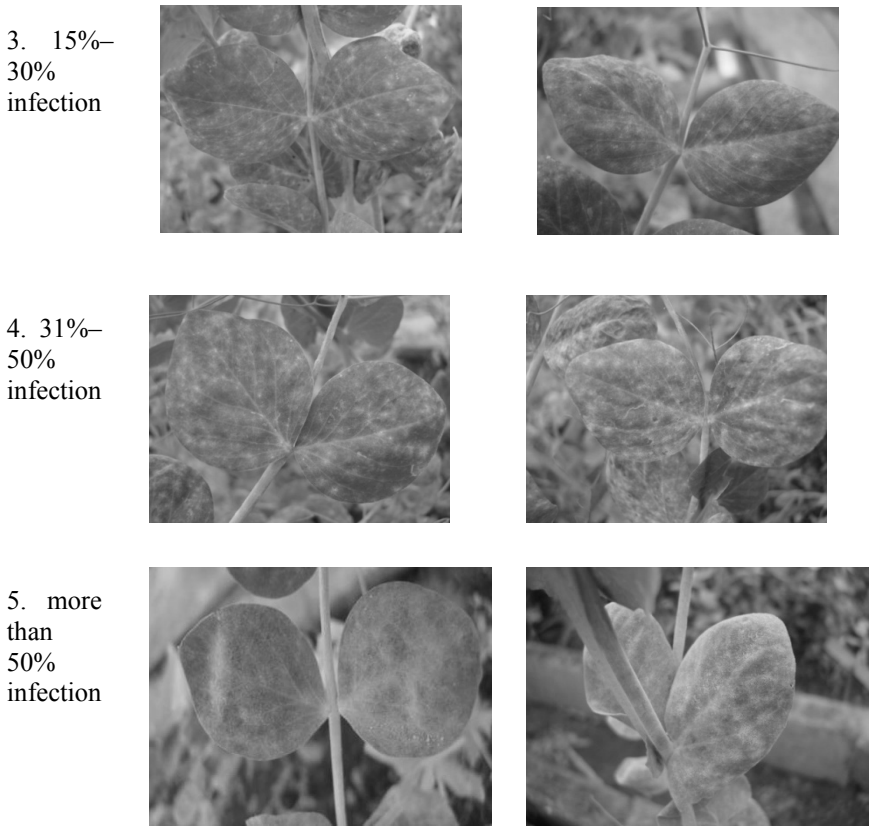

Fig. 3.1. Powdery mildew rating scale.

Greenhouse experiment

To further assess the effectiveness of *B. subtilis* and other organic-based products, combinations of the potential treatments were also done. Three microplots measuring 1 × 5 m each were divided into six to contain the six treatments, namely EYCO plus garlic, *B. subtilis* + EYCO, *B. subtilis* + pepper extract, and *B. subtilis* + baking soda. EYCO alone was used as the standard check. The different treatments were replicated three times and arranged randomly in the microplots. All the necessary cultural practices in the commercial production of garden peas were employed uniformly in all treatments. Similar to the field trial, the treatments and disease

assessments were done a month after planting and thereafter weekly using the same rating index.

Results and Discussion

Field evaluation

The effect of the different treatments on the development of the powdery mildew disease is shown in Fig. 3.2 below. Apparently, EYCO alone was the most effective in inhibiting the powdery mildew infection as reflected by area under the disease progress curve value (AUDPC) value of 58.95%. This was followed closely by *B. subtilis* (isolate 1) alone, silicon, and Virtuoso, which were comparable to the standard fungicide, Sulfur. *Verticillium* sp., on the other hand, showed slightly higher powdery mildew infection, but this was significantly lower than the untreated control. On the other hand, plants sprayed with *B. subtilis* (isolate 2) and baking soda were comparable to the untreated plants. Significant control of the disease, however, did not give the corresponding increase in pod yield, as all the treatments produced a comparable yield (see Fig. 3.3 below).

EYCO is widely adopted by Korean farmers for the control of various pests and diseases because of its simple preparation at home, cheaper price (about a quarter of the price of the chemical pesticide), and safety for organic and environment-friendly agriculture (Jee et al. 2005). When the EYCO mixture was applied on lettuce plants, there was a 89.6%–96.3% control of powdery mildew infection, which was comparable to the standard fungicide, Azoxystrobin. Fungal mycelia and conidia were severely distorted or shrunken when sprayed with EYCO. Consequently, it was assumed that the EYCO acts directly against fungal pathogens rather than induce disease resistance on the plant. On the other hand, Gubler (2012) reported that Serenade, a commercial preparation of *B. subtilis*, when sprayed on the plant destroys fungal pathogens, thus preventing the powdery mildew infection. The product has some effect in killing the powdery mildew organism but is not as effective as the oils or sulphur. On the other hand, powdery mildew of cucumber caused by *Sphaerotheca fuliginea* was suppressed by the addition of 100–200 mg/L of silicon (potassium silicate) in hydroponic solution (Schuerger & Hammer 2003). However, an increase in cucumber yield was not observed. In similar studies, the addition of silicon is also thought to enhance plant resistance through the build up of phytoalexins (Fawe et al. 1998), silicify epidermal cells which reinforces physical barriers (Kim et al. 2002), and activate

PR–genes (Rodriguez & Datnoff 2005). However, these reports varied considerably as to the type of plant which mainly differs in accumulating silicon, their effects on specific plant pathogens, and environmental conditions.

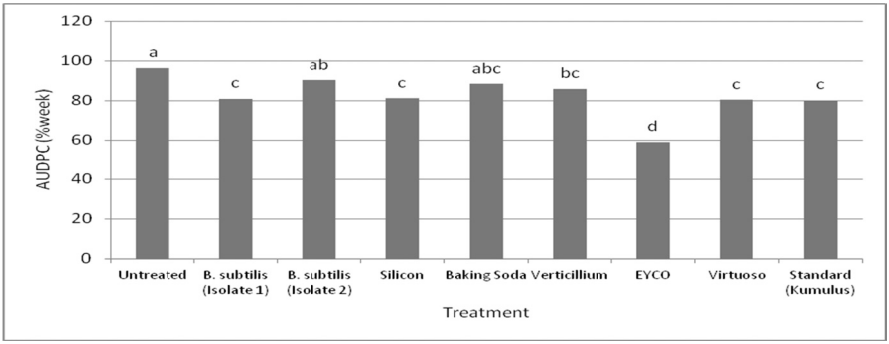

Fig. 3.2. Effect of *B. subtilis* and other organic-based products on powdery mildew infections in garden peas under field conditions

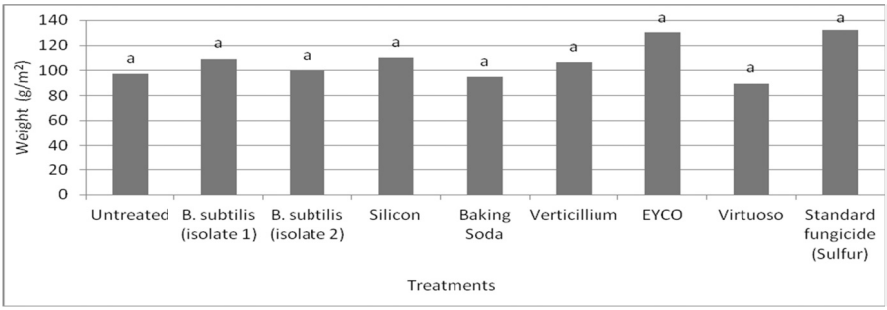

Fig. 3.3. Effect of *B. subtilis* and other potential organic-based products on garden pea yields

Greenhouse evaluation

Results show that EYCO alone gave the lowest powdery mildew infection comparable to EYCO + garlic having AUDPC values of 142.8% and 174.07%, respectively (Fig. 3.4). On the other hand, plants treated with EYCO + *B. subtilis* and *B. subtilis* + baking soda were comparable to those treated with EYCO + garlic. The lowest efficacy was noted in *B. subtilis* + pepper, which was comparable to the untreated control. In terms

of yield, no significant differences were noted among the treatments (see Fig. 3.5 below). However, the addition of *B. subtilis* and other organic-based products on EYCO slightly improved plant growth and vigour. Plants treated with EYCO + garlic, EYCO + *B. subtilis*, *B. subtilis* + pepper and *B. subtilis* + baking soda had bigger leaves and more robust and longer stems compared to plants treated with EYCO alone. The increase in effectiveness of EYCO when combined with other substances allowed in organic agriculture like *B. thuringiensis*, neem oil (azadirachtin), extracts of plant materials such as hot pepper seed or gingko leaf, sulphur and copper hydroxide has been reported (Jee et al. 2005). For example, on the control of thrips in lettuce, the mixture of EYCO and 20 ppm of azadirachtin or hot pepper seed extract increased the control value from 66.3% to 82%, while EYCO alone has less than 40% control value.

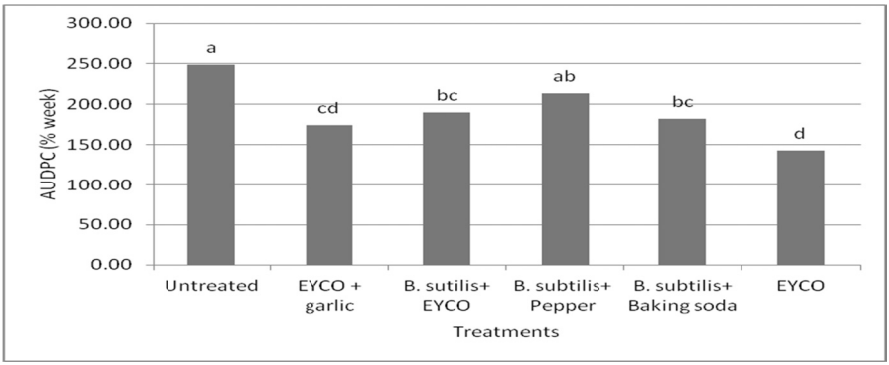

Fig. 3.4. Effect of *B. subtilis* and organic-based product combinations on powdery mildew infection under greenhouse conditions

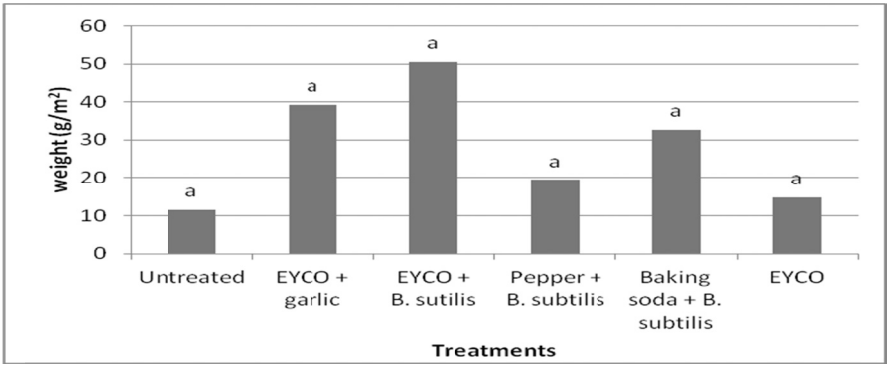

Fig. 3.5. Effect of *B. subtilis* and organic-based product combinations on garden pea yields

Conclusion

B. subtilis (isolate 1), isolated from powdery mildew-infected garden pea leaves, sprayed at weekly intervals, provided a good control of powdery mildew in garden peas at a level equivalent to that of the standard fungicide. This demonstrates the potential of *B. subtilis* for controlling powdery mildew infection. Combining *B. subtilis* (isolate 1) with EYCO and baking soda did not only suppress the disease infection significantly under greenhouse conditions, but also improved plant growth and vigour when compared to plants treated with EYCO alone. This could be due to the growth-promoting properties of the biocontrol agent. Shortening the spraying interval and combining it with other organic-based products may further improve its effectiveness. Additional studies are therefore recommended to verify the efficacy of *B. subtilis* as an alternative to synthetic chemicals against powdery mildew of garden pea in organic production systems.

Acknowledgment

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CHAPTER FOUR

IMPROVING THE YIELD OF GLUTINOUS WHITE CORN BY DISTANCE OF PLANTING AND USE OF BIOCONTROL AGENTS FOR MANAGEMENT OF ASIAN CORN BORER, *OSTRINIA FURNACALIS* GUENEE

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Abstract

Preliminary bioassay studies under laboratory conditions identified the *Beauveria bassiana* strain Bb 101-OFL as a potential fungal isolate for the control of the Asian corn borer, *Ostrinia furnacalis* Guenee. This *B. bassiana* strain caused up to 90% Asian Corn Borer (ACB) mortality at a dose of 1×10^9 spores/ml. The effectiveness of *B. bassiana* Bb 101-OFL under field conditions was assessed in combination with other cultural practices such as planting distance of glutinous white corn as determined by the agronomic and yield responses and the yield of glutinous white corn. The laboratory experiment was carried out at the University of Philippines, Los Baños (UPLB), while the field experiment was undertaken at the RET Experimental Farm, CLSU, from March to August 2011 and June to September 2011, respectively. The treatments in the field experiment were distance of planting as the main plot (60 × 10, 60 × 20, and 60 × 30 cm) and use of biocontrol agents (*B. bassiana* and *Bacillus thuringiensis*) as the subplots in comparison with the standard check and

control following the split plot experiment in Randomized Complete Block Design with three replications. Results from the field screening showed more ACB in corn plots planted at a distance of 60 cm × 20 cm. Other parameters such as entrance hole and tunnel count were not affected by the different distances of planting. In terms of ear characteristics, significantly longer (16.99 cm), bigger (4.08 cm) and heavier (122.81 g) ears were noted in corn plots with 60 cm × 30 cm planting distance, whereas the lowest plant survival as well as the shortest (13.92 cm), smallest (3.88 cm), and lightest ears (79.60 g) were noted from 60 cm × 10 cm planting distance. Application of *B. bassiana*, Bb 101-OFL, effectively suppressed the population of ACB, and did not harm the natural enemies present in the plots. Moreover, plants treated with *B. bassiana* produced significantly longer (16.06 cm), bigger (4.06 cm), and heavier ears (113.07 g) and resulted in a yield increase of 29.7% relative to the untreated control. In conclusion, *B. bassiana* is more effective against ACB than the *M. anisopliae* under laboratory screening. The potency of *B. bassiana* in suppressing ACB under field experiments was comparable to Bt and chemical control (Lannate 40 SP).

Introduction

Corn (*Zea maize* L.) is one of Asia's most important crops and is considered a staple food for nearly eighty million people. However, the average yield in Asia is only 1.2 to 1.4 metric t/ha, less than half of the average world yield of 3.9 metric t/ha (FFTC 1999). In the Philippines, corn is one of the major cereal crops grown, with over a million Filipino farmers depending on it as their main source of income and employment. Families growing the crop account for some 15% of total agricultural households, while 20% of Filipinos depend on corn as food (PCARRD 1999). Corn is a versatile crop aside from its uses as human food, feed for livestock and raw materials for the processing of other industrial products. Because of great demand in the market as a fresh green, and as a grain or processed food, continued improvement of the yield and quality of glutinous corn to better serve the consumers and uplift the livelihood of the producers is needed.

The Asian corn borer (ACB), *Ostrinia furnacalis* Guenee, is the major corn insect pest species in the Philippines and Asia, causing low yield of corn all year round. The damage caused by ACB exerts a heavy toll on corn production resulting in 20%–80% yield loss. ACB feeds on the stem, leaves and corn ears and causes complete crop failure during severe infestations (Mostoles et al. 2008). In the past, corn borer was primarily

controlled by synthetic insecticides, whereas in the 1950s farmers used chlorinated compounds that were broadly toxic, and hence ecologically disruptive (PCARRD 1985).

The use of entomophatogenic fungi is an option of primary consideration in the management of corn borer and other insect pests of corn, because their positive effects on natural enemies are more permanent and non-hazardous to human beings and the environment. Hence, biological control practice should remain at the forefront of the emerging technology on Integrated Pest Management (IPM) in corn, considering similar efficacy with other control methods such as cultural, physical and even with chemical control in terms of corn yield. The entomopathogenic fungi commonly used are *Beauveria bassiana* (Balsamo) Veuillemin and *Metarhizium anisopliae* (Metsch.) Sorokin. These fungi are economically important because of their potential as biological control agents against a variety of insect pests (Saini 2008). The objectives of the study are: (a) to identify an effective fungal isolate and its corresponding concentration as a biocontrol agent against Asian corn borer to be used in field experiments; (b) to determine the effect of distance of planting and biocontrol agents on the agronomic characteristics and yield of glutinous green corn; (c) to assess the efficacy of biocontrol agents on the field population of Asian corn borer and other insect pests; (d) to determine the interaction effect of distance of planting and biocontrol agents on the yield of glutinous green corn.

Materials and Methods

Two activities were carried out in this study: Activity 1 was the laboratory screening of the efficacy of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against Asian corn borer, and Activity 2 was the field experiment on the effect of distance of planting and use of biocontrol agents on the yield of glutinous white corn.

Activity 1. Laboratory screening for entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, highly virulent to *O. furnacalis*

Different isolates of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, isolated from lepidopterans, were obtained from the Insect Pathology Laboratory, Crop Protection Cluster, College of Agriculture, University of the Philippines at Los Baños, Laguna by Dr. Barbara L. Caoili. The isolates were mass-produced in the Potato Dextrose

Agar (PDA) medium. On the other hand, a commercially-formulated Bt product, Halt, was used as the reference material in this experiment.

Screening of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* against ACB

Ten isolates of *M. anisopliae*, namely Calaca1, Calaca2, WL1-2a, W21, W22, KAA1, KAA2, KAA3, PM15, Ma2i, and two isolates of *B. bassiana*, the Bb 101 and Bb1, were initially tested in 30 larvae each at 1×10^8 spore/ml concentration. Among these isolates, Bb 101, WL1-2a and Calaca2 were found to cause mortality to ACB. By Koch's postulate test, the spores on infected ACB larvae were re-isolated, cultured in PDA media, and re-infected to ACB larvae to confirm if the infection was caused by *B. bassiana* and *M. anisopliae.* The effective *B. bassiana*, Bb 101, was re-isolated and labelled Bb 101-OFL, while the *M. anisopliae* was WL1-2a (WL1-2a-OFL) and Calaca 2. These isolates were mass-produced in PDA and used in determining the potent concentration of spores against ACB for field screening.

Mass production of the entomophatogenic fungal isolates

Isolates of entomopathogenic fungi *Metarhizium anisopliae* (WL1-2a, Calaca 2) and *Beauveria bassiana* (Bb 101) were mass-produced in the Insect Pathology Laboratory using the PDA medium (see Appendix A for the preparation of the PDA medium). Cultures of the two fungal isolates were incubated in Petri dishes and then sealed with Parafilm™ and kept under room temperature in the Insect Pathology Laboratory. After three days, the spores of the selected fungal isolates were sub-cultured onto PDA by streaking in flat bottles using a sterile loop. Inoculation was done inside the isolation chamber and kept at room temperature for 14–30 d in preparation for the production of stock conidia suspension.

Production of stock conidia suspension and counting of spores

The conidia were harvested by scraping them off with a sterile wire loop with 10 mL 0.05% Triton X-100. The conidial suspension was transferred in umber bottles, and filtered through sterile cheese cloth. The suspended spore solution was homogenized by blending with a vortex mixer for 1–2 min.

Fungal spore concentration was determined through the use of a hemocytometer under a compound microscope. Serial dilutions of the

conidia suspension were prepared in 0.05% Triton X-100 until a countable number of spores was attained. A 200 µl sample of the spore suspension was placed at the edge of the cover glass hemocytometer, allowing it to be drawn rapidly into the space between the cover glass of the ruled area of the slide. The preparation was allowed to stand for 1–2 min so that the spores could settle to the bottom. The number of spores counted in the five squares was totalled and the number per millilitre calculated using the formula:

No. of spores/ml = Total No. of spores counted in five squares × 10^4 × df

where; df = dilution factor

Efficacy test on ACB mortality

To test the pathogenecity of each of the fungal isolate, 10 larvae of 7 d old ACB were placed in each sauce cup and starved for 4–8 h and allowed to stay for 2 min in Petri dishes with different concentrations of fungal isolates from *B. bassiana* and *M. anisopliae* along with an untreated control. The different concentrations used for each entomopathogenic isolates are summarized in Table 4.1 below.

Table 4.1. Different concentrations used for each entomopathogenic isolate

Beauveria bassiana	***Metarhizium anisopliae***	
	WL1-2a-OFL	**Calaca2**
1×10^5 spores/ml	1×10^3 spores/ml	1×10^3 spores/ml
1×10^6 spores/ml	1×10^4 spores/ml	1×10^4 spores/ml
1×10^7 spores/ml	1×10^5 spores/ml	1×10^5 spores/ml
1×10^8 spores/ml	1×10^6 spores/ml	1×10^6 spores/ml
1×10^9 spores/ml	1×10^7 spores/ml	1×10^7 spores/ml
Untreated control	1×10^8 spores/ml	1×10^8 spores/ml
	1×10^9 spores/ml	
	$1x10^{10}$ spores/ml	

The control group was treated only with Triton X-100 solution. All treatments were replicated five times with 10 larvae per replicate. The number of dead larvae was observed daily from 1 d to 10 d after infection. The concentration found to have the highest mortality was used in the field

experiment. Based on the results, only *B. bassiana* at concentration of 1×10^9 spores/ml was evaluated for the field experiment

Activity 2. Distance of planting and application of *B. bassiana* against ACB on glutinous white corn

An area of 2,052 sq. m. used in the study was thoroughly prepared by alternate ploughing and harrowing using a tractor. The treatments in the field experiment were planted at a distance of (60 × 10, 60 × 20, and 60 × 30 cm) in the main plot and biocontrol agents *B. bassiana* (20 ml/ L of water) and *Bacillus thuringiensis* (30 g/16 L of water) were used in the subplots, in comparison with the chemical control (27.5 g/16 L of water) and untreated control (no BCA application) following the split plot experiment in Randomized Complete Block Design with three replications. Each main plot was further subdivided into four subplots measuring 4.8 × 6.0 m representing the sub-treatment (Biocontrol agent). The distances between the blocks and subplots were 2.4 and 2.0 m, respectively.

Two to three seeds per hill of IES variety of glutinous corn were sown. The distance between hills was followed using the distance as indicated in the different main treatments. Eight furrows were assigned per plot. A distance of 60 cm between rows was maintained in all plots. Seven days after seed emergence, thinning with more than one seedling was carried leaving one vigorous plant per hill. Missing hills were replanted using the pre-germinated seedlings grown in a plastic tray to complete the desired population in the main plot. Two rows of buffer plants between blocks and plots were planted to avoid contamination in each treatment during spraying of treatments.

The rate of fertilizer used was 180–60–60 kg N,P_2O_5,K_2O/ha. Complete fertilizer (14–14–14) was applied at 14 DAS at a rate of 1.23 kg per plot and Urea (46—0–0) at the rate of 0.75 Kg per plot at 30 DAS in all treatments. Furrow irrigation was employed in the study, the first right after sowing of seeds and then at 21 days after emergence (DAE). After this there was no further irrigation due to frequent rainfall throughout the duration of the study.

To control weed growth, a pre-emergence herbicide (Atrazine) was sprayed 1 DAS after the first irrigation, followed by inter-row cultivation at 21 DAS to prevent the re-growth of weeds and for better soil aeration. Hand-weeding was done at 30 DAS before the last application of N fertilizer, and at 45 DAS.

B. bassiana was prepared using the stock solution 105.5 × 10^9 spores/ml by using the formula: $C_1V_1 = C_2V_2$. The *B. bassiana* was sprayed at the recommended spore concentration of 1 × 10^9 spores/ml based on the screening results. Where: C_1 = concentration of stock suspension, V_1 = amount of stock solution for dilution, C_2 = desired concentration (working concentration), and V_2 = amount of working concentration

Bt (Halt) and standard check (Lannate 40SP) were prepared following the recommended dosage per 16 L of water. The recommended dosage of Halt was 30 g and lannate 40SP was 27.5 g.

The plants were sprayed with treatment when there were three ACB per plant, at 35 DAS using a knapsack sprayer with 16-L capacity. This was followed at 42, 49, 56 and 63 DAS with a total of five sprayings. Treatment application was done at 5 to 6 o'clock in the afternoon to avoid high temperatures lethal to the fungal spores. The knapsack sprayer was thoroughly washed three times for the application of another treatment. Aside from the buffer plants as guard rows, spray guard using laminated plastic was provided to avoid treatment mists during application.

Twenty plants per treatment, which served as the sample plants, were randomly tagged at 25 DAS for monitoring ACB larval population and other insect pests. The leaves, tassel and stalk were inspected for the presence of arthropods a day before and three days after treatment application. The occurrence of natural enemies was likewise monitored at weekly intervals, starting at 35 DAS followed by 42, 49, 56, 63, and 70 DAS.

Harvesting was done at green corn stage (70 DAS). Twenty plants per plot were harvested for data on ear length, ear diameter, percentage of damaged ear and number of entrance holes and tunnels of ACB. The rest of the plants were harvested for the total yield.

All data collected were tabulated, consolidated and statistically analyzed regarding variance for Completely Randomized Design for laboratory screening while carrying out the Split Plot experiment in Randomized Complete Block Design for field experiment using SAS version 9.1 for Windows. Comparison among means was done using the Duncan Multiple Range Test (DMRT) to determine significant differences among treatment means at 5% level of significance.

Results and Discussion

Activity 1. Laboratory screening for entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, highly virulent to *O. furnacalis*

Pathogenicity of *Metarhizium anisopliae* against ACB

The two isolates of *M. anisopliae* (WL1-2a-OFL and Calaca 2) at different concentrations were found to be of low pathogenicity against ACB as shown by the very low mortality ranging from 6% to 16% and 9.33% to 26.67% in Trials 1 and 2, respectively. *M. anisopliae* was not recommended for field evaluation with this finding (see Tables 4.2, 4.3, 4.4 and 4.5 below). The low larval mortality could be attributed to many factors, such as larval stage of ACB, virulence of the isolate, amount of the fungus applied, and the prevailing conditions in the laboratory.

Table 4.2. Average percentage mortality of ACB larvae as affected by different concentrations of *M. anisopliae* (WL1-2a-OFL and Calaca2) in Trial 1 and Trial 2

Spore concentration	WL1-2a-OFL		Calaca 2	
	Trial 1	Trial 2	Trial 1	Trial 2
1×10^3	16	9.33	2.13	14.0
1×10^4	6	20.93	4.80	10.0
1×10^5	10	23.00	5.28	15.0
1×10^6	12	22.33	4.24	17.0
1×10^7	16	20.83	5.04	14.0
1×10^8	10	26.67	4.93	15.3

Pathogenicity of *Beauveria bassiana* against ACB

Presented in Table 4.3 below is the cumulative number of dead larvae of ACB as affected by the different concentrations of *B. bassiana*. From day 1 after post infection (DAI) to 10 DAI, there was an increasing number of dead larvae in all spore concentrations evaluated. At 10 DAI, the ACB larvae treated with the spore concentration of 1×10^9 significantly obtained the highest mortality of 90%, but the result was comparable with the concentration of 1×10^8 spore/ml with 80% mortality. Among the spore concentrations evaluated, 1×10^9 provided a more efficient action as shown by the high mortality rate even at an earlier time from infection having

50% mortality at 3 DAI. Furthermore, at 10 DAI the 1×10^5 spore concentration obtained a significantly lower mortality of 32%. This laboratory result conformed to the findings of Bing & Lewis (1993) wherein the fungus *B. bassiana* effectively suppressed corn borer with a mortality of 84%. On the other hand, larvae infected with *B. bassiana* (see Fig. 4.3 below) spores of *B. bassiana* that come into contact with the cuticle of the ACB larvae germinate and grow directly through the cuticle, then penetrate the body of the host and come out as white spores, known as white muscardine. The fungus proliferates throughout the insect's body, draining the insect of nutrients, eventually killing it about 48–72 h after spraying (FFTC 1999).

Activity 2. Distance of planting and application of *B. bassiana* against ACB on glutinous white corn

Population of Asian corn borer

The cumulative number of ACB 1 d before (1 DBTA) and 3 d after treatment application (3 DATA) and percent efficacy as influenced by biocontrol agents at different planting distances is presented in Table 4.4 below. The number of ACB larvae at 1 DBTA varied significantly among plant populations and use of biocontrol agent. This implies that although there was not yet any spraying of the biocontrol agents, the occurrence of ACB was already affected by the planting distance, wherein a higher population was noted at 60 cm × 20 cm distance of planting.

On the other hand, the cumulative number of ACB at 3 DATA was significantly affected by planting distance and biocontrol agents. A greater number of ACB larvae was noted at a planting distance of 60 cm × 20 cm than at 60 cm × 10 cm and 60 cm × 30 cm. A lower number of ACB larvae was noted on plants sprayed with *B. bassiana* than Bt (Halt), but the count was comparable with plants sprayed with Lannate 40SP. The interaction effect showed that the effect of *B. bassiana* remained the same at different planting distances, which means that it is effective even at varying plant populations per plot as shown by the comparable means from these treatment combinations.

Comparing *B. bassiana* with Bt and chemical control, the results showed a comparable number of ACB in *B. bassiana* sprayed plants with the chemical control and Bt in all the planting distances evaluated. This proved the efficiency of *B. bassiana* in controlling the ACB infestation in corn.

Table 4.3. Corrected cumulative mortality and average percentage mortality of ACB larvae as affected by different concentrations of *B. bassiana*

Concentration	Days After Post Infection										% Mortality
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
1×10^5	0	1.3	3.3	5.7	6.3	8.3	9.3	12.0	12.7	12.7	32.00 [d]
1×10^6	0	1.0	3.0	12.0	16.0	18.0	20.3	22.7	23.7	23.7	54.00 [c]
1×10^7	0	2.7	8.3	19.7	24.3	25.0	25.7	29.7	30.7	31.0	66.00 [bc]
1×10^8	0	1.7	17.0	27.0	30.3	32.0	32.7	37.3	37.7	37.7	80.00 [ab]
1×10^9	0	3.7	28.0	36.0	39.7	41.0	42.0	44.3	44.3	44.3	90.00 [a]

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.
Note: Average of 3 trials with 5 replications, n = 50 larvae.

In terms of percentage efficacy, *B. bassiana* was comparable with the chemical control (Lannate 40SP) and standard check (Bt). Application of *B. bassiana* significantly reduced the population of ACB by 66.7% compared to the untreated control. On the other hand, distance of planting and interaction effect did not reach any significant mean differences.

The study shows that the application of *B. bassiana* had higher efficacy in controlling the population of ACB comparable to the standard check and application of Bt. The result may be attributed to the effective suppression of *B. bassiana* on the population of ACB and the proper timing of application at different corn stages. This finding coincides with those of Obrycki et al. (1993) that the fungus *B. bassiana* reduces larval populations of the corn borer when applied to corn during whorl stage and pollen-shed stage. However, reduction of the population of ACB in untreated control could be caused by the higher number of natural enemies observed in the untreated control.

Effects of treatment on population of natural enemies

Spiders

The cumulative number of spiders was significantly higher on corn planted at a distance of 60 cm × 30 cm with 3.9 spiders per plant, compared to corn planted at a planting distance of 60 cm × 20 with 3.6 spiders. On the other hand, corn at a planting distance of 60 cm × 30 cm was comparable with a planting distance of 60 cm × 10 cm with 3.8 spiders. On the use of biocontrol agents, spraying of *B. bassiana* and Bt produced a significantly higher number of spiders, with the same mean of 3.8 spiders, than the chemical control (Lannate 40SP) with 3.1 spiders (see Table 4.5 below).

Table 4.4. Cumulative number of Asian corn borer per plant and percentage efficacy as affected by distance of planting and biocontrol agents and their interaction

Treatment		Observation*		
Distance of Planting (cm)	**Biocontrol Agent**	**1 DBTA**	**3 DATA**	**% Efficacy**
60 × 10	Control	24	22.2f	5.84
	Lannate 40SP	13	9.9ab	22.49
	B. bassiana	12	9.7a	18.06
	Bt (Halt)	13	10.4abcd	23.15
	Mean	**15.4 A**	**13.0 A**	**17.38**
60 × 20	Control	24	22.8f	4.87
	Lannate 40SP	13	10.9bcd	14.21
	B. bassiana	14	10.7abcd	20.72
	Bt (Halt)	14	11.4d	20.30
	Mean	**16.2 B**	**13.9 B**	**15.03**
60 × 30	Control	22	20.6e	7.79
	Lannate 40SP	13	10.8abcd	14.23
	B. bassiana	13	10.5abcd	16.92
	Bt (Halt)	13	11.2cd	11.08
	Mean	**15.1 A**	**13.2 A**	**12.50**
Mean Biocontrol agent	Control	**23.3 Z**	**21.8 Z**	**6.17 Y**
	Lannate 40SP	**12.8 X**	**10.6 XY**	**16.98 X**
	B. bassiana	**12.6 X**	**10.3 X**	**18.57 X**
	Bt (Halt)	**13.6 Y**	**11.0 Y**	**18.18 X**

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.
*Total of five sprayings
DBTA = Day before treatment application
DATA = Days after treatment application

Coccinellid beetles

Corn planted at a distance of 60 cm × 30 cm had a significantly higher number of coccinellid beetles than the other two planting distances (see Table 4.5 below). Moreover, the effects of biocontrol agents, *B. bassiana* and Bt, were comparable with each other in all observations. A significantly lower number of coccinellid beetles was noted from the chemical control treated plants (Lannate 40SP). Results showed that *B. bassiana* did not affect the population of coccinellid beetles. The findings

support the work of Thungrabeab & Tongma (2007), in that *B. bassiana* was found to be non-pathogenic to natural enemies (Coccinellidae) and a beneficial soil insect.

Table 4.5. Cumulative number* of natural enemies (spiders and coccinellid beetles) per plant as influenced by distance of planting and biocontrol agents

Treatment		Natural Enemies	
Distance of Planting (cm)	**Biocontrol Agent**	**Spiders**	**Coccinellid beetles**
60 × 10	Control	4.5	4.3
	Lannate 40SP	3.1	2.1
	B. bassiana	3.7	3.6
	Bt (Halt)	3.9	3.9
	Mean	**3.8AB**	**3.5 B**
60 × 20	Control	4.1	4.6
	Lannate 40SP	3.2	2.3
	B. bassiana	3.7	3.8
	Bt (Halt)	3.6	3.5
	Mean	**3.6B**	**3.5 B**
60 × 30	Control	4.4	5.1
	Lannate 40SP	3.2	2.8
	B. bassiana	4.0	4.1
	Bt (Halt)	4.1	4.3
	Mean	**3.9A**	**4.1 A**
Mean Biocontrol agent	Control	**4.3 X**	**4.7 X**
	Lannate 40SP	**3.1 Z**	**2.4 Z**
	B. bassiana	**3.8 Y**	**3.8 Y**
	Bt (Halt)	**3.8 Y**	**3.9 Y**

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.
* Total of six observations

(a) Plant damage number of entrance holes per plant at harvest

An insignificant difference was observed on the number of entrance holes as affected by the distance of planting. However, application of biocontrol agent significantly influenced the number of entrance holes per plant. Corn applied with biocontrol agents exhibited a significantly lower number of

entrance holes per plant than the untreated control. Furthermore, the effect of any biocontrol agents evaluated was comparable with the chemical control (see Table 4.6 below). This means that the *B. bassiana* used is as effective as the chemical control and the standard biocontrol agent Bt.

A significant difference in the number of entrance holes per plant was observed on the use of biocontrol agents but not on distance of planting and its interaction (see Table 4.6). Corn sprayed with *B. bassiana* has a comparable number of entrance holes per plant with that of Bt and chemical control treated plants. Likewise, the application of biocontrol agents significantly reduced the number of entrance holes per plant as compared with the untreated control. Reduction of the number of entrance holes per plant with the application of biocontrol agents could be attributed to the effective suppression of biocontrol agents on the population of ACB.

Number of ACB tunnels per plant at harvest

The same trend was observed on the number of tunnels with that of the number of entrance holes per plant. Corn applied with different biocontrol agents had a lesser number of tunnels per plant as compared with the control (Table 4.6). The number of tunnels observed on plants applied with *B. bassiana* was 5.78, which is comparable with the plants applied with Bt and chemical control with an identical mean of 5.44 tunnels per plant. The finding supports the work with Obrycki et al. (1993), in that application of *B. bassiana* on corn reduces the tunnelling of the corn borer due to the weakened larvae brought about by the colonization of *B. bassiana*.

Percentage damaged ears

The application of *B. bassiana* significantly reduced damaged ears by 39% relative to the untreated control and was comparable with the chemical control (Lannate 40SP) and standard check (Bt). On the other hand, planting distance and its interaction with biocontrol agents did not show significant mean variations among treatments (Table 4.6).

These results clearly indicate the efficiency of *B. bassiana* in controlling the infestation of ACB in glutinous white corn, thus preventing damage to corn ears.

Table 4.6. Number of entrance holes, ACB tunnels per plant and percentage of damaged ears at harvest as influenced by distance of planting and biocontrol agents

Treatment		Number of Entrance Holes	Number of ACB Tunnels	Damaged Ears (%)
Distance of Planting (cm)	**Biocontrol Agent**			
60 × 10	Control	9.00	7.67	66.67
	Lannate 40SP	6.67	5.00	45.00
	B. bassiana	6.67	5.00	38.33
	Bt (Halt)	7.33	5.33	36.67
	Mean	**7.42**	**5.75**	**46.66**
60 × 20	Control	9.33	6.67	68.33
	Lannate 40SP	8	5.67	48.33
	B. bassiana	8.33	5.67	46.67
	Bt (Halt)	8.33	5.67	45.00
	Mean	**8.50**	**5.92**	**52.08**
60 × 30	Control	9.33	7.67	56.67
	Lannate 40SP	6.67	5.67	41.67
	B. bassiana	8.33	6.67	31.67
	Bt (Halt)	7.33	5.33	43.33
	Mean	**7.92**	**6.33**	**43.33**
Mean Biocontrol agent	Control	**9.22 Y**	**7.33 Y**	**63.88 Y**
	Lannate 40SP	**7.11X**	**5.44X**	**45.00 X**
	B. bassiana	**7.78X**	**5.78X**	**38.88 X**
	Bt (Halt)	**7.67 X**	**5.44 X**	**41.66X**

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.

Leaf damage rating

Leaf damage rating was observed from vegetative stage to tasseling stage, from 35 to 49 DAS. At 60 × 10 cm planting distance, there was an increase in leaf damaged to 26%, while at 60 × 20 and 60 × 30 cm the increase was 28% and 31%, respectively. At 42 and 49 DAS, spraying of *B. bassiana* significantly reduced leaf damage from 11% to 17% comparable with the application of Bt and chemical control (Lannate 40SP) compared with the untreated control (see Table 4.7 below).

At 35 to 49 DAS, leaf damage rating was insignificantly different regarding the distance of planting and its interaction, but with highly significant difference in leaf damage on the use of biocontrol agents at 42 and 49 DAS (Appendix Table 128, 130). Hence, the application of biocontrol agents, *B. bassiana* and Bt, significantly reduced leaf damaged. Results coincide with the findings of Obrycki et al. (1993), in that the fungus *B. bassiana* reduces larval populations of the corn borer when applied to corn during whorl stage and pollen-shed stage; the reduction in the population of ACB resulted in a decrease in leaf damage.

Table 4.7. Leaf damage rating as affected by distance of planting and biocontrol agents

Treatment		**Observations**		
Distance of Planting (cm)	Biocontrol Agent	35 DAS	42 DAS	49 DAS
60 × 10	Control	3.27	4.52	5.13
	Lannate 40SP	3.30	4.06	4.47
	B. bassiana	3.17	3.83	3.93
	Bt	3.18	4.05	4.17
	Mean	**3.26**	**4.12**	**4.46**
60 × 20	Control	3.3	4.56	4.87
	Lannate 40SP	3.0	4.08	4.18
	B. bassiana	3.1	3.90	4.02
	Bt	3.1	4.16	4.43
	Mean	**3.16**	**4.17**	**4.39**
60 × 30	Control	3.17	4.28	5.22
	Lannate 40SP	2.93	4.05	4.13
	B. bassiana	3.30	4.13	4.60
	Bt	3.13	4.12	4.40
Mean	**Mean**	**3.16**	**4.14**	**4.62**

	Control	**3.26**	**4.46 X**	**5.08 X**
	Lannate 40SP	**3.10**	**4.06 Y**	**4.30 Y**
	B. bassiana	**3.22**	**3.95 Y**	**4.21 Y**
	Bt	**3.18**	**4.11 Y**	**4.36 Y**

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.

DAS = Days after sowing **Rating Scale:** 1= No obvious damage or injury on leaves, 2 = Pinhole damage common on a few leaves, 3 = Several leaves with pinhole and shot hole damage, 4 = Several leaves with elongated lesions, 5 = Numerous lesions on leaves with evidence of stalk penetration.

Plant damaged rating

Presented in Table 4.8 below is the plant damaged rating as influenced by the distance of planting and use of biocontrol agents. From 49 to 70 DAS, significant increase in plant damaged was observed. At 63 DAP, corn planted at a distance of 60 × 10 cm suffered the highest plant damage with a mean of 2.84, while a planting distance of 60 × 20 and 60 × 30 cm had comparable plant damage, with means of 2.74 and 2.68, respectively. Using biocontrol agents, *B. bassiana* at 49 and 56 DAP exhibited significantly lower plant damage comparable to the chemical control (Lannate 40SP).

There were no significant differences on distance of planting and interaction at 49, 56 and 70 DAP. However, at 63 DAP there were significant differences observed as influenced by the distance of planting, and from 49 to 70 DAP highly significant differences on plant damage were noted on the use of biocontrol agents (see Appendix Tables 132, 134, 136, and 138). Comparison among treatment means showed that the application of biocontrol agents significantly reduced plant damaged compared to the untreated control. Reduction of plant damage could be attributed to the biocontrol agents that effectively suppressed the number of ACB and possibly the endophytic relationship between *B. bassiana* and corn plants. Wagner & Lewis (2000) stated that after inoculation with a foliar spray of conidia, germinating hyphae grew randomly across the leaf surface. A few hyphae were observed within xylem elements. Because vascular bundles are interconnected throughout the corn plant, this may explain how *B. bassiana* travels within the plant and ultimately provides overall insecticidal protection.

Table 4.8. Plant damage rating as affected by distance of planting and biocontrol agents

Treatment		Days after Sowing, DAS			
Distance of plant--ing (cm)	Biocontrol Agent	**49**	**56**	**63**	**70**
60 × 10	Control	2.12	2.43	3.17	3.88
	Lannate 40SP	1.85	2.1	2.72	2.83
	B. bassiana	1.80	2.1	2.73	2.90
	Bt	1.93	2.2	2.75	2.93
	Mean	**1.92**	**2.21**	**2.84A**	**3.14**
60 × 20	Control	2.32	2.7	3.14	3.73
	Lannate 40SP	1.72	2.0	2.60	3.00
	B. bassiana	1.83	2.1	2.58	2.97
	Bt	1.88	2.2	2.63	3.03
	Mean	**1.94**	**2.22**	**2.74B**	**3.18**
60 × 30	Control	2.28	2.6	3.02	3.82
	Lannate 40SP	1.68	1.9	2.43	2.80
	B. bassiana	1.98	2.3	2.62	2.93
	Bt	1.88	2.2	2.63	2.90
	Mean	**1.96**	**2.23**	**2.68B**	**3.11**
Mean Biocontrol agent	Control	**2.24 A**	2.57 A	3.11A	3.81 A
	Lannate 40SP	**1.75 C**	2.01C	2.58B	2.88B
	B. bassiana	1.87BC	2.14BC	2.64B	2.93B
	Bt	1.90 B	2.18 B	2.67B	2.96 B

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.

DAS = Days after sowing **Rating Scale:** 1 = Plants with slight injury and little or no damage on stem, 2 = Plants with slight injury and broken or lost tassel, 3 = Plants with extensive leaf injury and broken tassels, 4 = Plants broken above the ear, 5 = Plants with extensive leaf injury and broken above the ear.

(b) Agronomic characteristics

Plant height (cm) at harvest

Plant heights at harvest as influenced by distance of planting and use of biocontrol agents are presented in Table 4.9. Plant height at different distances of planting ranged from 163.82 to 164.40 cm. With the

application of biocontrol agents, plant heights obtained ranged from 163.21 to 164.96 cm. These numerical differences, however, failed to show significant differences among treatments and in their interaction.

Ear height (cm) at harvest

The ear height, ranging from 87.02 to 87.16 cm, was not significantly affected by different distances of planting and with the application of biocontrol agents ranging from 87.01 to 87.20 cm. The results show that having comparable plant height would produce the same ear height (see Table 4.9 below).

Ear length (cm) at harvest

The length of ear at harvest varied significantly among planting distances and use of biocontrol agent. Ear length from wider spacing (60 cm × 30 cm) produced longer ears compared to closer distancing (60 cm × 20 cm and 60 cm × 10 cm). This is attributed to reduced competition for growth factors, such as soil nutrients and water as well as light, brought about not only by lower populations but also by wider spacing. With an equal amount of growth factors, lower populations tend to have more access to growth factors than in greater populations per unit area. On the other hand, ear length decreased in relation to increasing plant density. This may be due to the effects of interplant competition for incident photosynthetic photon flux density, soil nutrients and soil water (Sangoi 2000). Similar results have been reported by Akman (2002). In terms of the effect of biocontrol agents, Table 4.9 below shows that plants sprayed with *B. bassiana* produced significantly longer ears than the untreated control and were comparable with the standard check and chemical control.

Ear diameter (mm) at harvest

Ear diameter decreased with increases in plant density. The thicker ears were obtained from corn plants planted at distances of 60 × 20 and 60 cm × 30 cm with means of 4.02 and 4.08 cm, respectively, and the thinnest ears (3.88 cm) were obtained from plants planted at a distance of 60 cm × 10 cm (see Table 4.9 below). Konuskan (2000) reported that plant densities affected ear diameter and thinner ears were obtained at high densities, and similar results have been reported by Akman (2002). In terms of the use of biocontrol agents, plants applied with *B. bassiana*

produced comparable ear size with Bt (Halt) and chemical control (Lannate 40SP). The control produced significantly smaller ear diameters.

Plant stand at harvest (%)

The plant stand at harvest, as influenced by distance of planting and use of biocontrol agents, is shown in Table 4.9 below. Corn with a distance of 60 cm × 30 cm gave a significantly higher plant survival with a mean of 95.21% (114.25 plants), followed by 60 cm × 20 cm with a mean of 92.04% (165.67 plants). The lowest plant survival was observed on corn with a distance of 60 cm × 10 cm with a mean of 73.68% (265.25 plants). The plant stand is not affected by different biocontrol agents.

Statistical analysis showed that there was a significant difference in plant survival on distance of planting, but insignificant on the use of biocontrol agents and their interaction (see Appendix Table 148). Comparison among treatment means showed that as the plants became dense from 30 cm to 20 cm and 10 cm, plant survival was significantly reduced. Corn planted at a distance of 60 cm × 10 cm exhibited a very low plant survival, and because of this condition the stem lodging increased and competition between plants for nutrients, sunlight and space during their growth and development was very high. The results coincide with the findings of Tollenaar et al. (1997), in that when plant density is increased beyond the optimum plant density there is an increase in stem lodging. Also, there was intense interplant competition for incident photosynthetic photon flux density, soil nutrients and soil water (Lemcoff & Loomis 1994).

Average weight of ear (g)

The average ear weight significantly varied with treatments. Corn planted at a distance of 60 cm × 30 cm significantly produced the heaviest ears at 122.81 g. The lightest weight was obtained from corn planted at a distance of 60 cm × 10 cm with 79.60 g. On the use of biocontrol agents, application of *B. bassiana* significantly produced the heaviest ears with 113.07 g or 27.05% heavier ears, compared to the untreated control. Also, application of *B. bassiana* was comparable to Bt and chemical control (Lannate 40SP) with 111.57 g and 112.05 g, respectively (see Table 4.9 below). This could be attributed to the greater plant population per unit area wherein at this condition the plants compete for nutrients, sunlight, water and space for good growth and development, and effective suppression of *B. bassiana* for ACB.

Table 4.9. Agronomic characters as affected by distance of planting and biocontrol agents

Treatment		Plant height (cm)	Ear height (cm)	Ear length (cm)	Ear diameter (cm)	Plant stand (%)	Average weight of ear (g)	Computed yield per hectare* (ton)
Distance of plan--ting(cm)	Biocontrol agent							
60× 10	Control	164.85	87.05	13.29	3.72	73.24	53.10	6.48 c
	Lannate 40SI	165.50	87.10	14.03	3.94	73.42	87.53	10.71 a
	B. bassiana	164.47	87.08	14.31	3.94	74.04	88.50	10.93 a
	Bt (Halt)	162.80	86.88	14.06	3.92	73.98	89.26	11.01 a
	Mean	**164.40**	**87.02**	**13.92 C**	**3.88 B**	**73.68 C**	**79.60 C**	**9.78 A**
60× 20	Control	164.40	87.24	16.11	3.88	91.29	95.83	7.29 c
	Lannate 40SP	163.23	86.91	16.62	4.01	94.26	116.29	9.13 b
	B. bassiana	164.18	86.98	16.76	4.02	92.04	117.70	9.03 b
	Bt (Halt)	163.47	87.17	16.61	4.16	90.56	118.03	8.90 b
	Mean	**163.82**	**87.08**	**16.52 B**	**4.02 A**	**92.04 B**	**111.97 B**	**8.59 B**
60× 30	Control	165.62	87.32	16.70	4.02	94.72	98.50	5.18 d
	Lannate 40SP	165.32	87.01	17.08	4.07	95.56	132.33	7.02 c
	B. bassiana	163. 30	87.22	17.11	4.19	95.00	133.00	7.02 c
	Bt(Halt)	163.35	87.11	17.06	4.06	95.56	127.42	6.76 c
	Mean	**164.40**	**87.16**	**16.99 A**	**4.08 A**	**95.21 A**	**122.81A**	**6.50 C**
Mean								

Control	**164.96**	**87.20**	**15.36 Y**	**3.87 Y**	**86.42**	**82.48 Y**	**6.32 Y**
Lannate 40SP	**164.68**	**87.01**	**15.91 X**	**4.01 X**	**87.74**	**112.05X**	**8.96 X**
B. bassiana	**163.98**	**87.09**	**16.06 X**	**4.06 X**	**87.04**	**113.07X**	**8.99 X**
Bt (Halt)	**163.21**	**87.05**	**15.91 X**	**4.04 X**	**86.69**	**111.57X**	**8.89 X**

Means within column followed by similar letter(s) are not significantly different at 5% level by DMRT.
* Computed yield is based on plant stand.

Computed yield per hectare

Presented in Table 4.9 above is the ear yield per hectare as influenced by distance of planting and use of biocontrol agents. Corn planted at a closer spacing of 60 cm × 10 cm produced a significantly higher yield with a mean of 9.78 t/ha, while 60 cm × 20 cm and 60 cm × 30 produced a mean of 8.59 and 6.50 t/ha, respectively. On the use of biocontrol agents, a 29.7% increase in ear yield was obtained with the application of *B. bassiana* relative to the control. Application of *B. bassiana* and Bt was comparable to chemical control (Lannate 40SP). The interaction effect showed that the closer spacing of 60 cm × 10 cm with the application of biocontrol agents produced the highest yield.

The ear yield per hectare was significantly influenced by distance of planting, and high plant density could be attributed to a greater number of plants and number of ears produced per unit area. This is evident in the correlation analysis in Table 4.8, wherein the yield was positively correlated with the number of ears/ha, and although the individual ear weight was higher in plants at wider spacing it did not contribute significantly to the total yield per hectare. On the other hand, increase in yield using biocontrol agents could be attributed to their effective suppression on the number of ACB and damage caused.

Conclusion

B. bassiana is more effective against ACB than the *M. anisopliae* under laboratory screening. *B. bassiana* (Bb 101-OFL isolate) caused mortality up to 90% at a dose of 1×10^9 spores/ml, while *M. anisopliae* (WL1-2a-OFL and Calaca 2) caused less than 50% mortality. In field experiments, the potency of *B. bassiana* in suppressing ACB is comparable to Bt and chemical control (Lannate 40SP). On the other hand, corn planted at distances of 60 × 20 and 60 × 10 cm had high ear yields. To confirm *B. bassiana*'s potency against ACB, field trials should be done, and studies on different media for faster mass production of *B. bassiana* spores without losing virulence, specifically in long-term storage, should be done. Likewise, a study on the possibility of spore production for commercial purposes should be prioritized. Also, further studies should be conducted on proper planting distances for optimum plant density giving high yields.

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CHAPTER FIVE

DEVELOPMENT OF BROAD-SPECTRUM ACTINOMYCETES FOR BIOCONTROL AND PLANT GROWTH PROMOTION OF FOOD CROPS

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Abstract

The current investigation is aimed at identifying actinomycetes and their metabolites with multiple actions against insect pests and pathogens, including plant growth promotion (PGP). We characterized 137 actinomycetes, isolated from 25 different herbal vermi-composts, for their antagonistic potential against charcoal rot in sorghum (caused by *Macrophomina phaseolina*) and wilt in chickpea (caused by *Fusarium oxysporum* f. sp. *ciceri* [FOC], respectively) by dual culture assay. Of the isolates, the three most promising *M. phaseolina* antagonistic strains (CAI-21, CAI-26 and MMA-32) and the five most promising *F. oxysporum* f. sp. *ciceri* (FOC) antagonistic strains (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) were further evaluated for their antagonistic and PGP potential by blotter paper assay, greenhouse and field conditions. All eight strains were characterized for their physiological traits (tolerance to salinity, temperature, pH and compatibility to antibiotics and fungicides) and further evaluated in the field for their PGP on rice. Ten strains (CAI-8, CAI-13, CAI-70, CAI-85, CAI-87, CAI-132, CAI-133, CAI-155, SAI-25 and BCA-508) were also found to be effective in suppressing *Helicoverpa*, *Spodoptera* and *Chilo* spp. under laboratory and greenhouse conditions. The sequences of the 16S rDNA gene of all eighteen strains matched with *Streptomyces*, but the species appeared to be different. This study confirms

that the selected *Streptomyces* strains have broad-spectrum biocontrol and PGP properties.

Introduction

Plant pathogens such as *Rhizoctonia bataticola*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Phytophthora* spp. have a broad host range, affecting several crops such as sorghum, chickpea, groundnut and pigeon pea leading to significant yield losses. For example, the dry root rot of chickpea caused by *R. bataticola* is not only a serious threat to chickpea but also to sorghum where its morph form *Macrophomina phaseolina* causes charcoal rot. On the other hand, some of the insect pests such as *Helicoverpa armigera*, *Spodoptera litura* and *Chilo partellus* (sorghum stem borer) cause serious damage to a number of food crops in dry-land agriculture, including chickpea, pigeon pea, groundnut and sorghum. The management of these key pathogens and insect pests was mainly addressed through chemical means for several decades, leading to the development of insecticidal resistance to a range of chemicals (Kranthi et al. 2002) and environmental contamination (Rao et al. 2009). Due to the broad host range of these biotic constraints, the farmers are finding it difficult to grow these crops profitably. Chemical control of these pathogens and insect pests is possible, but resource-poor farmers of semi-arid tropics who own small farms (75% of Indian farmers own 1.4−2.4 ha [Chadha et al. 2004]) cannot afford expensive chemical inputs such as fungicides and pesticides. Also, with increasing concern over environmental pollution as a result of injudicious usage of synthetic chemicals, there is a need for environment-friendly methods of pest management. Breeding for host-plant resistance is one of the promising areas; however, extensive screening of chickpea, groundnut and sorghum germplasm in the past has resulted in the identification of lines with only low to moderate levels of resistance to these biotic constraints (Pande et al. 2010; Das et al. 2008). Hence, there is an urgent need to identify alternate, environmental-friendly management options to control these important pathogens and insect pests.

Broad-spectrum antifungal and anti-insect pest biocontrol organisms are required for use in different cropping systems and for the control of multiple diseases and pests in a single crop. It is difficult to breed cultivars with resistance to a wide range of pathogens and pests in any crop species. Hence, we initiated a holistic approach to identify biocontrol agents that are effective against multiple pathogens and pests. A few of the available biocontrol agents mostly belonging to *Pseudomonas* spp. show a wide spectrum antifungal activity by virtue of volatile and diffusible antibiotics

(Haas & Keel 2003; Viji et al. 2003). Bacterial strains from diverse habitats of groundnut with broad spectrum antifungal activity have been isolated, identified and applied as seed treatment for control of collar rot in groundnut with or without Thiram (Kishore et al. 2005). Secondary metabolites of *P. aeruginosa* possess antifungal, plant-growth promoting, and biocontrol activities (Bano & Musarrat 2003). Spinosad (a product of soil actinomycete, *Sacchropolyspora spinosa*) causes significant reduction in the population of *H. armigera* and other pests (Mandour 2009; Wang et al. 2009). The main objective of the current investigation is to develop and evaluate broad-spectrum biocontrol agents with multiple actions against pathogens (charcoal rot in sorghum and wilt in chickpea caused by *M. phaseolina* and *F. oxysporum* f. sp. *ciceri* [FOC]) and insect pests (*H. armigera*, *S. litura* and *C. partellus*) so that one bio-control application can address more than one problem.

Materials and Methods

Preparation of herbal vermicompost

Foliage of 25 different botanicals (see Table 5.1 below) were collected from an International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) farm and were air-dried at room temperature (30 ± 2°C). The container for vermicomposting was constructed by cutting a 200 L plastic barrel into two halves. A metal grill was placed at the bottom of the barrel and the air-dried foliages of herbals were composted on top of the grill with earthworms (*Eisenia foetida*). When the herbal compost was ready, in about 2 months, about 100 g of the sample was collected and stored in a refrigerator at 4°C for further studies.

Isolation of actinomycetes

Ten grams of herbal vermicompost were suspended in 90 ml of physiological saline (0.85% of NaCl) in a flask and placed on an orbital shaker (at 100 rpm) at room temperature (28 ± 2°C) for 1 h. At the end of shaking, the vermicompost samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4–10^6 were plated on starch casein agar (SCA) by spread plate technique and incubated at 28 ± 2°C for 4 d. The most prominent colonies were isolated and maintained on SCA slants at 4°C for further studies.

Antifungal activity of the actinomycetes

Actinomycetes were evaluated for their antifungal activity against FOC and *M. phaseolina* by dual-culture assay. A fungal disk (*M. phaseolina*/ FOC) of 6 mm diameter was placed on one edge (1 cm from the corner) of the glucose casamino acid yeast extract (GCY agar) plate, and actinomycete isolate was streaked on the other edge of the plate (1 cm from the corner), followed by incubation at 28 ± 2°C for 4 d, or until the pathogen covered the entire plate in the control plate. Inhibition of fungal mycelium (halo zone) around the actinomycete colony was noted as positive and the inhibition zone was measured.

Enzymatic activities and secondary metabolite production by the actinomycetes

Siderophore production was determined according to the methodology described by Schwyn & Neilands (1987) and Gopalakrishnan et al. (2011b). Colloidal chitin was freshly prepared and used in the chitin agar as per the standard protocols of Hirano & Nagao (1988), and the chitinase production assay was conducted as described by Gopalakrishnan et al. (2011a). The standardized protocols of Hendricks et al. (1995) and Gopalakrishnan et al. (2011b) were used to evaluate the cellulase production. Protease production was done as per the protocols of Bhattacharya et al. (2009) and Gopalakrishnan et al. (2011b). Hydrocyanic acid (HCN) production was estimated qualitatively by the sulfocyanate colorimetric method (Lorck 1948). Observations were recorded on a 0−3 rating scale (based on the intensity of the reddish brown colour) as follows (Gopalakrishnan et al. 2011b): 0 = no colour change; 1 = light reddish brown; 2 = medium reddish brown, and 3 = dark reddish brown. Indole acetic acid (IAA) production was done as per the protocols of Patten & Glick (1996) and Gopalakrishnan et al. (2011b). Quantification of IAA was done by measuring the absorbance in a spectrophotometer at 530 nm and expressed as µg/ml in the culture filtrate.

Physiological traits of the actinomycetes

This was carried out as per the protocols of Gopalakrishnan et al. (2012). In brief, actinomycetes were streaked on Bennet's agar with various concentrations of NaCl ranging from 0% to 16% at an interval of 2% and incubated at 28°C for 5 d. For pH, the actinomycetes were streaked on Bennet's agar adjusted to pH 5, 7, 9, 11, and 13 and incubated at 28°C for

5 d. For pH 3, Bennet's broth was inoculated with the three actinomycetes and at the end of a 5-day incubation the intensity of growth was measured at 600 nm in a spectrophotometer. The influence of temperature on the actinomycetes was measured by streaking on Bennet's agar and incubated at 20, 30, and 40°C for 5 d. For 50°C, Bennet's broth was inoculated with the three actinomycetes, and at the end of a 5-day incubation the intensity of growth was measured at 600 nm in a spectrophotometer. A total of seven antibiotics (chloramphenicol, kanamycin, trimethoprim, nalidixic acid, streptomycin, ampicillin and tetracycline) and six fungicides (thiram, bavistin, benlate, captan, benomyl and radonil at field application level) were studied for their resistance/susceptible pattern against the actinomycetes. The required quantities of antibiotics/fungicides were dissolved in sterilized Milli Q water and mixed into Bennet's agar just before being poured into the Petri plates. Upon solidification, the actinomycetes were streaked and incubated at 28°C for 5 d.

Antifungal (against *M. phaseolina*) activity of the actinomycetes

Determination of *in vivo* antifungal activity of the three actinomycetes against *M. phaseolina* (CAI-21, CAI-26 and MMA-32) was done by blotter paper assay (Nene et al. 1981). Inoculum of *M. phaseolina* was prepared by homogenizing a 5-day-old culture grown on potato dextrose broth at 28 ± 2°C. Two-week-old seedlings of sorghum (variety R16-susceptible to charcoal-rot) were dipped in the inoculum of *M. phaseolina* for 30 min and placed side by side on a blotter paper (45 × 25 cm) so that only the roots were covered. Actinomycete isolates were inoculated (5 ml/plant) separately into plants. Fifteen plants per replicate and three replications were made for each actinomycete. Positive and negative controls were made by inoculating the plants only with *M. phaseolina* and sterile water, respectively. The blotter paper was kept moist with sterilized water and incubated at 28 ± 2°C for 8 d with a 12-h day length provided by fluorescent lights (120 μmol/m^2/s). The disease symptoms of the charcoal-rot (black-coloured microselerotia infection on the root surface) were recorded on a 0−4 rating scale (0 represents no visible charcoal-rot symptom, while 4 represents maximum disease symptoms), and the percentage of infected roots in actinomycete inoculated treatments compared with the control was calculated.

Antifungal (against *M. phaseolina*) and PGP activity of the actinomycetes on sorghum under greenhouse conditions

The three antagonistic actinomycetes against *M. phaseolina* (CAI-21, CAI-26, and MMA-32) were evaluated in a greenhouse. A total of 4 treatments (three actinomycetes + *M. phaseolina* inoculated-positive control) were made with six replications. *M. phaseolina* inoculum was mass multiplied on sorghum grains (R16). A pot mixture (800 g) was prepared by mixing red soil, sand and FYM at 3:2:2 and filled in 8" plastic pots followed by inoculation with *M. phaseolina* inoculum (20% of pot weight, 200 g/pot). Water (100 ml) was added to each pot to wet the potting mixture, and the pots were covered with polythene sheets. The whole set up was incubated at 32 ± 2°C in a greenhouse for 15 d for charcoal-rot symptoms to develop. Two weeks later, surface sterilized and sprouted seeds were transferred into test actinomycetes for an hour before being sown in the pots (six seeds/pot but thinned to three after one week). Booster doses of actinomycetes (5 ml/seedling, 10^8 cfu/ml) were applied twice (at 15 and 30 d after sowing) by the soil drench method. Growth parameters including root length, root dry weight, shoot dry weight, shoot root ratio, percentage of root and shoot dry weight increased over the control and the disease incidences were determined at day 60 after sowing. For evaluating the PGP potential of the three actinomycetes, the above-explained greenhouse experiment was repeated without applying *M. phaseolina*. However, one new treatment was included in which only water was added and the positive control (only *M. phaseolina* inoculated) was removed. Growth parameters including root length, root volume, root dry weight, shoot dry weight, shoot root ratio, and % root and shoot dry weight and length increase over the control were determined at day 60, after sowing.

Antifungal activity (against FOC) of the actinomycetes on chickpea under wilt-sick field conditions

The five most potential antagonistic actinomycetes against FOC (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) from the *in vitro* and greenhouse studies were further evaluated individually for their antagonistic potential in *Fusarium* wilt sick field at ICRISAT, Patancheru, during the 2009−10 cropping seasons. The field had been maintained as a wilt sick plot from 1980. Each actinomycete was inoculated by two different methods: M1 = inoculation of the seeds by soaking in the respective actinomycete culture for 1 h and M4 = inoculation of the seedlings after emergence with the

respective actinomycete culture (5 ml/seedling, 10^8 cfu/ml). Thus, the combination of actinomycete isolates × two methods of inoculation constituted 10 independent treatments in addition to one positive control, where no actinomycete was inoculated. Each treatment was replicated three times in a randomized complete block design (RCBD) and the plot size was 3 rows of 2 m long with a row spacing of 30 cm and a plant-to-plant spacing of 10 cm. Chickpea seeds of a highly susceptible cultivar to *Fusarium* wilt, JG-62 (acquired from Legumes Pathology Division, ICRISAT) were surface-sterilized with sodium hypochlorite (2.5% for 5 min) and rinsed with sterilized water (8 times) before being sown into the field. During the cropping season, a maximum temperature range of 30.1°C and 34.3°C, and a minimum temperature range of 9.2°C and 16.2°C, were recorded. Incidence of *Fusarium* wilt disease (number of plants showing wilt symptoms to total number of plants in a plot) was recorded on 17, 21, 24 and 28 DAS until the susceptible check showed 100% mortality. The actinomycete population was also enumerated, as explained earlier, from the rhizosphere soils at 28 DAS for all treatments.

PGP activity of the actinomycetes on rice under field conditions

The experiment was laid out in a completely randomized block design with three replicates and with subplot sizes of 10 × 7.5 m. Rice was grown by the rice intensification (SRI) method proposed by the Central Rice Research Institute (crri.nic.in). The eight actinomycetes (CAI-21, CAI-26, MMA-32, CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) were grown on a starch casein broth at 28°C for 5 d and further evaluated for their PGP traits. Control contained no actinomycetes. A nursery was established adjacent to the experiment field. Twelve-day-old single seedlings were uprooted from the nursery, their roots dipped in the respective actinomycete strains broth (containing 10^8 cfu/ml) for 45 min and transplanted at a spacing of 25 × 25 cm. Rice plants were inoculated with the actinomycetes (1000 ml) once in 15 d until the flowering stage along with the irrigation water. The recommended dose of NPK (120, 60, and 40 kg/ha, respectively) was supplied through compost, vermicompost and organic manures mixed with cow dung and straw. The plots were weeded by Cono-weeder at 10, 20 and 30 days after transplanting (DAT). Water management was done as recommended for the SRI method. After panicle initiation, all the plots were kept flooded with a thin layer of water (1−2 cm), and all were drained 15 d before harvest. The crop was harvested manually and all required observations were made. Root samples were collected from the top 15 cm soil profile and analyzed for root length

density, volume, and dry weight. Soil samples were collected from a 0−15 cm soil profile at 75 DAT and at harvesting. These were analyzed for soil chemistry (% organic carbon, available phosphorous and total nitrogen) and biological analysis (dehydrogenase activity, microbial biomass nitrogen and microbial biomass carbon).

Evaluation of actinomycetes for their entomopathogenic traits

A total of 96 actinomycetes were screened for their entomopathogenic traits against *Helicoverpa armigera* (Hubner), of which 10 were found to be promising. All 10 isolates were tested for their efficacy against 2nd and 3rd instar larvae of *H. armigera* (repeated thrice), 3rd instar larvae of *Spodoptera litura* (F.) (repeated twice), and seven-day-old larvae of sorghum stem borer, *Chilo partellus* (Swinhoe) (repeated thrice). The actinomycetes were cultured in starch casein broth for 8 d at 28°C. At the end of the incubation, the cultures were centrifuged at 10,000 g for 10 min and the supernatants were concentrated on a rotary evaporator at 35°C and assayed. Biomass was extracted with acetonitrile and concentrated on a rotary evaporator at 35°C and assayed. The efficacy of actinomycete cultures were tested in a diet impregnation bioassay, a detached leaf bioassay, and a greenhouse experiment (Sharma et al. 2005).

Molecular identification of the actinomycetes

Pure cultures of the actinomycetes were grown in starch casein broth until log phase (4 d) and genomic DNA was isolated. The amplification of the 16S rDNA gene was done by using universal primer 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') and 27F (5'- AGA GTT TGA TCM TGG CTC AG-3'). The PCR product was sequenced at Macrogen Inc. Seoul, Korea. The sequences obtained were compared with those from the GenBank using the BLAST program, aligned using the Clustal W software, and phylogenetic trees were inferred using the neighbour-joining method in the MEGA version 4 program (Tamura et al. 2007).

Results

Selection of antagonistic actinomycete isolates against *M. phaseolina* and FOC

A total of 137 actinomycetes that produced pigments and inhibited the adjacent colonies in the SCA plate were isolated from the 25 different

herbal vermicompost samples (see Table 5.1 below) and further screened for their antagonistic potential against *M. phaseolina* and FOC by *in vitro* dual-culture assay. A maximum diversity of actinomycetes was found in vermi-composts prepared with chrysanthemum while no actinomycetes were found in vermicompost prepared from Datura (see Table 5.1 below). Of the 137 actinomycete isolates, only 79 were found to have the antagonistic potential against *M. phaseolina* and 33 against FOC. Of the 79 positive isolates against *M. phaseolina*, three (CAI-21, CAI-26 and MMA-32) were found to be more promising, whereas of the 33 positive isolates against FOC, five (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) were found to be more promising. Hence, these eight isolates were selected for further characterization studies.

Table 5.1. Actinomycetes population and diversity of the 25 different herbal vermicomposts used in this study

S. No	Vermi-composts of	Scientific names of the herbals	pH of the vermi-composts	Actinon--ycetes count*	Actinomycetes diversity#
1	Rice straw	*Oryza sativa*	7.0	7.41	6
2	Gliricidia foliage	*Gliricidia sepium*	8.1	7.53	6
3	Neem foliage	*Azadirchta indica*	7.8	7.51	8
4	Adhatoda foliage	*Adhatoda vasica*	8.3	7.12	4
5	Annona foliage	*Annona squamosa*	7.9	7.74	7
6	Chilli foliage	*Capsicum annuum*	8.1	6.83	2
7	Calotrophis foliage	*Calotrophis gigantea*	8.1	7.04	3
8	Chrysanthemum foliage	*Chrysanthemum morifolium*	7.6	7.40	12
9	Datura foliage	*Datura metal*	7.7	0	0
10	Garlic foliage	*Allium sativum*	8.2	7.12	4
11	Ginger foliage	*Zingiber officinale*	8.8	7.46	7

12	Sweet potato foliage	*Ipomoea batatas*	7.8	7.27	6
13	Jatropha foliage	*Jatropha curcas*	8.1	6.82	2
14	Jatropha seed	*Jatropha curcas*	7.4	6.88	1
15	Bitter guard foliage	*Momordica charantia*	8.1	7.29	3
16	Drum stick foliage	*Moringa oleifera*	7.8	6.68	2
17	Oleander foliage	*Nerium indicum*	8.0	7.25	5
18	Onion foliage	*Allium cepa*	8.7	6.33	1
19	Parthenium foliage	*Parthenium hysterophorus*	8.0	7.51	8
20	Turmeric foliage	*Curcuma aromatica*	9.1	7.20	5
21	Pongamia foliage	*Pongamia pinnata*	7.3	7.40	6
22	Yellow oleander foliage	*Thevetia peruviana*	8.0	7.17	6
23	Tobacco foliage	*Nicotiana tabacum*	8.4	2.00	2
24	Tridax foliage	*Tridax procumbens*	8.3	6.83	3
25	Vitex foliage	*Vitex negundo*	7.5	7.12	2

* expressed in Log_{10} values; # expressed in numbers

Enzymatic activities and secondary metabolite production by the actinomycetes isolates

When the three *M. phaseolina* and five FOC antagonistic actinomycetes were evaluated for their enzymatic activities and secondary metabolite production, all eight isolates produced siderophore, HCN and IAA (except KAI-90), whereas only five isolates produced chitinase (except CAI-21, CAI-121 and CAI-127), cellulase (except CAI-24, CAI-121 and CAI-127) and protease (except CAI-121, KAI-32 and KAI-90) (see Table 5.2 below). Isolate CAI-121 produced the maximum IAA with 43.7 µg/ml of culture filtrate, 8−10 times higher than the other positive isolates (see Table 5.2 below).

Table 5.2. Enzymatic activities and secondary metabolite production by the actinomycetes

Isolate	Production score for^					IAA# (µg/ml)
	Siderophore	Chitinase	Cellulase	Protease	HCN*	
CAI-21	1	0	3.8	3	3	1.13
CAI-26	2	1	2.5	4	3	1.17
MMA-32	3	1	2.2	2	2	4.66
CAI-24	3	2	0	3	3	5.90
CAI-121	3	0	0	0	2	43.70
CAI-127	4	0	0	3	3	3.50
KAI-32	3	2	3	0	3	2.30
KAI-90	3	4	3	0	3	0

^ = rating scale for siderophore, chitinase, protease and HCN was as follows: 0 = Negative; 1 = Positive; 2 = 1--3 mm; 3 = 4--6 mm; 4 = >7 mm; * = hydrocyanic Acid; # = indole acetic acid.

Physiological traits of the actinomycetes

All eight actinomycetes were able to grow in NaCl up to 6% and they were all able to grow at pH values between 5 and 13 (acidic to highly alkaline) and temperatures between 20°C and 40°C (see Table 5.3 below). However, the optimum conditions for good growth were 0%−4% NaCl, 7−13 pH and temperatures of 20°C −30°C (see Table 5.3 below). All eight strains were highly resistant to ampicillin (>100 ppm), sensitive to nalidixic acid (50−100 ppm) and highly sensitive to Kanamycin (except CAI-21 and CAI-26), chloramphenicol, streptomycin and tetracycline (< 50 ppm; see Table 5.3 below). When the actinomycetes were evaluated for their fungicide tolerance at field application level, they were found to be tolerant to Bavistin (@2500 ppm) and sensitive to all the other tested fungicides (see Table 5.3 below).

Table 5.3. The physiological traits of the actinomycetes

Traits	CAI -21	CAI -26	MM A-32	CAI -24	CAI -121	CAI -127	KAI -32	KAI -90
Salinity (%)								
0	+++	+++	+++	+++	+++	+++	+++	+++
2	+++	+++	+++	+++	+++	+++	+++	+++
4	+++	+++	+++	++	++	+++	+++	++
6	++	++	++	+	+	++	++	+
8	+	+	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
pH								
3	-	-	-	-	-	-	-	-
5	+++	++	+	++	+	++	+	++
7	+++	+++	+++	+++	++	+++	+++	+++
9	+++	+++	+++	+++	+++	+++	+++	+++
11	+++	+++	+++	+++	+++	+++	+++	+++
13	+++	+++	+++	+++	+++	+++	+++	+++
Temperature (°C)								
20	+++	+++	+++	+++	+++	+++	++	++
30	+++	+++	+++	+++	+++	+++	+++	+++
40	++	++	++	+++	+++	+++	++	+++
50	-	-	-	-	-	-	-	-
Antibiotics resistance pattern (ppm)								
Ampicillin	1000	800	800	200	100	100	200	200
Chloramphenicol	50	50	50	25	25	25	25	25
Kanamycin	100	100	50	1	1	1	1	1
Nalidixic acid	100	100	50	25	50	50	50	50
Streptomycin	25	25	10	50	50	50	50	20
Tetracycline	5	5	25	10	20	20	50	50
Fungicide tolerance (ppm)*								
Thiram @ 3000	-	-	+	-	-	-	-	-
Bavistin @ 2500	+++	+++	+++	+++	+++	+++	+++	+++
Benlate @ 4000	-	-	-	-	-	-	-	-
Captan @ 3000	+	+	+	-	-	-	-	-

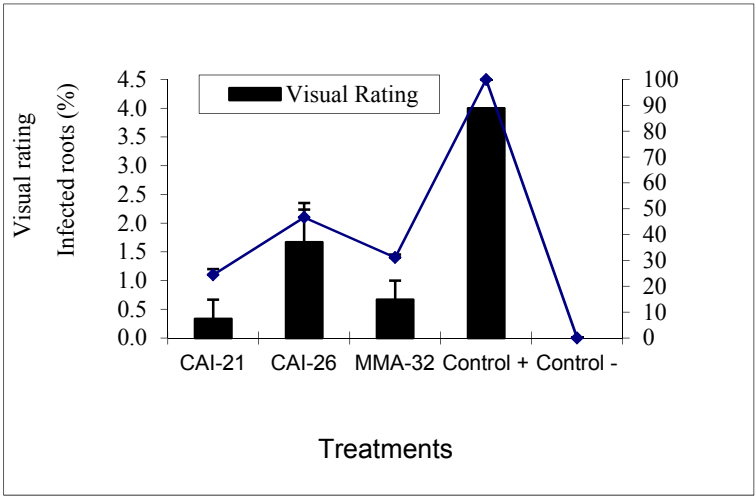
Benomyl @3000	+	+	-	-	-	-	+	+
Radonil @ 3000	-	-	-	-	-	+	+	-

+++ = good growth; ++ = medium growth; + = poor growth; - = no growth; * = at field application level.

Antifungal (against *M. phaseolina*) activity of the actinomycetes

When the three actinomycetes (CAI-21, CAI-26 and MMA-32) were evaluated for their *in vivo* antifungal potential against *M. phaseolina* by blotter paper assay, very little disease symptoms (rating < 2) and lesser root infection (47%−77% lesser than the control) were observed in the actinomycete-treated sorghum roots (see Fig. 5.1 below).

Fig 5.1. Influence of the three antagonistic actinomycetes (CAI-21, CAI-26 and MMA-32) on *M. phaseolina* by blotting paper assay



Antifungal (against *M. phaseolina*) and PGP activity of the actinomycetes on sorghum under greenhouse conditions

The actinomycetes were further evaluated for their *in vivo* antagonistic potential against *M. phaseolina* in greenhouse on sorghum crop. It was not possible to get the charcoal rot disease under positive control; however,

PGP characteristics were noticed. All the three actinomycetes increased shoot weight, root weight, root length and shoot root ratio compared to control (see Table 5.4 below). All three actinomycetes increased both sorghum shoot biomass (33%−53%) and root biomass (12%−21%) over the control (see Table 5.4 below). The highest increase of both shoot and root biomass (45% and 21% respectively) was found in CAI-26. No negative effect was found in any of the isolates.

In order to confirm the PGP traits, all three actinomycetes were further evaluated in greenhouse but without inoculating with *M. phaseolina* on sorghum crop. All three isolates increased sorghum shoot (10%−34%) and root biomass (29%−57%) (see Table 5.4 below). Root length (4%−60%) and root volume (16%−64%) were also found to be greater in comparison to the control (see Table 5.4 below). Among the three actinomycetes, MMA-32 increased all the parameters by at least 25%, i.e. shoot dry mass (27%), root dry mass (57%), root length (60%), and root volume (64%), over the control (see Table 5.4 below).

Antifungal activity (against FOC) of the actinomycetes on chickpea under wilt sick field conditions

When the five potential FOC antagonistic actinomycetes were evaluated in wilt sick field conditions, a reduction of *Fusarium* wilt incidence (4%−19%) was observed at 28 DAS over the control, where no actinomycetes were inoculated (see Fig. 5.2 below). In the control, 100% disease incidence could be noticed by 20 DAS itself. Reduction of wilt disease incidence was found to be at maximum with CAI-24 that was up to 25% at 24 DAS and 15% at 28 DAS. The next one being KAI-90 with 22% reduction at 24 DAS and 19% at 28 DAS with the M1 (seed inoculation) method. The other three isolates (CAI-121, CAI-127 and KAI-32) showed lower levels of reduction of wilt disease incidence (up to 18% at 24 DAS and 10% at 28 DAS) over the control (see Fig. 5.2 below). At 30 DAS, when the population of actinomycetes was enumerated from the rhizosphere soils, no actinomycete was found in the control plots, whereas actinomycetes (up to 10^6 log values) were found in actinomycete-inoculated plots (see Fig. 5.3 below).

Table 5.4. Antifungal (against *M. phaseolina*) and plant growth promotion (PGP) activity of the actinomycetes on sorghum under greenhouse conditions

Treatment	Shoot weight (g)	Root weight (g)	Root length (cm)	Shoot-root ratio	% increase over control	
					Shoot weight (g)	Root weight (g)
CAI-21	3.08	0.84	15.9	3.74	53	12
CAI-26	2.92	0.91	17.1	3.24	45	21
MMA-32	2.68	0.85	15.3	3.17	33	13
Control	2.01	0.75	15.0	2.74		
SE±	0195*	0.061***	1.55***	0.220***		
LSD (5%)	0.554	0.173	4.40	0.624		
CV%	17	17	23	17		

PGP potential

Treatment	Shoot weight (g)	Root weight (g)	Root length (cm)	Root volume	Shoot-root ratio	% increase over control			
						Shoot weight (g)	Root weight (g)	Root length (cm)	Root volume
CAI-21	7.36	0.88	42.11	17.02	8.58	31	40	4	16
CAI-26	6.1.6	0.81	57.55	21.23	7.65	10	29	42	45
MMA-32	7.14	0.99	64.68	23.89	7.24	27	57	60	64
Control	5.61	0.63	40.39	14.61	8.95				
SE±	0.256^{NS}	0.063^{***}	6.517^{*}	2.490^{NS}	0.851^{***}				
LSD (5%)	0.728	0.181	18.513	7.074	2.420				
CV%	9	21	33	35	22				

Note: Values are means of six replications and data calculated per plant after 60 DAS; * = Statistically Significant at 0.05; ** = Statistically Significant at <0.01; *** = Statistically Significant at < 0.001, NS = Not Significant; SE = Standard Error; LSD = Least Significant Difference; CV = Coefficient of variance.

Fig 5.2. Antifungal activity (against FOC) of the actinomycetes on chickpea under wilt sick field conditions

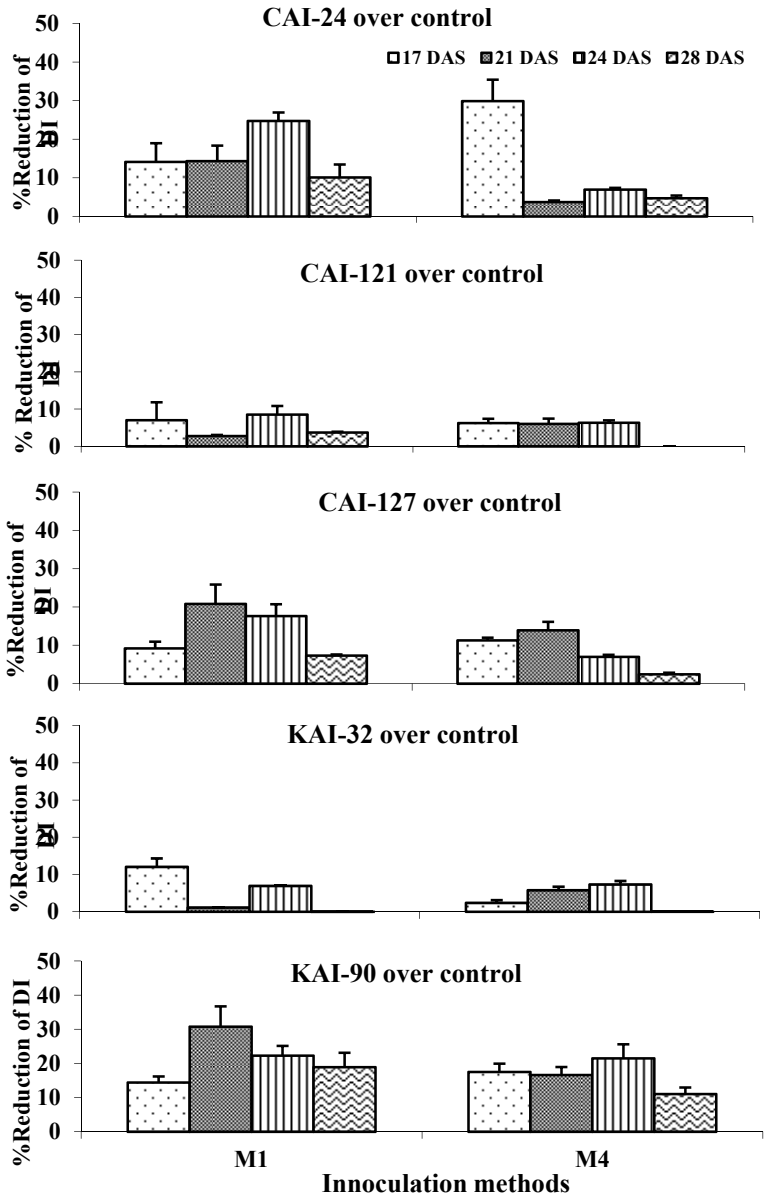
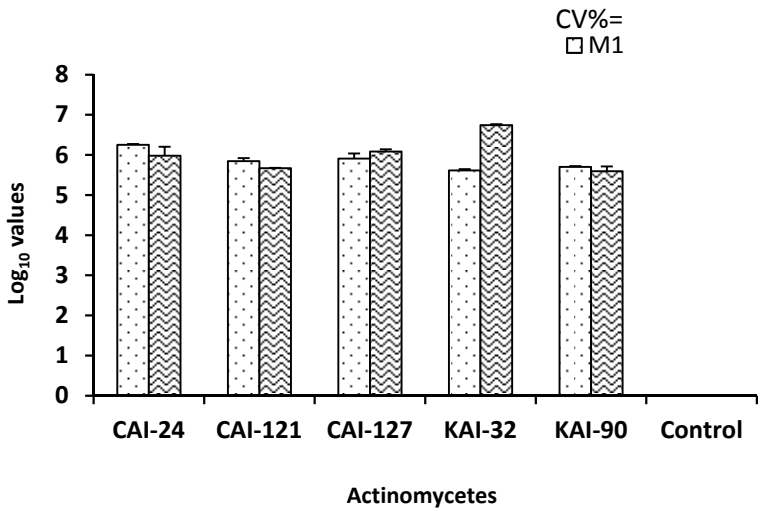


Fig. 5.3. Population of actinomycetes at the end of the field experiment in the Fusarium-infested field at ICRISAT in the actinomycetes-inoculated soils over the positive control (where no actinomycetes were inoculated).
Note: M1 = seed treatment; M4 = actinomycetes applied after seedling emergence



PGP activity of the actinomycetes, antagonistic to *M. phaseolina*, on rice under field conditions

Under rice field conditions, the actinomycetes antagonistic to *M. phaseolina* CAI-121, CAI-126, and MMA-32 significantly enhanced root length (m/m^2), volume (cm^{-3}/m^2), and dry weight (g/m^2) 1,000 seed weight (g), stover yield (g/m^2), grain yield (g/m^2), and total dry matter (g/m^2) over the control (see Table 5.5 below). Grain and stover yield were enhanced by 9%−11% and 11%−22%, respectively, over uninoculated controls (see Table 5.5 below). Root length, volume and dry weight were also increased by 39%−65%, 13%−30% and 16%−24% respectively (see Table 5.5 below). Among the three actinomycetes, CAI-21 caused greater increases of the root system and yield than the other two isolates (see Table 5.5 below). The available P, total N and organic carbon % were significantly higher in the top 15 cm of rhizosphere soils of actinomycete-treated plants (by 13%−34%, 30%−53%, and 26%−28% respectively) at harvesting than those of controls (see Table 5.5 below). The biological activities (microbial biomass carbon, microbial biomass nitrogen and dehydrogenase activity) in

the top 15 cm rhizosphere soils were also found to be significantly higher in the actinomycete-inoculated treatments at harvest over the controls (27%−83%, 23%−43% and 34%−151%, respectively; see Table 5.5 below).

PGP activity of the actinomycetes, antagonistic to FOC, on rice under field conditions

Under field conditions on rice, the five biocontrol potential (against FOC) actinomycetes CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90 significantly enhanced root length (m/m^2, 3%−15%), root volume (cm^3/m^2, 1%−35%), root dry weight (g/m^2, 2%−55%), stover and grain yield (g/m^2, 6%−25% and 0.2%−10%, respectively), total dry matter (g/m^2, 3%−18%), and test seed weight (g) over the control (see Table 5.6 below). The available P, total N and organic carbon % (chemical activities) were also found to be significantly enhanced in the top 15 cm of rhizosphere soils of actinomycete-treated plants (by 67%−122%, 32%−53% and 0%−13%, respectively) at harvesting compared to those of the controls (see Table 5.6 below). The soil biological activities (microbial biomass carbon [$\mu g/g^1$ soil, 0.5%−41%], microbial biomass nitrogen [$\mu g/g^1$ soil, 7%−52%], and dehydrogenase activity [$\mu g/TPF/g^1$ soil $24/h^1$, 2%−75%]) in the top 15 cm rhizosphere soils were found to be significantly higher in actinomycete-inoculated treatments (except CAI-24) at harvest, over the control (see Table 5.6 below).

Table 5.5. PGP activity of the actinomycetes, antagonistic to *M. phaseolina*, on rice under field conditions

Influence on roots and yield parameters

Treatment	Root length (m/m^2)	Root volume (cm^3/m^2)	Root dry weight (g/m^2)	100 seed weight (g)	Grain yield (g/m^2)	Stover yield (g/m^2)	Total dry matter (g/m^2)
A1 (CAI-21)	5453 (65.2)	1338 (30.0)	103.2(23.8)	17.02	15.9	957 (10.6)	2116
A2 (CAI-26)	5194 (57.4)	1299 (26.2)	97.4 (16.9)	21.23	15.9	942 (8.9)	2216
A3 (MMA-3)	4596 (39.3)	1159 (12.6)	96.7 (16.0)	23.89	15.6	939 (8.5)	2106
Control	3299	1029	83.3	14.61	15.1	865	1906
Mean	4636	1206	95.2		15.6	926	2086
SE±	271.5**	60.8*	3.9*	2.490^{NS}	0.80^{**}	14.4^{*}	10.1*
LSD (5%)	939.6	210.5	13	7.074	0.37	56.7	61.7
CV%	10	9	7	35	1	3	1

Influence on soil chemical and biological parameters

Treatment	Available P (ppm)	Total N (ppm)	Organic carbon (%)	Microbial biomass C (µg/g^{1} soil)	Microbial biomass N (µg/g^{1} soil)	Dehydrogenase activity (µg/TPF g^{1} soil 24 /h^{1})
A1 (CAI-21)	142 (33.9)	2978(53.1)	1.47 (25.6)	2149 (49.4)	70 (37.2)	156 (151.6)
A2 (CAI-26)	119 (12.2)	2823 (26.2)	1.50 (28.2)	1824 (26.8)	63 (23.5)	134 (116.1)
A3(MMA-3)	120 (13.2)	2535 (12.6)	1.50 (28.2)	2634 (83.1)	73	83 (38.8)
Control	106	1945	1.17	1438	51	62
Mean	122	2570	1.41	2011	64	109
SE±	5.7*	141.9*	0/052*	183.9*	2.1***	12.6*
LSD (5%)	22.2	491.2	0.179	636.2	7.8	43.7
CV%	8	10	6	16	6	20

* = Statistically significant at 0.05, ** = Statistically significant at 0.01, *** = Statistically significant at 0.001.

Table 5.6. PGP activity of the actinomycetes, antagonistic to FOC, on rice under field conditions

Influence on root and yield parameters

Treatment	Root length (m/m^2)	Root volume (cm^3/m^2)	Root dry weight (g/m^2)	100 seed weight (g)	Grain yield (g/m^2)	Stover yield (g/m^2)	Total dry matter (g/m^2)
CAI-24	2087	396	30.5	18.9	587	584	1170
CAI-121	3263	513	36.9	18.9	583	637	1221
Cai-127	3470	692	49.5	18.6	619	754	1373
KAI-32	3652	627	54.3	19.3	640	754	1394
KAI-90	3592	581	44.8	18.9	587	693	1279
Control	3182	507	35.1	18.6	582	671	1183
SE±	3208	553	41.9	18.9	600	671	1270
LSD (5%)	52.3***	8.7***	1.02***	0.03***	9.7**	27.8**	31.9**
CV%	7	7	10	1	3	7	4

Influence on soil chemical and biological parameters

Treatment	Available P (ppm)	Total N (ppm)	Organic carbon (%)	Microbial biomass (µg/g^{1} soil)	Microbial biomass N(g/g^{1} soil)	Dehydrogenase activity (µg/TPF/g^{1} soil 24/h^{1})
CAI-24	133	2456	1.49	1715	60	94
CAI-121	117	1992	1.47	3293	65	113
CAI-127	115	2644	1.52	2875	88	194
KAI-32	122	2142	1.66	4020	65	135
KAI-90	129	2160	1.62	2946	62	136
Control	87	1190	1.47	2861	58	111
Mean	117	2231	1.53	2952	66	131
SE±	5.2*	63.1*	0.032*	150.5*	3.7***	9.3*
CV%	6	4	3	9	10	12

* = Statistically significant at 0.05, ** = Statistically significant at 0.01, *** = Statistically significant at 0.001.

Evaluation of actinomycetes for their entomopathogenic traits

Of the 96 actinomycetes evaluated for their entomopathogenic traits against *H. armigera*, only 16 were found to have a mortality of more than 70% on 2nd instar larvae (data not shown). The culture filtrates of ten actinomycetes (CAI-8, CAI-13, CAI-70, CAI-85, CAI-87, CAI-132, CAI-133, CAI-155, SAI-25 and BCA-508) also showed more than 70% mortality on 3rd instar larvae (see Table 5.7 below). Of the ten actinomycetes, six (CAI-8, CAI-13, CAI-70, CAI-85, CAI-87 and SAI-25) recorded 100% mortality on 3rd instar larvae of *H. armigera* (see Table 5.7 below). Of the ten promising actinomycetes against *H. armigera*, nine (except CAI-132) were further tested (three times) against 3rd instar larvae of *S. litura* and *C. partellus*. All nine isolates were found to have induced mortality between 38% and 77% for *S. litura* and 100% for *C. partellus* (see Table 5.7 below).

Table 5.7. Evaluation of actinomycetes for their entomopathogenic traits

Isolate	% mortality of the larvae at six days after treatment		
	H. armigera	***S. litura***	***C. partellus***
CAI-8	100	73	100
CAI-13	100	77	100
CAI-70	100	54	100
CAI-85	100	46	100
CAI-87	100	38	100
CAI-132	73	ND	ND
CAI-133	73	55	100
CAI-155	98	100	100
SAI-25	100	57	100
BCA-508	86	59	100
Control	9	0	0
Mean	85	56	90

ND = Not done

Molecular identification of the actinomycetes

In order to determine the identity of the eight potential PGP and antagonistic (against *M. phaseolina*) and ten entomopathogenic actinomycetes, their 16S rDNA was sequenced and analyzed. A neighbour-joining dendrogram was generated using the sequences from the eighteen actinomycetes (1400 bp)

and representative sequences from the databases. Phylogenetic analysis of 16S rDNA sequences of the eighteen actinomycete matched with *Streptomyces* but with different species (see Table 5.8 below).

Table 5.8. Identification of the eighteen broad spectrum actinomycetes based on 16S rDNA analysis

Sl. No.	Isolate No	Closely matched with
1	CAI-21	*Streptomyces albus*
2	CAI-26	*S. champavathi*
3	MMA-32	*S. roseoviolaceus*
4	CAI-24	*S. anulatus*
5	CAI-121	*S. setonii*
6	CAI-127	*S. setonii*
7	KAI-32	*S. setonii*
8	KAI-90	*S. africanus*
9	CAI-8	*S. cremeus*
10	CAI-13	*S. albolongus*
11	CAI-70	*S. caeruleatus*
12	CAI-85	*S.* spp.
13	CAI-87	*S. cyaneofuscatus*
14	CAI-132	*S. caeruleatus*
15	CAI-133	*S. carpaticus*
16	CAI-155	*S. cyaneofuscatus*
17	SAI-25	*S.* spp.
18	BCA-508	*S. cyaneofuscatus*

Conclusion

Three actinomycetes promising action against charcoal rot disease in sorghum caused by *M. phaseolina* (CAI-21, CAI-26 and MMA-32) and five actinomycetes (CAI-24, CAI-121, CAI-127, KAI-32 and KAI-90) promising action against Fusarium wilt disease in chickpea caused by FOC were demonstrated for their biocontrol potential under greenhouse and field conditions. All eight actinomycetes were also demonstrated for their plant growth promotion (PGP) potential under field conditions on rice grown by SRI methods. Hence, it can be concluded that the eight biocontrol potential actinomycetes also have PGP traits. In addition, another set of ten actinomycetes (CAI-8, CAI-13, CAI-70, CAI-85, CAI-87, CAI-132, CAI-133, CAI-155, SAI-25 and BCA-508) were also

demonstrated for their entomopathogenic traits against not only *H. armigera* but also *S. litura* and *C. partellus*, the key insect pests of many crops including pigeon pea, chickpea, cotton, tomato and sorghum. Thus, this study confirms that the selected actinomycetes (*Streptomyces* spp.) have broad-spectrum biocontrol and PGP properties. These actinomycetes, therefore, are likely to be potential candidates for the discovery of novel secondary metabolites which may be of importance for various PGP and biocontrol applications. Furthermore, identification of the mechanisms of action of these organisms may lead to the discovery of novel phenomena in PGP and biocontrol.

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CHAPTER SIX

STUDIES ON POPULATION DENSITY OF DIFFERENT PGPRS IN TURMERIC RHIZOSPHERE SOILS FOR BIOCONTROL ACTIVITY

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Abstract

Turmeric (*Curcuma longa* L) is an herbaceous annual plant belonging to the family Zingiberaceae in which curcumin has been identified as the active principle compound. The quality of turmeric rhizomes is affected by various pests and diseases, which in turn reduce the quality of rhizomes. Turmeric is an important exportable spice for India. In most foreign countries, turmeric price is fixed based on the appearance of rhizomes and curcumin content. In order to get the maximum yield in turmeric, planters used to apply a variety of inorganic chemical fertilizers and agrochemicals for controlling pests and diseases. Furthermore, constant and indiscriminate applications of agrochemicals and mineral fertilizers pollute the environment and soil, and are hazardous to animal and human health. Organic farming is a crop production method which encourages sustainable agriculture by enhancing the biological cycles in nature. The present study envisages studying the population density of different known plant growths promoting rhizobacteria (PGPR) strains in turmeric rhizosphere soil samples. The antagonistic activity of PGPRs was studied by performing tests against rhizome rot pathogen *Pythium aphanidermatum*. PGPRs have been proved to be efficient biocontrol agents in controlling various plant diseases and significantly enhancing the plant growth. The results reveal

that PGPRs such as *Bacillus*, *Pseudomonas*, *Trichoderma* and *Streptomyces* spp. were identified and present predominantly in the soils of turmeric plantations. The population diversity was further correlated with the soil nutrient status. The microbial population of PGPRs and soil nutrient contents were carried out both in the rhizosphere and non-rhizosphere soils. The results indicated that the microbial population was found to be greater in rhizosphere than in non-rhizosphere soils, and there was a positive correlation between microbial population and soil nutrient contents.

Introduction

Turmeric (*Curcuma longa* L) is a herbaceous annual plant belonging to the family Zingiberaceae. It has a large oval rhizome with sessile cylindrical tubers containing curcumin content and is commonly used as a flavouring and colouring agent, as well as a preservative in biomedical applications, for example. In India, turmeric is grown in all 18 states, of which Tamil Nadu is the largest producer. Andhra Pradesh, Tamil Nadu, Karnataka, Orissa and West Bengal are also major turmeric-producing states. It has been well studied in Asian, African and European countries due to its economic importance. It is native to tropical South Asia, but is now widely cultivated in the tropical and subtropical regions of the world (Reddy 2009). India occupies the first position in area, with 1,75,300 ha, and also in production, moving 7,94,400 tonnes during 2009–10. The other major countries exporting turmeric are China, Myanmar and Bangladesh. Indian turmeric fetches a premium price due to its superior quality in terms of high curcumin content, size and organoleptic characters in the international market. India has occupied around 60% of the world trade in turmeric.

Curcumin has been identified as the active principle of turmeric. It has anti-cancer and anti-inflammatory properties as well as a potent antioxidant capacity, its mechanisms of action including inhibition of several cell signalling pathways, effects on cellular enzymes, immuno-modulation, and effects on angiogenesis and cell-cell adhesion, which are well documented (Aggarwal et al. 2003).

The important turmeric varieties grown in India are: Alleppey Finger (Kerala), Erode and Salem turmeric (Tamil Nadu), Raja pore and Sangli turmeric (Maharashtra), and Nizamabad Bulb (Andhra Pradesh). In Tamil Nadu, the important varieties cultivated are Erode local, BSR-1 and 2, PTS-10, Roma, Suguna, Sudarsana and Salem local. Among these varieties, 70%–75% comprise the traditional varieties (Kandiannan et al.

2008). The high usage of agrochemicals has made soil infertile, led to the accumulation of toxic chemicals in the soil and food products, as well as an imbalanced nutrient cycling and ecosystem. In order to maximize the agricultural productivity with minimum soil loss, a cheaper and safer method is necessary. All these criteria can be achieved through the application of microbial inoculants like PGPRs (Dinesh et al. 2010). These microorganisms are known to possess a vast range of capabilities by producing growth-promoting substances, enzymes, vitamins, organic aids and bioactive compounds, enhancing the plant nutrients, biological N_2 fixation, phosphorous/potassium-solubilization, and crop protection against stress and disease conditions. PGPRs have been proved as efficient biocontrol agents in controlling various plant diseases besides enhancing the plant growth significantly.

The quality of turmeric rhizomes is affected by various pests and diseases. Furthermore, constant and indiscriminate applications of agrochemicals and mineral fertilizers pollute the environment and soil, and are hazardous to animal and human health (Raveendra et al. 2007). Organic farming is a crop production method which encourages sustainable agriculture by enhancing the biological cycles in nature. The present study studies the population density of different known PGPR strains in turmeric rhizosphere soil samples. The antagonistic activity of PGPRs was studied by performing prevention tests of the rhizome rot pathogen *Pythium aphanidermatum*.

Materials and Methods

Soil samples were collected from different turmeric planting districts of Tamil Nadu, India such as Coimbatore, Dharmapuri, Erode, Namakkal, and Villupuram for the estimation of nutrient contents and enumeration of different PGPRs. These samples were allowed to air dry at room temperature and various parameters like soil pH, total organic carbon (Walkley & Black 1934), total nitrogen (AOAC 1990), and available phosphorous (Jackson 1973) were determined. The population density of different PGPRs such as *Bacillus*, *Pseudomonas*, *Trichoderma* and *Streptomyces* species were enumerated in the above soil samples using nutrient agar (NA), King's B, *Trichoderma* selective medium (TSM), and casein nitrate agar (CNA) media, respectively.

The pathogen *P. aphanidermatum* was isolated from infected rhizomes of turmeric plants collected from different agroclimatic zones covering different turmeric planting districts of Tamil Nadu. The culture was identified and deposited at the Centre for Advanced Studies in Botany,

University of Madras, Chennai, followed by Microbial Type Culture Collection Centre (MTCC), Chandigarh. Enumeration and isolation of different PGPRs in the soil samples were performed by the serial dilution plate technique. Morphological characterizations such as Gram's staining, endospores and motility test, and biochemical characterizations such as pigment production, starch hydrolysis, casein hydrolysis, catalase test, nitrate reduction, indole production, gelatin hydrolysis, and hydrogen sulphide production were carried out (Williams & Wilkins 1994).

The influence of abiotic factors such as pH (4.0–6.5) and temperature (5°C–40°C) and various nutrient factors such as carbon, nitrogen, amino acid and vitamin sources on the growth of various PGPRs were identified. Six different carbon compounds—glucose, fructose, maltose, sucrose, starch and cellulose—and four nitrogen compounds—ammonium nitrate, sodium nitrate, potassium nitrate, and casein hydrolysate—were added by replacing starch and potassium nitrate, respectively, in the basal medium. On the other hand, sources of amino acid like alanine, methionine and arginine, and vitamins such as thiamine, riboflavin, and biotin were also selected and supplemented to the basal medium to enhance the growth potential of PGPRs. The inoculated plates were incubated for 10–15 d depending on the nature of the experiment to record the growth rate of PGPRs (Ponmurugan et al. 2007).

The study was carried out by following the dual culture (Huang & Hoes 1976) and antibiosis (Dennis & Webster 1971) techniques. In the case of the dual culture study, a mixture (50:50%) of PDA and NA / King's B/ TSM / CNA media containing plates were inoculated with *P. aphanidermatum*, as well as PGPRs strains on diametrically opposite points. The radial growth of the pathogen and antagonist were measured at 24 h intervals. For the antibiosis study, petri dishes containing mixture malt extract medium amended with secondary metabolites of PGPRs were subsequently inoculated with *P. aphanidermatum*. Radial growth was measured periodically and the percentage inhibition was calculated.

Results and Discussion

Soil fertility is influenced by its physical, chemical and biological properties, including the cultivation of crops. In general, beneficial microorganisms in soil decompose various organic substances and thereby improve soil tilth and fertility, which in turn enhance the plant growth. Many beneficial microorganisms promote plant growth by mutualistic or symbiotic relationships by fixing atmospheric nitrogen or mobilizing phosphorous or solubilizing potassium to a greater extent (Jayanthi et al.

2001). The rhizosphere is a region of dynamic process initiated by root exudations and the release of organic nutrients, and is influenced by a host of factors like soil features, environmental conditions, cultural practices, and soil microbial interactions (Baby et al. 2002). Among the beneficial, mutualistic non-symbiotic microorganisms, the *Bacillus subtilis*, *Pseudomonas fluorescence*, *Trichoderma atroviride* and *Streptomyces* species are very important in turmeric plant growth in terms of enhancing the yield potential and maintaining the soil health. The results reveal that the total number of various PGPR populations in turmeric soil samples was higher in Erode followed by Coimbatore, compared to the Dharmapuri and Vizhupuram districts. Moreover, among the PGPRs, bacterial PGPRs such as *B. subtilis* and *P. fluorescence* were found to be higher than actinomycetes (*Streptomyces* spp.) and fungal PGPRs like *T. atroviride*. Similarly, of the bacterial PGPRs, *P. fluorescence* was the highest (14.5×10^5) population in the soils of Erode, while *B. subtilis* was the lowest (9.72×10^5 cfu/gm soil dry wt) population.

The population density of PGPR strains was positively correlated with soil nutrients like total organic matter, available nitrogen, phosphorous and potassium content (see Table 6.1 below). Moreover, the population density of PGPRs was found to be higher in rhizosphere than in non-rhizosphere soil samples collected from turmeric fields. The same trend was registered in population density which was found to be greater in soil samples obtained from Erode followed by Coimbatore, and lowest in the Dharmapuri and Vizhupuram districts. The soil edaphic parameters, like soil reaction (pH), electrical conductivity (Ec), clay, silt, sand content and water-holding capacity, varied at differing levels between turmeric planting districts; however, there was a strong relationship between these parameters and the population diversity of PGPRs. Similar observations were reported in tea soils by Baby et al. (2002) and Ponmurugan et al. (2011), who observed a correlation between beneficial microorganisms like nitrogen fixers, phosphate solubilizers, antibiotic producing microorganisms (actinomycetes), and biocontrol agents and nutrients in the soil.

A total of 1,500 strains of PGPRs were isolated from turmeric soil samples and subjected to screening for their antagonistic activity by following the paper discs method. Further, the morphological and biochemical characteristics of the PGPR strains were studied and the results were presented in Table 6.2. The morphological characterization showed that the isolates were found to be Gram's positive organisms. The cells were coiled rods and the endospores were free to light in nature. The biochemical characterization results indicated that most of the strains were

efficient in hydrolyzing starch, gelatin and casein. Indole production was strictly negative for some strains but the catalase test was positive for the rest of the strains. The production of hydrogen sulphide and nitrite reduction showed positive results in the majority of the isolates (see Table 6.2 below). These results coincided with the report of Ravel et al. (2000) and Ponmurugan et al. (2011).

The growth of PGPR strains in the basal medium adjusted with different pH and nutrient sources revealed that a better growth was recorded in a pH at 5.5. This pH level may be correlated with the soil pH. The optimum temperature for the growth of PGPR strains was 25°C followed by 20°C. Among the different carbon and nitrogen sources tested, maltose and potassium nitrate, respectively, were the best supplementary sources. Of the different vitamin and amino acid sources evaluated, biotin and alanine, respectively, were found to be limiting substrates for the utmost growth of PGPR strains (see Table 6.3 below). The production of an array of antifungal metabolites has been known to be influenced by components of medium and cultural conditions such as pH, temperature, carbon, nitrogen and other sources (Augustine et al. 2004).

The antagonistic potential of various PGPR strains was studied against a rhizome rot disease-causing pathogen *P. aphanidermatum*, which revealed a remarkable percentage of inhibition of pathogen growth. Among the two different methods of antagonistic activity studied, antibiosis was better than hyperparasitism in terms of good growth inhibition of the pathogen, in which 83.23%–92.19% and 75.56%–84.56% were registered with antibiosis and hyperparasitism, respectively. These results coincided with the reports of Zahner et al. (1979) and Ponmurugan et al. (2007; 2011), who observed the inhibition zone of *Phomopsis theae* against *Streptomyces* spp. The growth inhibition of the pathogen is due to the production of secondary metabolites by the antagonists (Thangapandian et al. 2007). According to Demain & Fang (1995), the most widely accepted theory is that antibiotics are used to compete with other organisms in a nutrient-depleting environment (Rovel et al. 2000; Augustine et al. 2004), along with the production of exopolysaccharide compounds, diffusible pigments and enzymes such as lipase, caseinase, gelatinase, cellulose and amylase by the antagonists (Ponmurugan et al. 2011). In the present study, most strains of PGPRs were found to be of potential antagonists against rhizome rot pathogen. Based on these results, it can be further inferred that the isolated PGPR strains can be used as soil inoculants to prevent the growth of soil-borne pathogens like *P. aphanidermatum* in turmeric soils.

Table 6.1. Population density of various PGPRs and nutrient statuses in turmeric soils of Tamil Nadu, India

Name of planting districts	Population Density				Soil reaction (pH)	Organic carbon (%)	Total nitrogen (ppm)	Available phosphorous (ppm)
	BS	PF	TA	SS				
Coimbatore	8.84	12.7	4.88	6.72	7.80	2.55	2734	14.72
Dharmapuri	8.54	10.3	2.57	3.53	6.92	1.87	1777	13.22
Erode	9.72	14.5	6.75	8.57	7.81	2.77	2844	16.83
Namakkal	7.73	11.4	1.75	5.35	6.82	0.85	1146	12.80
Villupuram	5.00	10.0	3.44	3.90	7.20	1.68	1305	13.55
SE ±	1.00	2.24	1.03	2.24	0.54	0.82	150.47	02.33
CD at P = 0.05	2.57	3.08	3.55	3.07	1.38	1.02	253.37	03.47

BS: *Bacillus subtilis* (cfu × 10^5 cfu/gm soil dry wt)
PF: *Pseudomonas fluorescence* (cfu × 10^5 cfu/gm soil dry wt)
TA: *Trichoderma atroviride* (cfu × 10^3 cfu/gm soil dry wt)
SS: *Streptomyces species* (cfu × 10^4 cfu/gm soil dry wt)

Table 6.2. Morphological, physiological and biochemical characterization of various PGPRs

Parameters	Strains of various PGPRs			
	Bacillus	*Pseudomonas*	*Streptomyces*	*Trichoderma*
Cell morphology	Coiled rods	Rods	Coiled rods Rods Spiral spore chain	Cluster of spore chain
Gram's staining	++	++	++	
Pigment production	+	-	+	-
Starch hydrolysis	++	-	+	++
Casein hydrolysis	++	-	+	++
Catalase test	+	++	++	+
Nitrate reduction	++	++	++	++
Indole production	+	-	+	-
Gelatin hydrolysis	++	+	++	-
Hydrogen sulphide production	++	++	++	-
Presence of endospores	++	++	++	-
Nature of endospores	Free cells	Tight free cells	Tight free cells	-

++ Prominent growth, + Moderate growth, - No growth

Table 6.3. Effect of biotic and nutrient factors on the growth of silicon solubilizing bacteria

Parameters	Strains of various PGPRs			
	Bacillus	*Pseudomonas*	*Streptomyces*	*Trichoderma*
Optimum pH	5.0	5.0	5.5	5.5
Optimum temperature (°C)	25	25	20	25
Glucose *	++	+	+	++
Fructose *	++	+	+	++
Maltose *	++	++	++	++
Sucrose *	++	+	-	-
Starch *	++	+	++	++
Cellulose *	++	++	-	-
Ammonium nitrate **	++	+	++	+
Sodium nitrate **	++	+	+	+
Potassium nitrate **	++	++	++	++
Casein hydrolysate **	++	+	+	+
Alanine #	++	++	++	++
Methionine #	+	+	+	+
Arginine #	+	+	+	+
Thiamine ##	+	-	+	+
Riboflavin ##	+	+	+	+
Biotin ##	++	++	++	++

* Carbon sources, ** Nitrogen sources, + Moderate growth ++ Prominent growth - No growth, # Amino acid sources, ## Vitamin sources

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CHAPTER SEVEN

ROLE OF PGPR IN THE BIOREMEDIATION OF HEAVY METAL IONS AND PLANT GROWTH-PROMOTION OF WHEAT AND PEANUT GROWN IN HEAVY METAL CONTAMINATED SOIL

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Abstract

Heavy metal contamination of agriculture soil is a significant environmental problem and has several disadvantages for human health and agriculture. Plant soil root ecosystem is an important interface of soil and plants and plays a significant role in the bioremediation of contaminated soil with heavy metals, in which rhizobacteria are known to affect heavy metal mobility and availability to the growing plant through the release of chelating agents, acidification, phosphate solubilization and redox changes, and therefore have the potential to enhance the bioremediation processes. Bioremediation strategies with appropriate heavy metal-adapted rhizobacteria have received considerable attention. In this chapter, some recent advances in the rhizobacteria in bioremediation of heavy-metal contaminated soils will be presented. In addition, excessive metal concentrations in contaminated soils result in decreased soil microbial activity and soil fertility, and yield losses. Use of plant growth-promoting rhizobacteria (PGPR) capable of growing in the presence of a variety of heavy metals offers a sustainable and eco-friendly solution as a multifaceted bio-inoculant, disease protectant and effective means of biosorption of heavy metals from contaminated agricultural soil. PGPR capable of producing siderophores that chelate iron as well as other heavy

metals like cadmium, lead, nickel, arsenic, aluminium, magnesium zinc, copper, cobalt and strontium other than iron, can help in adsorbing/ absorbing heavy metals from soil and will have the advantage of bioremediation besides plant growth-promotion and phytopathogen suppression. The present study describes a new method of screening of siderophores complexed with different metals where siderophore producing microbes chelate the respective metal ions from dye solution, causing colour change. Siderophore producing *Alcaligenes* sp. STC1 and *Pseudomonas aeruginosa* RZS3 strains were found to remove various heavy metal ions at the batch scale. The bioremediation potential of these strains were superior (99.52% and 99.76% of $ZnCl_2$) to the chemical ion chelators like Ethylene Diamine Tetra Acetic Acid (92.40% removal) and Citric Acid (83.98% removal). Siderophores produced by these stains were found with heavy metals like $MnCl_2.4H_2O$, $NiCl.6H_2O$, $ZnCl_2$, $CuCl_2$ and $CoCl_2$ compared to $FeCl_3.6H_2O$. These isolates were also capable of promoting plant growth in wheat and groundnut seeds sown in heavy-metal contaminated soil under pot assay conditions. Seed bacterization of wheat (*Triticum aestivum*) and groundnut (*Arachis hypogaea*) enhanced seed germination, root height, shoot length and chlorophyll content.

Introduction

The advent of various modern industries, including fertilizer and pesticide industries, and the liberal use of these agrochemicals have greatly contributed to the extensive contamination of heavy metal ions in agriculture fields causing serious agro-environmental pollution and even threatening human life. Soils contaminated by heavy metals are likely to induce a corresponding contamination in harvested crops (Nan et al. 2002), and crops in or close to contaminated sites can uptake and accumulate these metal ions, leading to their biomagnifications through the food chain and resulting in increased toxicity (Gupta & Mohohaptra 2003). Consumption of these heavy-metal contaminated crops exerts potential risks to humans and animals causing the malfunction of organs and chronic syndromes (Gupta & Gupta 1998). Most of these are proven to be carcinogenic even at a slightly higher concentration.

In this regards, use of PGPR, capable of adsorbing heavy metal ions, has been suggested as an eco-friendly, sustainable, attractive and economical alternative to the commonly applied physicochemical remediation methods approach for bioremediation. PGPR present in plant soil root ecosystem plays a significant role in the bioremediation of contaminated soil with heavy metals, in which the plant growths

promoting rhizobacteria (PGPR) are known to chelate these heavy metal ions, and thus can play a vital role in the bioremediation of heavy metal ions (Das et al. 2007). The application of PGPR with the potential of adsorbing heavy metals from fields will have the twin advantages of bioremediation and plant-growth promotion.

PGPRs secrete a variety of metabolites including the production of siderophores, a small, molecular weight, extracellular organic compound secreted under iron-starved conditions for the solubilization and transport of iron. Though they are specific ferric iron chelators they can also bind to other metals such as divalent heavy metals and actinides because of potentially high metal-siderophore stability constants. Siderophores have been found to operate with complex heavy metals like cadmium, lead, nickel, arsenic (III, V), aluminium, magnesium, zinc, copper, cobalt and strontium. This approach can be used in removing many toxic metals from the soil (Nair et al. 2006).

The present research focuses on the production of siderophores by heavy metal *Alcaligenes* sp. RZS3 and *Ps. aeruginosa* RZS3, use of siderophores as washing agents *vis-a-vis* chemical chelators to remove heavy metal from contaminated soil, and also focuses on plant growth at pot level.

Materials and Methods

Isolation of heavy metal resistant bacteria

Two bacterial isolates initially labelled as *Alcaligenes* sp. and *Ps. aeruginosa* obtained from garden soil were checked for their ability to produce siderophores in a succinate medium. The isolated bacterial strains were tested for their resistance to heavy metal ions through a dilution method (Cervantes et al. 1986). Freshly prepared nutrient agar were amended independently with various heavy metal salts like $MnCl_2.4H_2O$, $NiCl.6H_2O$, $ZnSO_4.7HO$, $ZnCl_2$, $FeSO_4$, $CuSO_4$, $CuCl_2$, $HgCl_2$, $FeCl_3.6H_2O$, $AgNO_3$ and $COCl_2$, and a 10 mM stock solution of each heavy metal was used (Sambrook et al. 1989), being further diluted at various concentrations ranging from 100 μM to 2000 μM, inoculated from log phase cultures.

Biochemical characterization and 16s rRNA sequencing of isolates

These isolates were initially subjected to various biochemical tests as per the procedure and protocols of Bergey's manual of systematic bacteriology (Kerters & Dey 1984). The pre-sterilized hi-carbohydrate biochemical kits (KB 002 and KB 009, Hi Media, Mumbai, India) were used for the biochemical identification of the isolates. Partially identified cultures were subjected to a 16s rRNA gene profile. The standard phenol-chloroform method was used for the extraction of genomic DNA (Sambrook et al. 1989). The 16S rRNA genes of the isolate were amplified by PCR (Pidiyar et al. 2002) and sequenced on an automated DNA sequencer (ABI377) using the Big Dye terminator kit (Applied Biosystems) (Hauben et al. 1997). Results were compared with the public databases (NCBI) to determine the identity and homology of the isolate.

Heavy metal quantification

The isolates *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3 were cultivated independently in a tris-minimal medium with different levels of $MnCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$ and $CuCl_2$ in the range of 2–4 mg/L, respectively, at 28°C and 120 rpm. In order to establish the correlation between the amount of heavy metal ions added and amount of heavy metal ions absorbed by the isolate, the residual concentration of each metal ion was estimated on an atomic absorption spectrophotometer (Model S-Series 711869 v1.26; Make: Thermo, USA) at the respective wavelength (Shao & Sun 2007; Diels 1997).

Screening, production, detection and quantification of siderophore

Growth and siderophore production was carried out in an 500 mL Erlenmeyer flask containing 100 ml succinate medium (Meyer & Abdallah 1978). For this purpose, *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3 (6×10^6 cells mL^{-1}) were grown independently in a succinate medium at 28 $\pm$ 2°C at 120 rpm for 24–48 h. Following the incubation, cell density was measured at 620 nm by using a double beam UV-Visible spectrophotometer (1240, Shimadzu, Japan). The detection and estimation of siderophore were performed after centrifugation (15 min at 5,000 g × cm at 4°C) and a cell free supernatant was assayed for the presence of siderophore by using the Chrome Azurol Sulphonate (CAS) test and a

CAS agar assay (Schwyn & Neilands 1987). The CAS shuttle assay (Payne 1994) was used to quantify the amount of siderophore produced by the *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3.

Extraction and estimation of siderophore

After 48 h, 100 ml of inoculated succinate medium was centrifuged (5,000 g × cm for 15 min), and a pH of cell-free supernatant was adjusted to pH 6.0 with concentrated HCl and kept overnight at 4°C. This was extracted thrice with equal volumes of ethyl acetate (Schwyn & Neilands 1987). The acetate extract was then evaporated and the crude siderophore thus obtained was gravimetrically quantified; the subsequent yield of siderophore was reported as mg/100 ml.

Screening for complexation of siderophore with heavy metal ions

On the basis of the principle of the CAS method, a modified method was developed to screen the ability of siderophore to complex with various metals ions. Each CAS agar containing various metal ions (1 mM metal stock in 10 mM HCl) like $MnCl_2.4H_2O$, $NiCl.6H_2O$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, $HgCl_2$ and $AgNO_3$, instead of Fe (III), was separately inoculated with *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3 and incubated at 28 $\pm$ 2°C for 24 h and observed for change in colour of CAS from blue to orange/yellow/red.

Evaluation of siderophore in the bioremediation of heavy-metal-spiked soil

Siderophore, EDTA (chemical chelator), and citric acid (organic chelator) were evaluated for their efficiency to remove heavy metal ions from the soil matrix at the batch scale. Two soil samples for the present study were collected from a local garden soil area and from heavy metal spiked soil. For the second set, the garden soil was artificially spiked with heavy concentrations of heavy metals (5,000 mg/kg) like $MnCl_2.4H2O$, $NiCl_2.6H2O$, $ZnSO_4.7H2O$, $ZnCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$, $CuCl_2$, $HgCl_2$, $AgNO_3$, and $CoCl_2$. Soil was shaken for 3 d on a rotary orbital shaker and then spread in trays for air-drying and sieved following its ageing for 45 d. Each heavy metal contaminated soil (10 g) was separately mixed with 100 ml of siderophore, EDTA (0.01M) and CA (0.01M) (Abumaizar & Smith 1999) as a washing solution and succinate medium as control, and the pH

of all the washing agents was set to 7.0 ± 0.05. Extraction of heavy metals from contaminated soil involves the dissolution of the metal-mineral bond followed by the dispersion of the pollutant metal in the washing liquid. After three successive washings of heavy-metal-contaminated soil with extractants which included siderophore, 0.01 M citric acid and 0.01 M EDTA, with SAM as control, the samples were shaken at 120 rpm and withdrawn after 24 h, and then centrifuged at 5,000 rpm for 10 min. The concentration of heavy metals in supernatant was estimated by an atomic absorption spectrophotometer (Model: S-Series 711869 vl.26; Make: Thermo, USA)

Bioefficacy test

In the case of bacterized seeds, a significant increase in plant growth was observed in individual heavy metal spiked soil as compared to the control seeds. Seed bacterization of wheat and peanuts with *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3 showed a much more pronounced effect on seed germination, shoot height, root length, number of leaves and chlorophyll content of wheat and groundnut. The population densities from rhizosphere soil also showed an abundance of PGPRs.

In the presence of *Alcaligenes* sp. coated seeds grown in $MnCl_2$-spiked soil, a 40% increase in the germination of wheat and a 33.3% rise in the germination of peanut seeds grown in $MnCl_2$, $NiCl_2$, $ZnSO_4$, $ZnCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$ and $CuCl_2$ spiked soil was recorded. A percentage increase in the shoot length of wheat plants was recorded as 42.28% in the presence of $CoCl_2$ and 25.80% for peanuts with $NiCl_2$, $ZnSO_4$, $FeSO_4$, $CuSO_4$ and $CuCl_2$. Increase in the root length of wheat was noted as 31.57% in the presence of $FeCl_3$ and $CuCl_2$, whereas a 45.0% increase in groundnut root length was observed with $MnCl_2$, $NiCl_2$ and $ZnSO_4$. The number of wheat leaves increased by 25.0% in the presence of $MnCl_2$, $ZnSO_4$, $ZnCl_2$ and $FeSO_4$, while the leaves of peanuts increased by 50.0% in the presence of $MnCl_2$. The chlorophyll content of wheat and peaplants in the presence of $CuSO_4$ rose by 36.84% and 35.29%, respectively.

Wheat and peanut seeds bacterizied with *Ps. aeruginosa* and grown in $MnCl_2$, $ZnSO_4$, $ZnCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$ and $CuCl_2$ showed 20.0% and 33.3% increases, respectively. The percentage increase for the shoot length of wheat and peaplants was recorded as 42.28% and 35.80% in the presence of $FeSO_4$ and $NiCl_2$, respectively. The hike in the root length of wheat was noted as 27.77% in the presence of $NiCl_2$, $ZnCl_2$, $FeSO_4$, $FeCl_3$, $CuSO_4$ and $CuCl_2$, whereas a 40.0% increase in peanut root length was observed with $MnCl_2$ and $FeCl_3$ spiked soil. The number of wheat leaves

increased by 25.0% in $MnCl_2$, $NiCl_2$, $ZnSO_4$, $ZnCl_2$, $CuSO_4$, $CuCl_2$ and $CoCl_2$ spiked soil, while the leaves of peanuts increased by 40.0% in the presence of $MnCl_2$ and $FeCl_3$. The chlorophyll content of wheat and peanut plants in the presence of $ZnSO_4$ and $CuCl_2$ increased by 29.41%.

Root colonization

The root or seed colonization of PGPR is considered an important parameter of plant-growth promotion. For this purpose, the population densities of isolates on the rhizoplane of wheat and peanuts were determined by taking the plate count of soil (10^{-5} dilution used) and the number of colonies (cfu/gm soil) as the means of root and seed colonization.

Results and Discussion

Isolation of heavy metal resistant bacteria

The maximum tolerable concentrations of heavy metals for *Alcaligenes* sp. RZS2 was 2,000 μM for $MnCl_2$ and 1,600 μM for $ZnCl_2$. *Ps. aeruginosa* RZS3 tolerated high concentration, i.e. 1,800 μM of $ZnCl_2$ and 1,600 μM for $MnCl_2$, and 1,400 μM for $ZnSO_4$ and $FeCl_3$. Metal ions like $HgCl_2$ and $AgNO_3$ inhibited the growth of both isolates. The sensitivity of isolates towards $HgCl_2$ and $AgNO_3$ is attributed to the antimicrobial effects of these ions.

Biochemical characterization and 16s rRNA sequencing of isolates

Preliminary phenotypic characterization showed that the siderophore-producing isolates were Gram-negative straight, mobile rod, had the fermentative metabolism of glucose, L-arabinose, mannose and ribose, and also utilized the citrate. They also synthesized the fluorescent pigment on nutrient agar. Preliminary phenotypic characterization identified the isolate as *Alcaligenes* sp. and *Pseudomonas* sp., and partial gene sequencing of 16S rRNA of these isolates showed 98% and 99% similarity with *Alcaligenes* sp. STC1 and *Ps. aeruginosa*. The 16S rRNA gene sequence of these isolates was submitted to the Gene bank under the names *Alcaligenes* sp. RZS2 and *Ps. aeruginosa* RZS3 with accession numbers HQ443704 and HQ453360, respectively.

Heavy metal quantification

Alcaligenes sp. RZS2 and *Ps. aeruginosa* RZS3 adsorbed more than 90% of the added heavy metal ions. *Alcaligenes* sp. RZS2 showed 96.55% absorption of $CuCl_2$ (see Fig. 7.1a below), while *Ps. aeruginosa* RZS3 showed 90.16% absorption of $ZnCl_2$ (see Fig. 7.1b below).

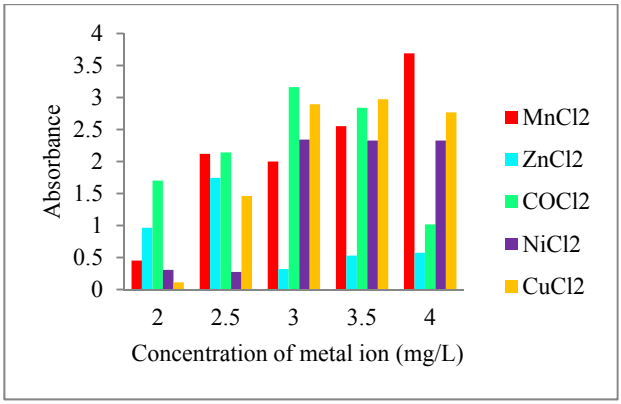

Fig. 7.1a. Absorption of heavy metal by *Alcaligenes* sp.

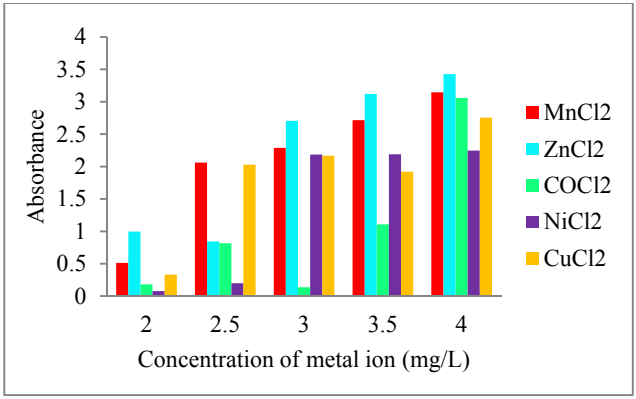

Fig. 7.1b. Absorption of heavy metal by *Ps. aeruginosa*

Screening, production, detection and quantification of siderophore

In shake flask studies, change in the colour of succinate medium from colourless to fluorescent green after 24 h showed siderophore production by the isolates. This was confirmed by a CAS test, where the addition of CAS to a cell-free supernatant changed the colour of CAS from blue to orange (see Fig. 7.2a below). Change in the colour of the CAS reagent is due to the fact that the siderophores present in the supernatant chelate the iron from CAS reagent, resulting in colour change (Schwyn & Neilands 1987; Milagres et al. 1999). *Alcaligenes* sp. excreted the highest amount (92.61%) of siderophore, while *Pseudomonas* sp. produced the lowest amount (43.22%) of siderophore, comparatively.

Extraction and quantification of siderophore

The siderophore yield was estimated by solvent extraction, and crude siderophore production was reported to be 1.7 mg/100 ml for *Alcaligenes* sp. RZS2 and 0.9 mg/100 ml for *Pseudomonas* sp. RZS3 of succinic acid medium.

Screening for complexation of siderophore with heavy metal ions

Siderophore, though selective for complexation with Fe (III), also bound to divalent metals like Mn^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} with varying capacities. Change in the colour of modified CAS from blue to orange indicated the chelation of that metal ion from CAS agar plate, confirming the ability of siderophores of *Alcaligenes* sp. and *Pseudomonas* sp. to chelate/bind metal ions other than Fe (III). The order of complexation of metals with siderophore produced by both organisms in CAS assay was Cu^{2+}, Ni^{2}, Mn^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Ag^{2+}. Aiken et al. (2003) reported that siderophores bind divalent metals like Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Sn^{2+} and Pb^{2+} with varying capacities. A similar test for uranyl complexation was reported by Renshaw et al. (2003). Amongst all the CAS agar plates, the one prepared with Cu instead of Fe (III) showed maximum colour change.

The heavy metal cations are structurally very similar, and the cations Mn, Fe, Co, Ni, Cu and Zn have ionic diameters between 138 pm and 160 pm (Weast 1984). Siderophores have a high affinity for ferric iron, and they can also form complexes with metals other than ferric iron. Because

of the similarity in size and charge between Al and Fe, aluminum readily forms complexes with siderophores (Garrison & Crumbliss 1987), and these aluminum-siderophore complexes can be transported into the cell (Emery 1971). Bhattacharya (2010) investigated the ability of siderophores to chelate other metal ions such as Cr, Cd, Mn, Cu, Pb, Hg, Mo, Ni, Ag and Zn, observing changes in λmax and extinction coefficients, confirming that siderophores form complexes with metal ions. Neubauer (2000) and Kiss & Farkas (1998) also reported that siderophores can form stable complexes with metals that are of environmental concern, such as Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np. Although the bacterial siderophores have the potential to sparingly mobilize soluble metals, the efficiency of siderophore-producing bacteria to either mobilize or immobilize heavy metals from soils is dependent on several factors, such as the binding form of the heavy metals present, the charge of the siderophores, as well as the pH of the soil and its mineral composition and organic content (Neubauer 2002).

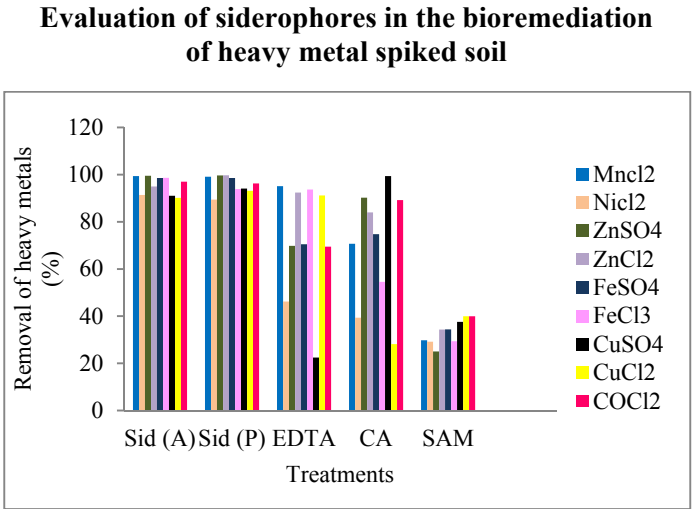

Fig. 7.3. Effect of sequential washing of heavy-metal-contaminated soil by washing agents

Among the various washings applied for the removal of heavy metal ions from spiked soil, siderophore wash effectively removed the heavy metals. The efficiency of siderophore for heavy metal removal was superior (99.52% and 99.76% removal of $ZnCl_2$) compared to the chemical ion

chelators like ethylene diamine tetra acetic acid (92.40% removal of $ZnCl_2$) and citric acid (83.98% removal of $ZnCl_2$).

Bioefficacy test

In the case of bacterized seeds grown in heavy metal spiked soil, a significant increase in plant growth was observed over the control (non-bacterizied) seeds sown in heavy metal spiked soil as well as untreated soil. A significant increase in seed germination, shoot height, root length, number of leaves and chlorophyll content was evident in wheat and peanut. Peanut seeds bacterized with *Alcaligenes* sp. RZS2 resulted in a 40.12% increase in germination in the presence of $MnCl_2$ spiked soil and 33.3% with $NiCl_2$, $ZnSO_4$, $ZnCl_2$, $FeCl_3$, $CuSO_4$ and $CuCl_2$ spiked soil, respectively. The 20.0% and 33.30% germinations were recorded with wheat seeds sown in the presence of $MnCl_2$, $ZnCl_2$, $FeCl_3$, $CuSO_4$ and $CuCl_2$ spiked soil and peanut seeds sown in the presence of $MnCl_2$, $ZnSO_4$, $ZnCl_2$, $FeSO_4$ and $CuCl_2$ spiked soil, and bacterized with *Pseudomonas aeruginosa* RZS3, respectively.

Wheat seeds bacterized with *Alcaligenes* sp. RZS2 resulted in 42.28% more shoot length in the presence of $CuSO_4$ spiked soil which, along with peanut seeds and soil spiked with $MnCl_2$, $ZnCl_2$ and $FeCl_3$, resulted in a 23.33% increase. Increases of 31.57% and 45.0% in root height in $FeCl_3$, $CuCl_2$, $MnCl_2$, $NiCl_2$ and $ZnSO_4$ spiked soil and a 25% increase in the number of leaves were recorded for *Alcaligenes* sp. RZS2 in the presence of $MnCl_2$, $ZnSO_4$, $ZnCl_2$ and $FeSO_4$ spiked soil.

A 50.0% increase in number of leaves of peanut seeds was observed when peanut seeds were inoculated with *Alcaligenes* sp. RZS2 in the presence of $MnCl_2$-spiked soil. The results of inoculation of *Alcaligenes* sp. RZS2 with wheat seeds showed a substantial increase to 29.41% of chlorophyll content of the leaves in the presence of the soil spiked with $HgCl_2$. Results with peanut seeds increased by 35.29% of chlorophyll content of the leaves in the presence of soil spiked with $ZnSO_4$, respectively.

PGPR can significantly increase the growth of plants in the presence of heavy metals including nickel, lead and zinc (Burd et al. 2000; Grichko et al. 2000). However, microbial iron-siderophore complex can be taken up by plants as an iron source. Therefore, efficient siderophore-producing bioinoculants could be ideal candidates in metal-contaminated soil. Siderophore-simulated significant root and shoot growth of mung bean to the extent of 16.48% and 28.80%, respectively, in the presence of $CdCl_2$ (110 μM) has been reported. Increase in root and shoot growth by 20%

Table 7.1. Influence of *Alcaligenes* sp. on wheat and peanut seeds in heavy-metal-spiked soil (100 μM)

% increase in germination of seeds											
	$MnCl_2$	$NiCl_2$	$ZnSO_4$	$ZnCl_2$	$FeSO_4$	$FeCl_3$	$CuSO_4$	$CuCl_2$	$CoCl_2$	$AgNO_3$	$HgCl_2$
Wheat	40.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	nil
Groundnut	33.3	33.3	33.3	33.3	33.3	33.3	33.3	33.3	nil	nil	nil
% increase in shoot length of plant											
Wheat	28.57	24.24	24.24	16.66	24.24	21.87	7.40	21.87	42.28	24.24	13.79
Groundnut	23.33	25.80	25.80	23.33	25.80	23.33	25.80	25.80	20.68	23.33	20.68
% increase in root length of plant											
Wheat	18.75	13.33	18.75	23.52	27.70	31.57	27.70	31.57	23.52	23.52	23.52
Groundnut	45.0	45.0	45.0	42.10	38.88	42.10	38.88	42.`10	38.88	38.88	35.29
% increase in number of leaves of plant											
Wheat	25.0	Nil	25.0	25.0	25.0	nil	nil	nil	nil	nil	nil
Groundnut	50.0	33.39	40.0	40.0	40.0	40.0	40.0	40.0	33.33	25.0	25.0
% increase in chlorophyll content of leaves											
Wheat	21.87	↓	25.58	04.0	↓	↓	36.84	18.51	↓	↓	29.41
Groundnut	08.30	15.38	Nil	15.38	nil	15.38	35.29	↓	31.25	26.66	18.51
% increase in cfu											
Wheat	41.07	39.26	75.0	31.24	71.87	63.73	38.12	22.65	34.43	21.42	02.94
Groundnut	68.51	70.68	56.77	36.64	31.08	67.72	22.72	29.16	↓	99.90	54.46

Table 7.2. Influence of *Ps. aeruginosa* on wheat and peanut seeds in heavy metal (100 μM) spiked soil

% increase in germination of seeds											
	$MnCl_2$	$NiCl_2$	$ZnSO_4$	$ZnCl_2$	$FeSO_4$	$FeCl_3$	$CuSO_4$	$CuCl_2$	$CoCl_2$	$AgNO_3$	$HgCl_2$
Wheat	20.0	nil	Nil	20.0	20.0	20.0	20.0	20.0	↓	↓	↓
Groundnut	33.3	↓	33.3	33.3	33.3	↓	↓	nil	nil	Nil	nil
% increase in shoot length of plant											
Wheat	21.08	24.24	21.87	21.87	42.28	19.35	19.35	16.06	26.47	13.79	16.06
Groundnut	34.28	35.80	30.80	23.33	25.80	23.33	25.80	23.33	23.33	20.68	20.68
% increase in root length of plant											
Wheat	23.52	27.77	23.52	27.77	27.77	27.77	27.77	27.77	23.52	23.52	23.52
Groundnut	40.0	33.33	25.0	25.0	33.33	40.0	25.0	25.0	25.0	25.0	25.0
% increase in number of leaves of plant											
Wheat	25.0	25.0	25.0	25.0	nil	nil	25.0	25.0	25.0	↓	↓
Groundnut	40.0	33.33	25.0	25.0	33.33	40.0	25.0	25.0	25.0	25.0	25.0
% increase in chlorophyll content of leaves											
Wheat	Nil	↓	29.41	17.24	↓	↓	↓	29.41	07.69	↓	↓
Groundnut	↓	04.34	42.10	26.66	↓	08.30	21.42	↓	31.25	26.66	↓
% increase in cfu											
Wheat	43.0	14.65	38.12	64.64	34.86	72.50	20.16	04.80	43.74	11.06	29.28
Groundnut	52.11	19.68	18.04	32.89	25.54	11.30	15.70	22.72	0.97	17.07	11.30

and 19.5%, respectively, in the presence of $(CH3COO)_2Pb$ (660 μM) was recorded by Tripathi et al. (2005). Sheng et al. (2008) demonstrated that heavy metal resistant, Pb-solublizing and plant growth-promoting endophytic bacteria grown in a Pb-contaminated site significantly increased the root dry weights from 23% to 37% and shoot dry weights from 12% to 39%.

Root colonization by isolates

A 75.0% increase in colony forming units (cfu/gm soil) was recorded with $ZnSO_4$ when wheat seeds were bacterized with *Alcaligenes* sp. RZS2. A 70.68% increase was recorded when soil was spiked with $NiCl_2$ and peanut seeds were bacterized by the same.

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PART II.

MACRO AND MICRO-NUTRIENTS: PGPR AND OTHER MICROBIALS IN SUSTAINABLE ORGANIC AGRICULTURE

CHAPTER EIGHT

INNOVATION FOR SUSTAINABLE AGRICULTURE WITH PRATHISTA NEW GENERATION ORGANIC FERTILIZERS

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Abstract

Organic farming today is very important for sustainable agriculture in the view of depletion of soil fertility, as well as environmental pollution due to the excessive application of inorganic fertilizers. Traditional organic fertilizers like cow dung and organic compost have a limited influence in this direction because of their low nutrient content and non-availability of the required quantities. A new generation of organic fertilizers produced by Prathista Industries, Hyderabad, India are aimed at developing all plant nutrient combinations (macro and micro) through fermentation technologies which are eco-friendly and bio-available in nature. Sustainability in agriculture will be successful only if the farmer adopts the activity of poultry and dairy to a small extent in association with regular crop production. Various experiments and demonstrations in farmers' fields have conclusively indicated that the organic inputs of Prathista Industries have resulted in increased crop productivity, milk yield in cattle, and egg production in poultry. The present article reviews the different aspects in this regard.

Excessive application of chemical fertilizers brings about micro climatic changes in the soil resulting in the depletion of soil health due to considerable reduction of microbial activity and organic carbon content. Nutrient uptake and utilization is much reduced. Adoption of organic farming is the only alternative to reverse the situation for reviving soil health. Organic agriculture promotes and enhances agro-ecosystem health,

including biodiversity, biological cycle and soil biological activity. Traditional organic manures like Farm Yard Manure (FYM) and Vermicompast have limitations of supply and in realizing equal yields compared to chemical fertilizers. Prathista Industries Limited is manufacturing certified organic inputs through fermentation technology utilizing different microbial cultures. The products are bioavailable, highly degradable, have longer shelf lives (3–5 years) and do not contaminate groundwater. This chapter reviews the bioefficacy results of the products on various crops like rice, sugarcane and vegetables.

A study conducted by ANGRAU on rice using Bio Zinc revealed that the grain yield was significantly greater in all the zinc-applied treatments either through soil or foliar applications than the control. Significantly higher yields (7,790 kg/ha) were obtained with soil application of ZnSO4 @ 10 kg/acre + spraying @ 3 ml/L at 45 days after transplanting (DAT), along with the recommended dose of fertilizers and soil application @ 5 kg/acre + spraying of bio zinc @ 3 ml/L at 45 DAT, and the recommended dose of fertilizers (7248 kg/ha) than control (4,319 kg/ha) and RDF alone (6,032 kg/ha), which also influenced the grain yield of rice over RDF alone (6,032 kg/ha). The favourable effect of zinc along with the recommended dose of fertilizers on yield might be due to tryptophnn, the precursor of growth promoting substances that lead to better growth and higher yields. This might be due to the direct contribution of nitrogen in terms of the synthesis of chlorophyll, protein and amino acids and phosphorus by stimulating root systems, leading directly to greater absorption and the translocation of nutrients.

In the investigation of the efficacy of Bio Potash formulation in paddy in comparison to Muriate Of Potash (MOP), an inorganic source of potassium and control (no potash application), an experiment comprising five treatments was laid out in a randomized block design with four replications, and observations were recorded for grain yield, yield component characters, and pest incidence. The results revealed a significant increase in grain yield, ear-bearing tillers/m^2 and filled grains per panicle with the application of bio-potash compared to control and RDF. Application of 50 kg/ha Bio Potash as basal followed by bio-potash spray @ 625 ml in 200 L of spray fluid per hectare at panicle initiation stage resulted in a significantly higher grain yield (36% over control and 14.41% increase over RDF). The further cost of the bio-potash treatment was observed to be equal to MOP application and hence may be recommended for use in paddy.

A field experiment was conducted to study the comparative performance of different organic manures (farm yard manure, vermicompost and press

mud cake) and Prathista Industries organic manures (Suryamin, Bio phos, Megacal, Bio potash and Organic NPK) on soil health and yield of sugarcane at the Agricultural Research Station, Anakapalle, India, with a variety 93 A 145 during 2009. The results reveal that the application of organic manures significantly increased the soil fertility over the initial status. Prathista Industries organic manures slightly improved soil fertility status in organic manure-treated plots compared to Prathista Industries organic manures added plots. Maximum cane and sugar yields with better stalk populations and number of millable canes were recorded in the Prathista Industries organic manure treated plots. Maximum cane yields (102.41 t/ha) and sugar yields (13.28 t/ha) were recorded in Prathista Industries organic manure treatments followed by vermicompost @ 5 t/ha (86.12 and 10.73 t/ha), farm yard manure @ 25 t/ha (85.17 and 10.93 t/ha), and press mud cake @ 7.6 t/ha (72.71 and 9.01 t/ha). The Highest Benefit Cost Ratio (BCR) of 2.42 was recorded in Prathista Industries organic manure treatments. The study showed that Prathista Industries organic manures are better substitutes for chemical fertilizers in sugarcane for sustained productivity and fertility in sugarcane (Kumari & Ramalakshmi 2010). The comparison of organic inputs with traditional organic manures in sugarcane also revealed maximum cane yield (102.0 Mt/ha) and sugar yield (13.2 Mt/ha), as well as highest cost benefit ratios (2.42), compared to farmyard manure (85.17 Mt/ha and 10.39 Mt/ha), press mud cake (72.17 Mt/ha and 9.01 Mt/ha) and Vermicompost (86.12 Mt/ha and 10.73 Mt/ha).

The effect of organic agri inputs on soil nutrient availability and increase in yield parameters in green gram (*Phaseolus aureus*) was studied at AGNR Agricultural University (ANGRAU). Bio Phos Granules and Bio potash Granules @ 75 kg/ha along with Bio Zinc Granules @ 12.5 kg /ha + FYM @ 5 t/ha were compared to RDF. The highest soil organic carbon content of 1.44% was record in the treatment (20N–40 P_2 O_5 and 20 K_2O) where "K" was applied through Bio Potash compared to inorganic fertilizer application (1.00). Bio phos alone in comparision with Bio potash Granules + Bio Zinc granules resulted in higher “K” in soil. The highest number of effects in nodules (12.0) was recorded in the treatment where P, K and Zn were substituted by organic sources. The highest seed yield (1425.6 kg/ha) was recorded in treatment with organic inputs (Anuradha & Ramesh 2010).

Trials were conducted at Dr. Y. S. R. Horticultural Universiy, Andhra Pradesh, India to study the effect of Bio phos granules @ 20 and 25 kg/acre as basal application and Bio phos liquid @ 2 ml/L (1, 2 and 3 sprays at 20 d after transplanting, pre-flowering and flowering stage) as

foliar application on growth and yield of brinjal cv. Bhagyamati, and to study the effect of Biophos granules @ 20 and 25 kg/acre as basal application in two split doses at 20–25 and 45–50 d after planting and 2 sprays of Biophos liquid @ 3 ml/L at 20–25 and 45–50 d after planting on growth and yield of onion cv. Agrifound Light Red during two successive years—2008 and 2009. In aubergines, among different treatments, recommended farming practice excluding P_2O_5+ basal application of Biophos @ 20 kg/ acre + 2 sprays of Biophos liquid @ 2 ml/L of water at two critical stages of crop duration was found to be significantly superior with regard to plant spread, number of fruits per plant and yield over other treatments with the application of generally available P_2O_5 fertilizer. The application of Bio phos granules is labour saving when compared to SSP. With regard to the efficacy of Bio phos granules, it is observed that 1 kg is equivalent to about 2 kg of single super phosphate to supplement the required P_2O_5 and can be an alternative to phosphatic fertilizer. The Cost Benefit Ratio (1:5.01) of the treatment of Bio phos @ 20 kg/acre + 2 sprays of Bio phos liquid @ 2 ml/L of water at two critical stages of crop growth and development compared to the control (1:4.2) is highly encouraging for the economic benefits to the farmer. In onions, among different treatments, a 50% recommended dose of DAP + two foliar applications of Bio phos liquid @ 3 ml/L was found to be significantly superior with regard to average bulb weight, yield per hectare, polar and equatorial diameter of bulb (bulb size) over other treatments with P_2O_5 from other sources of fertilizer (SSP, TSP, and Bio phos granules), followed by the treatment with 50% recommended dose of SSP + two foliar applications of Bio phos (3 ml/L). The highest cost benefit ratio of 1:2.69 was recorded with the treatment of 50% recommended dose of DAP + two foliar applications of Bio phos (3 ml/L) when compared to all the treatments and seems to be encouraging for the benefit to the farmer. Bio phos as foliar spray is effective when applied with other phosphatic fertilizers at half the recommended dose.

The effect of Prathista Industries veterinary feed additives / supplements of milk production in cows / buffalo revealed a significant increase in the milk yield by Cal Lacti powder @ 50 g/d with 12 L/d, followed by NG Cal liquid @ 50 ml/d (11.5 L/d) and CLG tonic liquid @ 100 ml/d (11.6 L/d) compared to control (10.5, 11, and 10.5 L of milk per day, respectively). The FAT and SNF percentage also increased, which helps the farmer economically. Similar trials conducted with farmers in the Venigandla village of Andhra Pradesh indicated an increase in the milk production in the range of 10.30%–15.17% per day compared to control (9 L/d). It was concluded that based on percentage increase of milk yield per

day and cost of feed supplement for the animals per day as per Maximu Retail Price (MRP) rate, feeding lactating animals with extra organic feed supplements containing calcium, phosphorous and electrolytes is cost effective and economically viable.

CHAPTER NINE

EVALUATION OF MIXED INOCULUM OF PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) STRAINS ON PEA AND MAIZE MIXED CROPPING SYSTEM WITH REDUCED DOSES OF CHEMICAL FERTILIZER

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Abstract

In recent years, use of PGPR has become one of the most attractive strategies for the development of sustainable agricultural systems due to their eco-friendly nature, lower consumption of non-renewable resources, and lower costs. In this study we have selected five potential PGPR strains (Bacillus sp. LM4-3, Azospirillus sp. LH12-3, Azotobacter sp. Lx191, Pseudomonas sp. Jm92, and Azospirillus brasilense LHS11) due to their known properties of nitrogen fixation, phosphate solubilization, production of phytohormones such as 3-Indoleacetic acid and antagonistic activity against various pathogens. These properties were previously tested using molybdenum blue spectrophotometry, high performance liquid chromatography (HPLC), acetylene reduction assay (ARA), and dual culture plate assays. Experiments were conducted at the Wuwei Institute

of Agricultural Sciences during 2010 in a field with mixed cropping of pea and maize. A randomized complete block design with a split-plot was used. There were four treatments: 100% NPK, PGPR inoculant +70% NPK, and PGPR inoculant +80% NPK on the basis of the reduction of 20%–30% fertilizer. Our results showed that the PGPR inoculants increased plant height, aboveground biomass, spike length, spike number, grain number, and economic yield of pea and maize. Treatment 3 was the first-grade combination, followed by treatment 2. Compared with the others, treatment 2 had no significant differences, while it increased the plant trait and yield. Based on our results, we conclude that this research is beneficial for the reduction of resource purchasing and could produce the yield of pea and maize at 370–560 RMB per hectare.

Introduction

There are huge areas of poor soil in China, in which the nutrient element is missing, especially nitrogen and phosphorus. To maintain a high yield of crops, more and more fertilizers, herbicides and insecticides are applied to agricultural products, which is the main way to increase the yields. However, in the long term, large amounts of simple chemical fertilizer will destroy the structure of soil and cause degradation of soil fertility and environmental pollution. The promotion of rhizobacteria (PGPR) has been proved to promote plant growth directly or indirectly via the biocontrol of host plant disease, production of phytohomones, or improvement of plant nutritional status (Click 1995). Therefore, the study and development of new fertilizers to replace or partly replace chemical fertilizer to ease the contradiction between supply and demand of fertilizer, and remove the disadvantage on the ecological environment to realize the sustainable development of agriculture and ecological security, are urgently needed.

In the present study, we have selected five potential PGPR strains to evaluate their mixed inoculum on a pea and maize mixed cropping system with reduced doses of chemicals.

Materials and Methods

The research was conducted at the Agricultural Science Research Institute of Wuwei in Gansu Province, China, located at the eastern end of the Hexi corridor, at an average altitude of 1,506 m, having an average annual rainfall of 156 mm, evaporation of 2,400 mm, an annual average temperature of 7.2°C, an annual frost-free period of 156 d, and sunshine hours of 2,945 h. The soils in the experiment were desert irrigation-

warping. The content of organic matter, total nitrogen and phosphorus content in soils were 12.52 g/kg, 0.68 g/kg and 1.41 g/kg, respectively.

The five growth-promoting strains, *Azotobacter* sp. Lx191, *Pseudomonas* sp. Jm92, *Bacillus* sp. LM4-3, *Bacillus* sp.LH12-3 and *Azospirillum* sp. LHS11, for producing inoculums, were previously isolated from the rhizosphere of wheat, maize and oats in arid fields. These stains were proven to stimulate oat growth under controlled conditions via *in vitro* and pot experiments (Yao et al. 2008; Rong et al. 2011). Inoculum was produced by adding mixed strains culture 100 mL in 500 g of sterilized peat especially by injection. The inoculum bags were incubated at 28°C for 10–15 d. The quality of inoculum was determined by the number of viable cells in the inoculated bags by the dilution plate count technique, and stored at room temperature for further use.

MZ-1 variety pea seeds were purchased from the local market, and the germination rate was 85%. The Maize (*zea mays L.*) seed variety Wuke II was provided by the Agricultural Science Research Institute of Wuwei, and the germination rate was 90%. All seeds had no pesticide coatings.

Seeds were dressed with mixed inoculum before sowing. In order to make the bacteria fully adhere to the surface of seeds they were kept in a cool place, away from light, for 2 h. The design of the experiment is presented in Table 9.1 below. Each treatment has three replicates, and random alignments were performed in this experiment. Each area was 3.2 m × 8 m. The inter-cropping of pea and maize was taken for two bands, with each band comprising two lines. The seeding rate, row spacing and plant spacing of peas were 270 kg/ha, 0.4 m and 0.4 m, respectively. For maize, it was 91.5 kg/ha, 4 m and 0.25 m, respectively.

The plant height and yield component of peas were determined. For maize, the plant height, aboveground biomass, economic yield and indexes of each growth period were determined.

Table 9.1. Field experiment design

Treatment		Fertilizer application
Treatment 1	NPK fertilizer only	Pea: urea 60 kg/ha, ammonium phosphorus 150 kg/ha Miaze: urea 300 kg/ha, ammonium phosphorus 247.5 kg/ha
Treatment 2	NPK fertilizer + inoculums	70% NPK fertilizer + inoculum
Treatment 3	NPK fertilizer + inoculums	80% NPK fertilizer + inoculum

Results and Discussion

The effect of inoculum on pea height and yield of inter-cropping of pea and maize at maturation stage is shown in Table 9.4 below. The plant height of maturation stage (PHMS), pob number, particle number and 1,000-grain weight made no significant differences in different treatments. However, the 1,000-grain weight of treatment 3 was higher than the control ($P < 0.05$), showing that 20–30 chemical fertilizer replaced by bio-fertilizer had no effect on the composite factors of pea.

Table 9.2. Effect of inoculum on height and composite factors of peas in the inter-cropping of pea and maize

Treatment	PHMS (mm)	Pob Number	particle number	1,000-grain weight	1,000-grain weight (g)
Control	871.3a	5.90a	4.3a	5.47b	218.13a
PGPR(A)+70%	877.3a	7.00a	4.7a	7.03ab	211.08a
PGPR(A)+80%	881.3a	7.10a	4.8a	7.60a	219.27a

a,b—Means with different letters are significantly different for treatment at $P<0.05$; PHMS—plant height of maturation stage.

Indicators such as plant height, tassel fresh weight, tassel length, tassel diameter and grain numbers per tassel of the mature period were measured. The results (see Table 9.3 below) show that there was a significant difference of tassel fresh weight between treatment 3 and the control. There was no significant difference in the rest of the growing

index among treatments. According to the results, the application of the microbial inoculant can substitute 20%–30% of the chemical fertilizer.

Table 9.3. Effect of inoculum on height and composite factors of maize in the inter-cropping of pea and maize

Treatment	PHMS mm)	Tassel fresh weight (kg/ha)	Tassel length (mm)	Tassel diameter (mm)	Grain number
Control	2145.0a	31370.22b	184.7a	47.9a	565a
PGPR(A)+70%	2114.0a	35356.64ab	189.3a	48.1a	586a
PGPR(A)+80%	2210.0a	35695.35a	192.0a	46.7a	602a

a,b—Means with different letters are significantly different for treatment at $P<0.05$; PHMS—plant height of maturation stage.

The impact of each treatment on the inter-cropping of pea and maize compound colony grain yield was analysed (see Table 9.4 below). The application of microbial inoculants can substitute 20%–30% of the chemical fertilizer, and the yield was stable. Compared with the control, the yield of treatments 2 and 3 increased 11.06% and 17.25%, respectively.

Table 9.4. Effect of inoculum on the grain yield of the inter-cropping of pea and maize

Treatment	**Intercropping grain yield (kg/ha)**
Control	9529.80b
PGPR(A)+70%	10583.40a
PGPR(A)+80%	11173.50a

a,b—Means with different letters are significantly different for treatment at $P<0.05$

In agricultural production, under the premise that it does not destroy the agricultural environment, high-yield and low investment is the ultimate goal of our pursuit. Thus, we carried out an analysis of economic benefits. The reduced cost of chemical fertilizer use per hectare was calculated using the local prices (diammonium phosphorus 50 kg, 150 RMB, urea 40 kg, 100 RMB). The results showed that under the inter-cropping mode of peas and maize, a 20% reduction in chemical fertilizer can save 398.4 RMB, and a 30% reduction can save 579.4 RMB.

The field experiment indicated that compound bio-fertilizer partially replacing chemical fertilizer had an effect on growth parameters of inter-cropping pea and maize, and that 80% NPK fertilizer + inoculum showed the best economic benefits. Numerous studies demonstrate that under similar growing conditions, utilizing bio-fertilizer taking the place of 20%–30% fertilizer can increase plant height, aboveground biomass and economic yield.

Regarding economic efficiency, pea and maize can save costs, and at the same time, in terms of ecological efficiency, partial application of bio-fertilizer instead of chemical fertilizer not only does not reduce crop production, but increases it to a certain extent. The most important point is that the long-term application of biofertilizer can improve soil conditions, enhancing utilization efficiency of fertilizer and reducing the adverse effect of chemical fertilizers, protecting the ecological environment.

Acknowledgement

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CHAPTER TEN

CELLULOLYTIC BACTERIA: ANTAGONISTS PATHOGENIC FUNGI CAUSING ROOT ROT OF SUGAR BEET

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Abstract

From the rhizosphere and rizoplana of the healthy sugar beet culture, 43 cellulolytic bacteria were isolated. Twelve strains that have expressed antagonism against phytopathogenic fungi were selected. Selected strains actively suppress the growth and development of pathogenic fungi of the genus *Fusarium* causing root rot, and seedling of sugar beet was installed. This study is on the growth promoting activity of two strains of cellulolytic bacteria 604 and 60(5)4, characterized by both a high antifungal activity and the growth promotion of sugar beet. Field tests have shown the great potential of using these strains. Inoculation seeds of the cellulolytic bacteria infestation of fusariosus seedling decreased by 2.0–2.4 times, and the prevalence of root crops is 2.5–3.0 times the control. Using bacteria significantly increased the resistance of plants to disease and increase of sugar beet yield amounted to 30–34 c/ha. On the basis of these strains, it is possible to develop biopreparations for the protection and stimulation of the growth of sugar beet.

Introduction

At present, in the Republic of Kazakhstan there is an acute problem in the improvement of the environmental situation and, in this regard, much attention is paid to agricultural biosafety**.** One of the most valuable commercial crops is sugar beet. At the same time, the sugar industry of Kazakhstan is working on imported raw materials, and a large number of finished product is imported into the country. Therefore, the government plans to expand the area under the crop and increase its production in the Republic. Lack of resources and simplified technology along with the phytosanitary conditions have deteriorated arable land, and change in the economic structure of the country has resulted in technological violations of sugar beet. Especially noticeable is a loss of the influence of biotic stress factors on irrigated lands where sugar beets are grown. For example, in 2010 the sugar beet crop losses from diseases caused by phytopathogenic microorganisms reached 30%. Regarding sugar beet in Kazakhstan, more than 20 species of pathogens have been recorded, the most common and most dangerous disease of root crops being caused by fungi of the genus *Fusarium* (Dzhanuzakov & Agataev 2001; Maui 2003; Abugaliev & Kostin 2007). According to several authors, this has reduced the root rot of the sugar beet harvest by 50% or more (Kazenas 2005; Agataev 2002). Therefore, the protection of plants against pathogens can improve the efficiency and stability of sugar beet.

The use of plant protection chemicals has several negative consequences, such as the formation of resistant pathogen strains, reducing the number of beneficial microorganisms in the microbial, and accumulation of toxic substances in the soil (Reddy et al. 2009). An alternative approach involves the creation of biological methods of plant protection from diseases through using biological products. The basis of such biological products is highly antagonistic strains of bacteria pathogens (Novikova 2003; Labutova & Suo et al. 2011).

In this respect, the use of cellulolytic bacteria with physiological and biochemical properties (high growth rate, simplicity to power sources, easy cultivation, etc.), providing them with high technology for the production of biomass, is promising. In addition, due to their biological characteristics, such as population stability and the ability to produce antifungal metabolites, they are active antagonists of fungi. In this regard, some of them may present antagonist activity against root rot causing pathogenic fungi.

The objective of this study was the selection of the rhizosphere and rizoplana healthy sugar beet cellulolytic bacteria as antagonists and growth

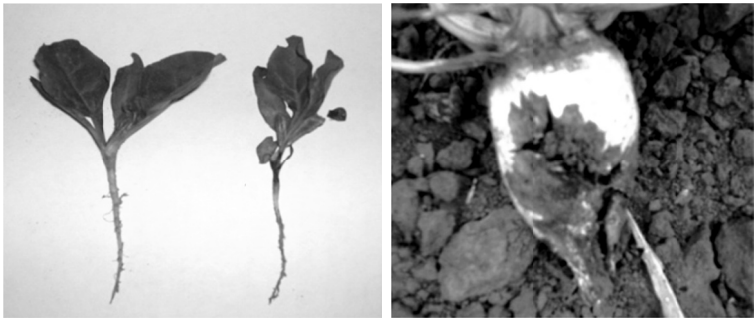
promoters and the selection of the most active strains in order to base biopreparations for plant protection against root rot on them.

Materials and Methods

Rhizosphere and rizoplana healthy plants of sugar beet from the Almaty region of Kazakhstan cellulolytic bacteria have been isolated and selected. The isolated cellulolytic bacteria from various natural substrates contain cellulose such as dead roots, leafs, bits of stem and soil. The pure cultures of cellulolytic bacteria were selected on the Hutchinson medium with sources of carbon native cellulose, such as wheat straw.

Phytopathogenic test cultures using fungi *Fusarium: Fusarium oxysporum M2* and three strains of *F. solani*: *F. solani (ros.), F. solani M3, F. solani M4* were isolated from diseased seedlings and sugar beet in 2010–11 in the Almaty and Zhambyl regions of Kazakhstan and identified by the authors (see Fig. 10. 1 below).

Cultivation of bacteria was carried out on the liquid and solid medium Hutchinson. Wheat straw was used as a source of cellulose. Fungi was grown on Czapek medium-7 and Saburo.



Healthy seedling diseased seedling root rot of root crops

Fig. 10. 1. The defeat of the roots of seedlings and root crops of sugar beet by root rot

A test culture was inoculated with pathogenic fungi melted and cooled to 40°C and poured into Petri dishes. After solidification of the agar medium it was cut into blocks. Cell suspension of bacteria was carried out in flooded wells, received at 1×10^6 cells/ml per 0.2 ml per well. The plates were incubated at 28 °C–30 °C in an incubator for 10 d or more. The results considered the largest areas of growth suppression in the test cultures.

The growth-promoting activity of bacterial strains were grown on medium Hutchinson for 5–7 d. Sugar beet seeds were inoculated with bacterial suspension at 1×10^6 cells per 1 g of seeds for 6–8 h at room temperature before planting. In the control the seeds were soaked in sterile water. After 7 d germination was determined, and after 10 d measurement of the aerial parts and roots was carried out. All results were statistically processed (Rokitsky 2007).

Results and Discussion

From the rhizosphere and rizoplana healthy sugar beet plants, 43 cultures of cellulolytic bacteria belonging to the genera *Cellulomonas*, *Bacillus*, *Brevibacterium* and *Flavobacterium* were isolated. From them, 18 strains were selected with antagonism against phytopathogenic fungi of the genus *Fusarium*, causing root rot of sugar beet. In a detailed study, 12 strains with increased antifungal activity were selected. Further work was done with these strains.

Data for the study of cellulolytic bacteria antagonistic activity against phytopathogenic fungi causing root rot of sugar beet is presented in Table 10.1 below.

Table 10.1. The antifungal activity of cellulolytic bacteria

Bacterial strains	The average diameter of fungal growth suppression zones, mm			
	F. oxysporum M2	*F. solani (ros.)*	*F. solani M3*	*F. solani M4*
21(8)	26 ± 1.3	25 ± 1.3	i.g.	20 ± 0.8
21N	0	15 ± 0.7	0	0
22T	27 ± 1.1	25 ± 1.2	20 ± 0.8	i.g.
60(5)4	27 ± 0.8	28 ± 0.6	i.g.	16 ± 0.5
80	0	23 ± 1.0	i.m.	i.m.
82	34 ± 2.1	27 ± 1.4	22 ± 1.0	i.g.
95	0	i.m.	i.m.	0
150	i.m.	i.m.	i.m.	18 ± 0.7
158	12 ± 0.3	i.m.	i.m.	20 ± 0.8
177	25 ± 0.5	i.g.	i.g.	22 ± 1.1
212(м)	12 ± 0.2	i.g.	0	0
604	28 ± 1.5	i.g.	i.g.	28 ± 1.6

Note: i.m.—complete suppression of formation of aerial mycelium throughout Petri dishes; i.g. —complete suppression of fungal growth throughout Petri dishes

From Table 10.1 it follows that all investigated strains have antifungal activity against fungal test organisms. The activity varies greatly depending on the strain of bacteria and fungal phytopathogen investigated. The antagonistic activity of bacteria against phytopathogenic fungi is manifested as a fungicidal or fungistatic activity. Fungistatic activity was expressed in the partial or complete suppression of the formation of aerial mycelium and sporulation of fungi or rarefaction growth in all Petri dishes, with fungicide as a complete or partial inhibition of fungal growth (Fig. 10.2). A number of variants also demonstrated discoloured pigmentation fungi.

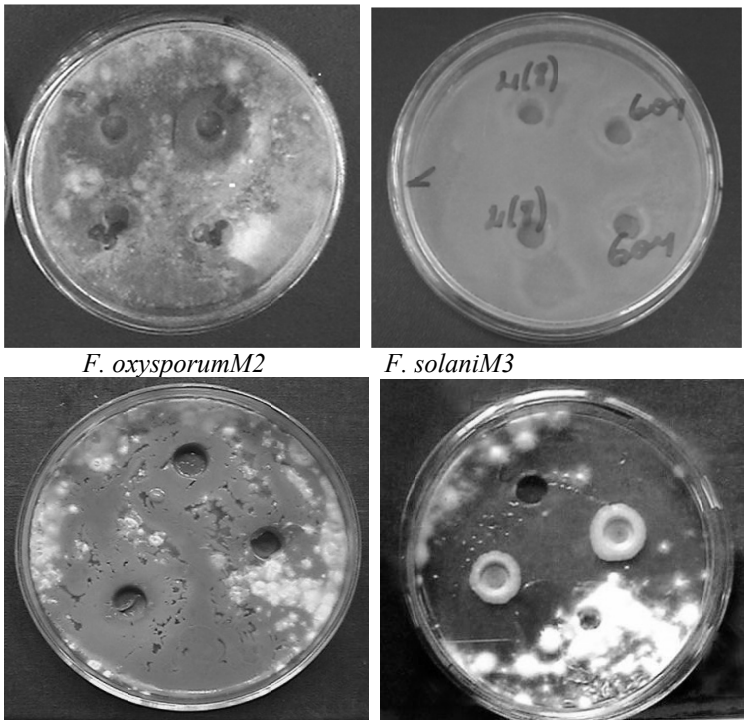


Fig. 10.2. Effect of strains of cellulolytic bacteria on phytopathogenic fungi of the genus *Fusarium*

It was established that 8 of the 12 cultures of cellulolytic bacteria strains inhibited the growth and development of the fungus *F. oxysporum M2*, and 3 strains did not have an antagonistic effect against this fungus.

All investigated strains possessed antifungal activity against fungus *F. solani (ros.)*. It was also shown that almost all the strains of bacteria inhibited the growth of pathogenic fungi *F. solani M3* and *F. solani M4*, with the exception of the three strains 21N, 212 (m) and 95 had no effect on these phytopathogens. According to the results obtained by the studies, the six strains were characterized by a high antagonistic activity to test cultures of pathogenic fungi: 21 (8), 22T, 82, 177 and 604. The study of the growth-promoting ability of selected strains of bacteria is presented in Table 10.2.

Table 10.2. Effect of cellulolytic bacteria strains on the germination and growth of sugar beet

Strains of the bacteria	Length of the stem, sm (Cm)	Root length, (Cm)	Germination (%)
Control	12.8 ± 1.2	8.8 ± 0.2	82.6 ± 2.4
21(8)	17.1 ± 1.3	10.2 ± 1.0	89.7 ± 2.3
22T	15.1 ± 1.2	9.7 ± 0.9	85.6 ± 2.2
60(5)4	18.3 ± 1.4	15.9 ± 1.1	89.9 ± 2.7
82	16.1 ± 1.0	14.2 ± 1.1	86.9 ± 2.1
177	14.1 ± 1.3	11.2 ± 0.9	89.1 ± 2.4
604	19.1 ± 1.5	17.9 ± 1.1	92.7 ± 2.6

On the received data, two strains of cellulolytic bacteria, 604 and 60 (5) 4, characterized by both a high antifungal activity and the ability for growth promotion of sugar beet, were selected. The effectiveness of the selected strains in the field were tested jointly with the Kazakh Scientific Research Institute of Plant Protection and Quarantine Ministry of Agriculture of the Republic of Kazakhstan. Seeds of sugar beet "Yaptushkovskaya 30," recommended for cultivation in the Almaty region of Kazakhstan, inoculated bacteria suspension and were plated in the fields. Standard seeds were treated with chemicals TMTD and Scor registered in Kazakhstan, serving as a control. The results are presented in Table 10.3 below.

Table 10.3. Effect of inoculation of sugar beet seeds to cellulolytic bacteria on germination, seedling infestation and yield

Variants	Density of germination, pieces/m^2	Disease, %		Yields, c/ha
		black leg	fusariusus root rot	
604	74.0	17.2	7.1	304.0
60(5)4	73,1	17.8	8.3	300.0
TMTD, 80% (6.0 kg/t)+ Scor (0.4 l/ha) (standard)	72.7	18.5	10.0	280.0
Control	68.9	39.0	21.0	270.0

The results of the field research show the high potential of cellulolytic bacteria to process sugar beet seeds. In the experiments, where the seeds were inoculated with cellulolytic bacteria, infestation of fusariosus root rot was 2.0–2.4 times lower than in the controls, and root crop disease decreased by 2.1–3.1 times as compared with the control. Using bacteria significantly increased the resistance of plants to disease, and sugar beet yield increase amounted to 30–34 c/ha.

Thus, the selected strains of cellulolytic bacteria 604 and 60(5)4 have antagonistic and growth-promoting activity and can be recommended for use in agriculture to protect products from sugar beet root rot. Further field testing should be conducted to refine the regulations of the strains (concentration, flow rate, etc.), biological activity and economic efficiency for the development of biopreparations based on them.

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PART III.

BIOACTIVE METABOLITES OF PGPR AND OTHER MICROBIAL INTERACTIONS IN THE RHIZOSPHERE

CHAPTER ELEVEN

SEARCH FOR 2, 4 DAPG POSITIVE GENE IN FLUORESCENT *PSEUDOMONAS* AND THEIR EXPLOITATION FOR SUSTAINABLE DISEASE MANAGEMENT

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Abstract

The plant-growth promoting rhizobacterias (PGPR) fluorescent *Pseudomonas* are known to produce an array of antibiotics such as phenazine, 2, 4-diacetyl phloro glucinol, pyoluteorin, and salicylic acid etc., capable of suppressing a broad spectrum of plant pathogens. Among them, 2, 4 DAPG is a major determinant in bio-control activity. This chapter is on the search for the presence of the 2, 4 DAPG gene among the isolates of fluorescent *Pseudomonas*, its formulations, compatibility with other pesticides and plant products, and utility as an integral component in the sustainable management of crop diseases. A total of 192 isolates collected from rhizospheres, including endophytic and phylloplane, were tested for bio-control efficacy. The isolates were further screened for molecular detection of the 2, 4 DAPG gene (phl gene) by PCR analysis using phl 2a and phl 2b primers. A total of eight isolates were identified as 2, 4 DAPG positive on the basis of their sequence analysis of 16S rDNA. Among the carrier materials, talc was best suited for both *P. putida* isolates with 11×10^7 cfu/g up to 300 d of storage. Among pesticides, carbendazim, hexaconazole and propiconazole showed compatibility, whereas indoxycarb

and novaluron were incompatible. The consortium of *P. fluorescens* and *Trichoderma viride* was found to be superior against pre-emergence damping off, powdery mildew, fruit rot, and wilt/root of chilli crop in addition to higher yield. The formulation was used for IPM demonstration for management of chilli diseases in 25 acres of farm fields for three years by the judicious use of pesticides, bioagents, botanicals and other IPM interventions. The average incidence of leaf spot, powdery mildew, root rot, wilt, fruit rot and aflatoxin contamination came down significantly. The number of pesticide sprays was brought down significantly with an overall average yield of 31.26 q/ha in IPM plot and 24.6 q/ha in a non-IPM plot, fetching a net gain of 54662 Rs/ha and preventing untold ecological damage. One spray of mancozeb followed by a *P. fluorescence* spray brought down the incidence of Alternaria blight of sesame, recording a yield of 7.81 q/ha.

Introduction

The plant growth promoting rhizobacteria (PGPR) promote plant growth by synthesizing growth stimulating plant hormones, inducing systemic resistance and suppressing pathogenic microorganisms. Fluorescent pseudomonads are known to produce an array of secondary metabolites, such as phenazine, 2, 4-diacetylphloroglucinol (2, 4 DAPG), pyoluteorin, hydrogen cyanide, and salicylic acid, etc., which make fluorescent pseudomonads target a broad spectrum of plant pathogens such as fungi, bacteria and nematodes (Borauh & Kumar 2002). The 2, 4-DAPG is the major determinant factor in the biological control of a wide range of pathogens which have been intensively studied. This antibiotic is a phenolic compound with a broad spectrum of toxicity against fungi, bacteria, nematodes, viruses and insects. *Phl* A, B, C, D genes are responsible for the production of 2, 4-DAPG in *Pseudomonas fluorescens* (Abbas et al. 2002). Hence, the molecular detection of the fluorescent *Pseudomonas* gene would go a long way in catering to the needs of broad-based plant protection. Once the potential PGPR possessing 2, 4-DAPG gene has been identified, the formulation plays a crucial role for devising biocontrol agents through seed treatment, seedling dip and foliar application. It is well known that the consortia of bioagents play an important role as opposed to a single bioagent, since this will not perform well at all times and in all kinds of environment. The strain mixtures permit a broad spectrum of action and enhanced efficacy, and may allow for a combination of various strains without genetic engineering. Therefore, the present study involving the molecular detection of the 2, 4-

DAPG gene, formulation, compatibility and exploitation in integrated pest management (IPM) has been undertaken.

Materials And Methods

Molecular detection of the 2, 4 DAPG gene among fluorescent *Pseudomonas*

Fluorescent *Pseudomonas* isolates collected from rhizospheres and phylloplanes of wheat, chickpea, maize, safflower, sunflower, cotton, onion, pigeon pea, chilli, aubergine, paddy, groundnut, beans and tomato crops were isolated on King's B agar by the serial dilution technique (Zinniel et al. 2002). For genomic DNA isolation of fluorescent *Pseudomonas* the kit method was followed and PCR amplification was done using *phl* 2a and *phl* 2b primers (Velusamy et al. 2006). The 2, 4-DAPG compound was isolated (Rosales et al. 1995) and 16S rDNA amplification was done using universal primer fD1 and Rp2, and the purified PCR product was sent for sequence analysis.

Formulation, shelf life and compatibility of fluorescent *Pseudomonas*

In order to determine the efficient formulation and shelf life of fluorescent *Pseudomonas*, talc, vermicompost, farmyard manure and King's B broth were used (Sivakumar et al. 2000). The compatibility of RPF-13 with fungides carbendazim, captan, hexaconazole and propiconazole, insecticides chlorpyriphos, carbofuran, indoxycarb, novaluron and imidacloprid, and plant extracts NSKE (Neem Seed Kernel Extracts), nimbicidine, pongamia, eucalyptus and *Prosofis juliflora* leaf extracts was tested at different concentrations with the poisoned food technique (Khan & Gangopadhyaya 2008). Different methods of application, such as soil, seed treatment, seedling dip and foliar spray of the bioagent, were tested on chilli with a talc-based formulation of PF4, RPF-13 and a consortium of PF4 and *Trichoderma viride* against the major diseases of chilli. The trial was also conducted on the management of the *Alternaria* blight of sesame.

Integration of PGPR in IPM demonstration

In order to integrate PGPR in IPM demonstration, a large-scale trial was conducted in farm fields for three consecutive seasons. The farmers'

beneficiaries were provided with various IPM inputs, including PGPR. The team of plant pathologists and entomologists supervised the demonstration in weekly visit and intervened with PGPR and other IPM inputs, bioagents, fungicides, pesticides and plant products. The observation on the incidence of disease was documented and the yield obtained in the IPM demonstration trial was compared with that of non-IPM practice.

Results and Discussion

Molecular detection of the 2, 4 DAPG gene in fluorescent *Pseudomonas*

Molecular detection of the *Phl* gene in potential isolates of fluorescent Pseudomonads was done by PCR analysis using primers, *Phl* 2a and *Phl* 2b. A total of eight isolates of fluorescent Pseudomonads were identified as 2, 4-DAPG positive, in which seven were isolated from crop rhizosphere and one isolate was endophytic in nature (see Fig. 11.1 below).

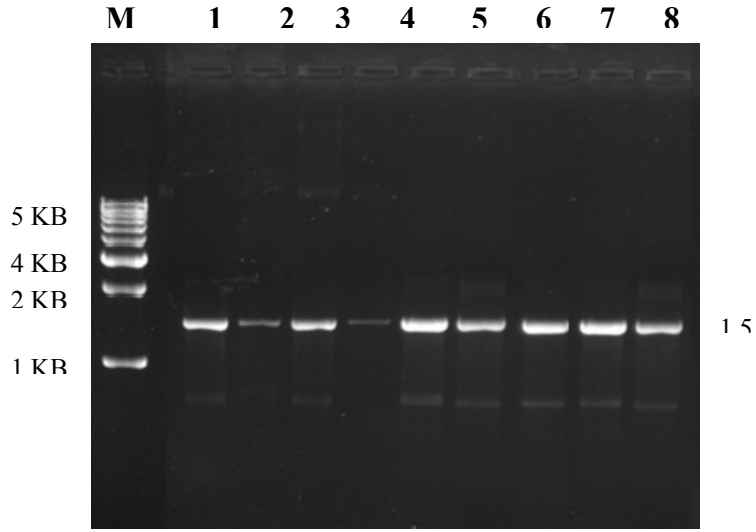

Fig. 11.1. 16s rDNA amplified 2, 4-DAPG gene of fluorescent Pseudomonads isolates

The 2, 4-DAPG producing fluorescent *Pseudomonas spp*. is the key component of the natural biological control of plant pathogens with a very broad-spectrum antagonistic activity against phytopathogens. Of the eight isolates, five are identified to be *Pseudomonas putida* along with one each of *Pseudomonas fluorescens* and two of *Pseudomonas aeroginosa.* There is a good chance of utilizing *P. putida* for biocontrol and PGPR. However, use of *Pseudomonas aeroginosa* will have limitations considering its association with human health hazards. Velusamy et al. (2006) reported a sub population of 27 strains of plant associated *Pseudomonas fluorescens* producing 2, 4-DAPG screened in a batch of 278 strains. Among the five 2, 4-DAPG^{+ve} isolates identified through 16S rDNA partial sequence analysis, two isolates were *Pseudomonas putida*—RP49 and RP27 isolated from pigeon pea rhizosphere—while another was *Pseudomonas aeroginosa*, i.e. RP3 from Sorghum rhizosphere.

Formulation and shelf life of fluorescent *Pseudomonas*

Development of the efficient formulation of potential native isolates of fluorescent *Pseudomonads* with a longer shelf life is indeed the pre-requisite for the management of plant diseases through biological means. The results indicate that for *P. putida*, talc was the best since it showed a uniform trend in maintaining the populations over the entire period of storage as compared to other substrates with 11×10^7 cfu/g (see Fig. 11.2 below).

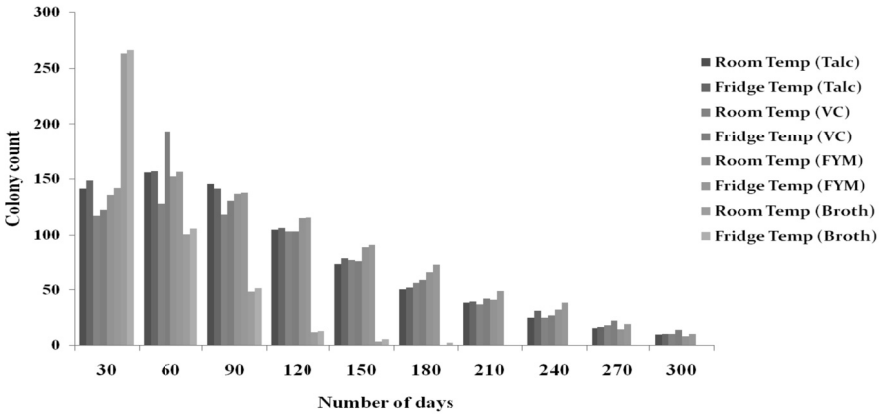

Fig 11.2. Effect of carrier materials/substrates and storage temperature on populations of *Ps. putida* (RPF-13) [cfu/g 1×10^7]

However, in the present investigation King's B broth recorded the highest mean populations of *P. putida* with 264.50×10^7 cfu/ml at 30 d of storage, but after a short period thereafter it declined very rapidly and lasted for 150 d at room and refrigerator temperatures. Vermicompost and FYM could encourage better populations of *P. putida* initially because of their physical properties, such as organic matter and moisture capacity, but could also maintain this until the end of the storage period of 300 d. In addition, their quality parameters differ with location as per the availability of raw materials and their composition, like organic carbon, method of preparation and their storage conditions. These reasons influence their limited use as carrier materials on a commercial basis for marketability.

Compatibility of fluorescent *Pseudomonas*

The results indicate the compatibility of *P. putida* with carbendazim, hexaconazole and propiconazole at both 0.1% and 0.2% concentrations. No colonies of *P. putida* were recovered with mancozeb and captan, revealing incompatibility. A combination of carbendazim with *P. putida* isolate is ideal for seed treatment. Considering the reduction of bioagent population in combination with mancozeb, an increased dosage of bioagent in seed treatment would be more helpful in suppression of plant diseases and thus limitation of incompatibility can be minimized. Laha & Venkataraman (2001) noted the compatibility of *P. fluorescens* with carbendazim while studying sheath blight in rice. *P. putida* was compatible with chlorpyriphos, carbofuran and imidachloprid at both the concentrations. Indoxacarb and novaluron were found to be incompatible with *P. putida* at both the concentrations in which no colonies were recovered. *P. putida* could give 70.00×10^{10} and 36.33×10^{10} cfu with carbofuran, resulting in 26.32% and 58.95% reductions over control at 0.1% and 0.2%, respectively, revealing a good compatibility at 0.1% (see Fig. 11.3 below).

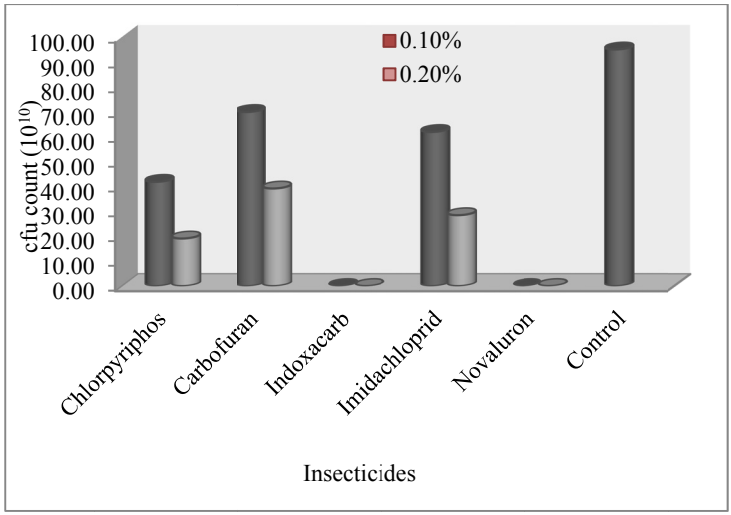

Fig. 11.3. Compatibility of *Ps. putida* (RPF-13) with insecticides

Combined application of *P. fluorescens* and carbofuran 3G significantly improved plant growth (Senthilkumar & Ramakrishnan 2004). Kumar et al. (2008) reported the compatibility of *P. fluorescens* with imidachloprid and carbofuran. The compatibility of *P. putida* was tested with plant extracts at 2.5% and 5.0% concentrations. *P. putida* showed incompatibility with nimbicidine and eucalyptus leaf extract at both the concentrations since no colonies were recovered. The results are highly encouraging especially with NSKE, which is compatible even at 5% concentration. NSKE, already a major component in integrated pest management (IPM). It is likely that a combination of NSKE and fluorescent *pseudomonads* will result in a better total crop protection against both plant diseases and insect pests.

Application of PGPR

Seed treatment of chilli with a consortium of *P. fluorescens* and *Trichoderma viride* recorded the highest germination and vigour index of 92.50% and 1601.71%, respectively, followed by *Pseudomonas putida* with 89.75% germination and 1423.78% vigour index. Among different treatments for the management of chilli diseases, a consortium of *P. fluorescens* and *T. viride* recorded the least pre- and post-emergence damping off, wilt/root rot incidence of 4.25% and 8.50% and 8.66%,

respectively, followed by *Pseudomonas fluorescens* (7.50% and 14.00%) for damping off and *Pseudomonas putida* (10.42%) for wilt/root rot incidence. In contrast, the chemical control recorded the minimum PDI for powdery mildew (14.78%) and fruit rot (14.68%), followed by a consortium of *Pseudomonas fluorescens* and *Trichoderma viride* with a PDI of 22.23% and 22.33% for powdery mildew and fruit rot, respectively. Among the different treatments, the maximum dry chilli yield was recorded by chemical control followed by a consortium of *Pseudomonas fluorescens* and *Trichoderma viride*.

Integration of PGPR in IPM demonstration

An observation on the incidence of disease and yield was recorded. The results revealed that the incidence of leaf spot was in the range of 13.47% to 29.71%, powdery mildew was from 31.33% to 42.72%, root rot/wilt was 4.83% to 14.45%, fruit rot was 10.15% to 29.76%, and aflatoxin contamination was 2% in IPM plots as against 15.17% to 32.71% leaf spot, 33.83% to 45.66% of powdery mildew, 6.74% to 20.75% of root rot/wilt, 13.80% to 31.48% of fruit rot, and 7% of aflatoxin contamination in non-IPM plots (see Table 11.1 below). The damage caused by insect pests, such as sucking pests and defoliators, also came down significantly. The incidence of disease came down and the number of sprays required for protection was significantly reduced by 30%, reducing the cost of protection. An average yield obtained in an IPM plot was 31.5 q/ha against 24.6 q/ha in non-IPM plots, aside from the reduction in cost of protection.

Table 11.1 Incidence of leaf spot, powdery mildew, root rot/wilt, fruit rot, and aflatoxin diseases in PGPR-mediated IPM and non-IPM plots for three seasons

Diseases	Percentage disease index (PDI)	
	IPM plots	**Non-IPM plots**
Leaf spot	13.47%–29.71%	15.17%–32.71%
Powdery mildew	31.33%–42.72%	33.83%–45.66%
Root rot/wilt	4.83%–14.45%	6.74%–20.75%
Fruit rot	10.15%–29.76%	13.80%–31.48%
Aflatoxin	2.0%	7.0%

In other words, the IPM plot gained a net profit of Rs. 54662/ha, as compared to non-IPM plots (see Table 11.2 below), indicating the involvement of PGPR as a foliar spray and seed treatment being an

integral component in IPM demonstration which helped in reducing pesticide sprays in chilli ecosystems, thus making it a green approach for sustainable and profitable disease management.

Table 11.2. Economics of chilli IPM and Non-IPM plots in three years 2008–2011

Location	Dry chilli yield* (qt/ha)	Total income (Rs/ha)	Cost of cultivation (Rs/ha)		Total cost (Rs/ha)	Net Profit (Rs/ha)
			Production cost	Protection Cost		
Nelahal						
IPM plots	31.26	236797	20950/-	12604/-	33554/-	203243/- ($ 4418)
Non-IPM plots	24.6	186941	18950/-	16410/-	38360/-	148581/- ($ 3230)

The best-performing fungicide, bioagent and plant extracts were integrated and evaluated for the *Alternaria* blight of sesame also under field conditions. Among the various treatments, one spray of mancozeb (0.2%) followed by one spray of *Pseudomonas fluorescens* at 35 and 55 d after planting resulted in Alternaria blight incidence of 21.66% with a yield of 7.87 q/ha as against check with a 59.39% incidence on a yield of 3.87 q/ha (see Table 11.3 below).

Many of the green revolution technologies in Asian countries have resulted in quantum leaps in crop yields. However, the achievement is at the cost of the social and ecological foundation of society. It is visualized that the use of PGPR as an integral component in crop protection technology would go a long way in realizing a millennial developmental goal.

Table 11.3. Integrated management of the *Alternaria* blight of sesame under field conditions Kharif 2009–10 and 2010–11

Treatment	Percentage disease index 2009–11			Yield (Q/ha)	B:C ratio
	Before Spray	After I Spray	After II Spray		
Two sprays of Mancozeb (0.2%)	10.00 (18.43)	12.88 (21.03)	19.67 (26.31)	7.81	3.75
Two sprays of *P. fluorescens* (0.5%)	9.78 (18.20)	13.44 (21.50)	22.22 (28.12)	7.03	3.51
Two sprays of *Prosopis juliflora* (5%)	10.55 (18.95)	14.55 (22.42)	29.77 (33.05)	5.64	2.84
One spray each of Mancozeb followed by *P. fluorescens*	11.11 (19.46)	13.11 (21.22)	21.66 (27.74)	7.87	3.86
One spray each of Mancozeb followed by *P. juliflora*	10.77 (19.16)	17.00 (24.34)	26.85 (31.16)	5.45	2.67
One spray of *P. fluorescens* followed by *P. juliflora*	11.44 (19.77)	14.44 (22.32)	31.59 (34.18)	4.66	2.33
Control	11.00 (19.37)	25.89 (30.58)	59.37 (50.41)	3.87	
SE m ±	0.31	0.47	0.81	0.16	
C.D. (5%)	0.96	0.66	2.50	0.49	

Acknowledgements

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CHAPTER TWELVE

ISOLATION, PARTIAL CHARACTERIZATION AND IDENTIFICATION OF RHIZOBACTERIA FOR GROWTH PROMOTION OF CORN (*ZEA MAYS L.*) AND TOMATO (*SOLANUM LYCOPERSICUM L.*) FROM SELECTED SITES IN THE PHILIPPINES

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Abstract

A total of 150 bacterial strains were isolated from rhizospheric soils in various parts of the Philippines. These were analyzed for nitrogen-fixing activity, phosphorus-solubilizing activity, indole-3-acetic acid (IAA), and gibberellic acid (GA) production. Results showed that out of 150 isolates, there were 43 nitrogen-fixers, three phosphorus-solubilizers, 39 GA producers, and 10 IAA producers. Four isolates (GTC-P4, AAP-GD4, GTC-LGDsf5 and GTC-LGDtA2) produced high amounts of IAA, ranging from 48.39 to 133.25 µg/ml, and these were selected for further analysis. These isolates were subjected to morphological and biochemical characterization, molecular identification and *in vivo* assay to test their effect on the growth of corn and tomato. AAP-GD4, GTC-LGDsf5 and GTC-LGDtA2 were closely identified as *Bacillus pumilus*, *Bacillus* sp., and *Bacillus cereus*, respectively. Biochemical and molecular procedures failed to identify GTC-P4, suggesting that it is a possible novel bacterium. GTC-LGDtA2 and GTC-LGDsf5 gave the highest values in the different

growth parameters (shoot height, root length and plant biomass) of corn and tomato, regardless of the level of inorganic fertilizer used. Other microbial inoculants, AAP-GD4 and GTC-P4, also gave statistically significant increases in different growth parameters of corn and tomato. Further characterization and field testing of plant growth promoting rhizobacteria (PGPR) are highly recommended to ensure the direct effects on corn and tomato production.

Introduction

Biofertilizers contain living microorganisms known to provide plants with healthy rhizospheres needed to ensure optimum plant growth (Mishra & Dadhich 2010). Generally, microorganisms used in biofertilizer production fall under plant-growth-promoting rhizobacteria (PGPR). Growth promotion is achieved through various mechanisms such as nitrogen fixation, solubilization of phosphorus, secretion of growth hormones, and others. The abundant presence of PGPR in soil leads to enhanced root growth, branching and/or root hair development, increased plant hormone concentrations, symbiotic nitrogen fixation, competitive suppression of pathogens, and/or solubilization of minerals (Shaukat et al. 2006; Richardson et al. 2009).

Application of biofertilizers ensures the sustenance of soil quality and agricultural productivity. Long-term use is seen to be more cost-effective, productive, eco-friendly and accessible to marginal and small-scale farmers compared to chemical fertilizers (Mahdi et al. 2010; Mishra & Dadhich 2010). The main aim of this chapter is to isolate and identify new microbial isolates with PGPR properties from soils and organic materials of the Philippines, then screen for their effectiveness for crop growth promotion, specifically corn and tomato production.

Materials and Methods

Microbial isolation from rhizospheric soil samples

Rhizobacteria were isolated from soil samples obtained from different parts of the Philippines and from various host plants, such as fern, bamboo, grass, corn, sugarcane, legume, sunflower and cogon grass (*talahib*).

Isolated bacteria were labelled based on their sources, medium of isolation and properties. Soil samples used in bacterial isolation were the following:

Sample A: Mindanao Mine Site
Sample B: Baguio Mine Site
Sample C: Bohol Bokashi 1
Sample D: Bohol Bokashi 2
Sample E: Fern Rhizosphere (U.P. Land Grant)
Sample F: Bamboo Rhizosphere (U.P. Land Grant)
Sample G: Grass Rhizosphere (U.P. Land Grant)
Sample H: Corn Rhizosphere (U.P. Land Grant)
Sample I: Sugarcane Rhizosphere (U.P. Land Grant)
Sample J: Legume Rhizosphere (U.P. Land Grant)
Sample K: Sunflower Rhizosphere (Batangas)
Sample L: Cogon grass Rhizosphere (Batangas)
Sample M: Sugarcane Rhizosphere (Batangas)
Sample N: Mango Rhizosphere (Batangas)
Sample O: Grass Rhizosphere (Isabela).

Isolation of nitrogen-fixing bacteria

Isolation was done using Dobereiner's medium (5g L-1 DL Malic acid; 4g L-1 Potassium Hydroxide [KOH]; 0.5g L-1 Yeast extract; 1ml L-1 10% NH_4Cl; 3ml L-1 Bromthymol blue; 0.1ml L-1 10% $MnSO_4 \cdot 7H_2O$; 0.5ml L-1 10% $MgSO_4 \cdot 7H_2O$; 0.2ml L-1 10% NaCl; 0.2ml L-1 10% K_2HPO_4; 0.1ml L-1 10% $CaCl_2$; 0.5ml L-1 10% $FeSO_4 \cdot 7H_2O$; and 15g L-1 Agar). After 3 d of incubation, colonies that grew were observed to have nitrogen-fixation activity, indicated by the change in medium colour. Single colonies were isolated and restreaked in the same media for confirmation.

Isolation of phosphorus solubilizing bacteria

Isolation was done using Pikovskaya medium (10g L-1 Glucose; 5g L-1 $Ca_3(PO4)_2$; 0.5g L-1 KCl; 0.2g L-1 $MgSO_4$; trace amounts of $MnSO_4$ and $FeSO_4$; 0.5g L-1 Yeast extract; and 15g L-1 Agar). After 3 d of incubation, colonies that grew and were observed to have a clearing zone exhibited phosphorus-solubilizing activity.

Auxin or indole acetic acid (IAA) production assay

Quantitative analysis of IAA was performed spectrophotometrically. Bacterial isolates were grown in 5 ml MS broth (2g L^{-1} glucose; 2g L^{-1} sucrose; 1g L^{-1} peptone; 1g L^{-1} Yeast extract; 0.5g L^{-1} K_2HPO_4; 0.1g L^{-1}

NaCl; 0.2g L^{-1} $MgSO_4$; 0.5g L^{-1} $(NH_4)2SO_4$; 0.02g L^{-1} $CaCl_2$; 0.01g L^{-1} $FeCl_3$; and 0.002g L^{-1} Na_2MoO_4), with and without 5,000 ppm L-Tryptophan (Merzaeva & Shirokikh 2010). Bacterial cultures were incubated in a rotary shaker for 48 h at room temperature. After shaking, 2 ml of the bacterial culture was obtained and centrifuged at 14,000 rpm for 12 min. The pellet was then discarded.

The IAA content was determined using the modified Salkowski reagent (Add 1 ml O.5 M ferric chloride to 50 ml 35% [w/w] Perchloric acid). Then, 2 ml of the Salkowski reagent was added to 1 ml supernatant. The solution was allowed to stand for 30 min before reading its absorbance at 530 nm using a UV-VIS Spectrophotometer. Commercially prepared IAA dissolved in water was used as a standard and a calibration curve with a range of 0 to 500 µg/ml was prepared (see Fig. 12.1 below).

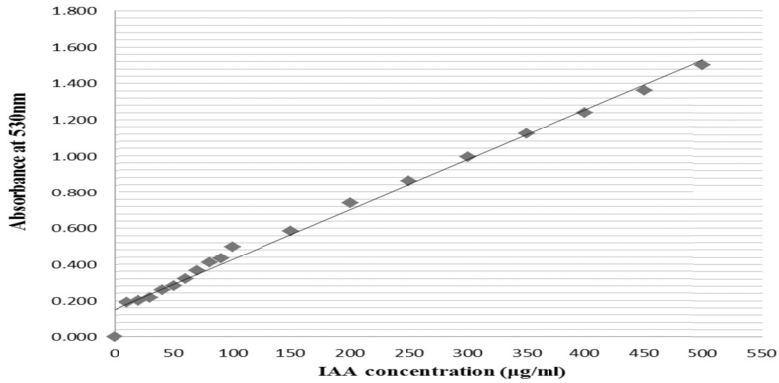

Fig. 12.1. Calibration curve of indole acetic acid

Gibberellic acid (GA) production assay

Bacterial isolates grown in a 5 ml nitrogen-free medium were incubated in a rotary shaker for 48 h at room temperature. After shaking, 2 ml of the bacterial culture was obtained and centrifuged at 14,000 rpm for 12 min. The pellet was then discarded. The presence of GA-like substances in the supernatant was determined by adding 1 ml of 2, 4-DNP reagent to 1 ml supernatant. The solution was boiled to 100°C for 5 min. After boiling, 1 ml alcoholic 70% KOH was added. An observable, positive wine-red colour change indicated the presence of GA, where the intensity of colour change is proportional to the amount of GA present.

Partial characterization of bacterial isolates

Colonial and cellular morphologies of the isolates were observed. On the other hand, biochemical characterization and identification of the bacterial isolates were done using the Biolog® GEN III Microbial Identification Kit. The GEN III MicroPlate™ test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. Biolog's Microbial Identification Systems software (e.g. Omnilog® Data Collection) was used to identify the bacterium based on its phenotypic pattern in the GEN III MicroPlate.

Molecular identification of bacterial isolates

Genomic DNA isolation was done using the cetyltrimethyl ammonium bromide (CTAB) method. The extracted genomic DNA was sent to Macrogen Inc. (Seoul, South Korea) for gene amplification and 16S rRNA gene sequencing. A Basic Local Alignment Search Tool (BLAST) was used to determine the identity of these bacterial isolates through obtained nucleotide sequences.

In vivo assay—Greenhouse trial

Two pot experiments were conducted to determine the top performing isolates for plant growth promotion using corn and tomato. The experiments were laid out following a Randomized Complete Block Design (RCBD) with two factors: chemical fertilizer and microbial inoculants. At the end of the trial, growth parameters of the plants such as shoot and root length were measured. Shoots and roots gathered were oven-dried and then weighed to obtain their respective dry weights.

Statistical analysis

All data were analyzed using Analysis of Variance (ANOVA) of two factors in a Randomized Complete Block Design, and means were compared using Duncan's Multiple Range Test (DMRT) and Least Significant Difference Test (LSDT) at $p < 0.05$ if ANOVA showed significant effects (Duncan 1955).

Results and Discussion

Microbial isolation from rhizospheric soil samples

A total of 150 bacterial strains were isolated from rhizospheric soil samples. The breakdown of the microbial collection is summarized in Table 12.1 below.

Table 12.1. Isolate codes and tally of bacterial isolates obtained from different rhizospheric samples

Source	Isolate Codes	Isolates grown in			Total Isolates
		Nutrient broth	Dobereiner's Medium	Pikovskaya Medium	
AAP Isolates					
Mindanao Mine Site	A1-A6	6	0	0	6
Baguio Mine site	B1-B3	3	0	0	3
Bohol Bokashi Sample 1	C1-C3	3	0	0	3
Bohol Bokashi Sample 2	D1-D9	9	0	0	9
Fern*	E1-E3	3	0	0	3
Bamboo*	F1-F5	5	0	1	14
	FH1-FH8	8			
Grass*	G1-G10	8	3	0	18
	GH1-GH7	7			
Corn*	H1-H9	6	5	1	16
	HH1-HH4	4			
Sugarcane*	I1-I6	5	2	0	12
	IH1-IH6	5			
Legume*	J1-J7	5	2	0	15
	JH1-JH8	8			

GTC Isolates					
Sunflower (Batangas)	LGDsf	0	5	0	5
Cogon grass (Batangas)	LGDt	0	8	0	8
Sugarcane (Batangas)	LGDsc	0	12	0	12
Mango (Batangas)	LGDm	0	2	0	2
Grass (Isabela)	P/N/S	19	4	1	24
Total		**104**	**43**	**3**	**150**

*Taken from University of the Philippines Land Grant, Pakil, Laguna

Indole-3-acetic acid (IAA) production assay

Using the calibrated standard curve of IAA, the amount of IAA produced by each of the ten selected bacterial strains was determined. A varying level of IAA was produced by each strain in the range 1.62 µg/ml to 133.25 µg/ml. The highest concentration of IAA was obtained from GTC-P4 (133.25 µg/ml) followed by AAP-GD4, GTC-LGDsf5, GTC-LGDtA2, GTC-LGDsf3, AAP-I6, AAP-HD7, AAP-HD4, AAP-A4 and AAP-G6 (1.62 µg/ml).

Gibberellic acid (GA) production assay

Thirty nine (39) bacterial isolates showed a positive result for GA production assay. Four of the determined IAA-producing bacterial isolates, which are AAP-HD4, AAP-GD4, GTC-LGDtA2 and GTC-LGDsf5, also showed GA producing activity.

Partial characterization and identification of selected bacterial isolates

Four bacterial isolates selected out of 150 bacterial isolates were morphologically and biochemically characterized. The four isolates GTC-P4, GTC-LGDsf5, GTC-LGDtA2 and AAP-GD4 were selected due to their capability of producing high amounts of IAA. Based on the results obtained, GTC-P4 bacterial isolate is an obligate aerobe, gram-negative, rod-shaped bacterium; it is also a nitrogen-fixing and phosphorus-solubilizing bacterium. On the other hand, GTC-LGDsf5 bacterial isolate is a facultative anaerobe, gram-negative, rod-shaped, nitrogen-fixing bacterium; GTC-LGDtA2 bacterial isolate is an obligate aerobe, gram-

positive, rod-shaped, nitrogen-fixing bacterium, while AAP-GD4 bacterial isolate is a facultative anaerobe, gram-positive, rod-shaped, nitrogen-fixing bacterium. AAP-GD4 and GTC-LGDtA2 were identified biochemically and molecularly as *Bacillus pumilus* and *B. cereus*, respectively. The Biolog® GEN III Microbial Identification kit was not able to identify GTC-P4 and GTC-LGDsf5. However, GTC-LGDtA2 was identified with the use of 16S rRNA sequencing and the closest match was *Bacillus* sp. On the other hand, no identification was obtained for GTC-P4 with the use of both biochemical and molecular techniques. This shows that GTC-P4 is a possible novel bacterium which needs to be further characterized.

The closest matches to AAP-GD4 and GTC-LGDsf5 were identified as *Bacillus* sp. through 16S rRNA sequencing with homologies of 99% and 100%, respectively. *Bacillus* sp. is a ubiquitous Gram-positive, aerobic, rod-shaped endospore-forming bacterium. It is the most abundant genus in the rhizosphere and has been widely studied as a plant growth promoting rhizobacteria capable of phytohormone production.

The closest match to AAP-GD4 was *B. pumilus* with 99% homology. *B. pumilus* is capable of producing high amounts of physiologically active gibberellins based on a study conducted by Maňero et al. (2008). A GA production assay of the isolates indeed showed that AAP-GD4 was determined to be a GA-producing bacterium.

On the other hand, GTC-LGDtA2's closest match was *B. cereus*, a plant-growth promoting rhizobacteria. Recently, it was also detected to be a promising bacterial isolate capable of IAA production and showed growth promotion in pigeon pea through seedling emergence, increase of shoot length, dry matter production, nodule number and nodule mass (Rani et al. 2011). It was also determined to possess an increased potential to degrade petroleum products (Idises et al. 2010). *B. cereus* is proven to be an effective plant-growth-promoting rhizobacteria, but strict precautionary measures are needed in handling this microbe. It is also considered to be an opportunistic pathogen causing food poisoning manifested by diarrhoea or emetic syndromes (Ivanova et al. 2003).

In vivo assay—Greenhouse trial

Pot experiments involving corn and tomato plants were used to determine the effects of microbial inoculation combined with different levels of inorganic fertilizer on different parameters of plant growth, such as plant height, root length and shoot and root biomasses. Microbial inoculants alone provide significantly lower results compared to those treated with

combined microbial inoculants and chemical fertilizer. This was further supported by the tomato pot experiment. This also indicated that crops are relatively dependent on inorganic fertilizer.

GTC-LGDtA2 and GTC-LGDsf5 gave the highest values of different growth parameters of corn and tomato, respectively, regardless of the level of inorganic fertilizer used. The growth promotion of GTC-LGDtA2 gave significant 57.22% and 49.18% increases in shoot and root biomass of corn, respectively, over the uninoculated control. On the other hand, GTC-LGDsf5 gave significant 34.24% and 48.84% increases in shoot and root biomass of tomato, respectively, over the uninoculated control. Other microbial inoculants, AAP-GD4 and GTC-P4, also gave statistically-significant increases in different growth parameters of corn and tomato.

Conclusion and Recommendations

The selected microbial isolates, identified as *Bacillus* sp., *B. pumilus* and *B. cereus*, were able to enhance growth of corn and tomato through their plant growth promoting activities. The presence of these isolates in the rhizospheres of corn and tomato provided a favourable condition for these crops to elicit optimum growth. However, a mixture of these isolates did not have a significant effect on the plant, possibly due to their inhibitory effect on one another.

The isolated microbial strains can still be utilized for further characterization and analysis of other possible plant-growth-promoting activities, such as biocontrol. These should also be further tested to ensure their effects on the agronomic yields of corn, tomato and other crops. Pot experiments were observed on the vegetative growth of the plant only, and to determine the effect of the microbial inoculants on the yields of corn and tomato, a field trial is recommended.

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CHAPTER THIRTEEN

BIOCHEMICAL CHARACTERIZATION OF NORMAL AND GALLED ROOTS OF BOTTLE GOURD AND SPONGE GOURD INFECTED WITH *MELOIDOGYNE INCOGNITA*

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Abstract

Experiments have been performed to study the effect of root-knot nematode *Meloidogyne incognita* infection on alterations in biochemical metabolites in 30, 45 and 60 d old bottle gourd and sponge gourd plants. Quantitative analysis of total carbohydrates, reducing sugars, free amino acids and proline content in normal as well as galled roots of host plants, showed that, besides total carbohydrate content, all metabolites studied have a greater concentration in diseased roots over healthy ones in both the host plants. A maximum decrease in total carbohydrate content was recorded in 45 d old infected roots. However, the concentration of various metabolites varies with the time of inoculation of host plants. Therefore, it can be concluded that altered biochemical reactions in diseased tissue perhaps result in enhanced metabolism after the infection of a parasite. Hydrolysis of reserved chemical metabolites is carried out by their respective enzymes to cater for the needs of growth and proliferation of the pathogen and provide susceptibility to the host, resulting in the reduced growth and low yield of plant product.

Introduction

It is widely recognized that plant parasitic nematodes alone or in combination with other pathogens comprise a major constraint to global

food production. These nematodes can damage almost all types of crops due to their subterranean habitat and cause more than $125 billion annual losses worldwide (Chitwood 2003). The interaction of plant parasitic nematodes with roots has been reported in altered cell metabolism. Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and low-yielding plants (Abad et al. 2003; Ahmed et al. 2009). The faster breakdown of proteins and conversely a slow absorption result increase the amount of amino acid in infected tissues. Root-knot nematodes cause measurable changes in the morphology and physiology of the host (Williamson & Gleason 2003). Root damage from the nematode results in stunted and chlorotic plants. A decreased amount of total carbohydrates, an increased amount of reducing sugar and total free amino acids have also been reported in root knot nematode infected-roots (Sharma & Sethi 1976; Basu & Sukul 1983; Sharma & Trivedi 1996).

Materials and Methods

Normal and root-knot nematode infected-roots of bottle gourd (*Lagenaria siceraria*) and sponge gourd (*Luffa cylindrica*) were collected from experimental plots at intervals of 30 d, 45 d and 60 d after nematode inoculation. The roots were dried in an oven at 40 °C and finely powdered for quantitative estimation of the following metabolities.

Total carbohydrate content

Total carbohydrate content was calculated according to Sadasivam & Manikam (1992) as follows.

The 100 mg of root powder was placed in boiling test tubes and hydrolysed with 5 ml of 2.5 N HCl by keeping it in a boiling water bath. The solution was neutralised with solid sodium carbonate until the effervescense ceased. The volume was made up to 100 ml and centrifuged.

0.2, 0.4, 0.6, 0.8, and 1 ml of the working standard (1 mg glucose/1ml distilled water) were taken into a series of test tubes and 0.2 ml of the sample solution and 1 ml of a BLANK with distilled water were also taken in three separate test tubes. Thereafter, the volume was made up to 1 ml in all the above taken test tubes. Then, 5 ml of 96% was slowly added and continuously shaken for approximately 10 min. All these test tubes were kept in a water bath at 25°C–30°C for 20 min. Finally, the Optic Density/Absorbance was determined at 490 nm, and the amount of total carbohydrate was calculated using the following formula

Absorbance corresponds to 0.1 of test = x mg of glucose

Reducing sugar content

Reducing sugars were estimated by following the Nelson-Somogyi method, modified by Sadasivam & Manikam (1992), as follows:

The 100 mg of root sample was taken and sugars were extracted with hot 80% ethanol, twice (5 ml each time). Supernatant was collected and evaporated by keeping it in a water bath at 80°C. Then, 10 ml water was added to dissolve the sugars. Then, 0.1 and 0.2 ml aliquot was pipetted out in separated test tubes. Finally, the volume was made up to 2 ml with distilled water. Then, 2 ml of distilled water was also pipetted out to set a blank. After this, 1 ml of alkaline copper tartrate reagent was added to each test tube, and then the test tubes were kept in boiling water for 10 min. After cooling to room temperature, 1 ml of Arseno-molybdic acid reagent was added to the tubes and the volume was made up to 10 ml. Finally, the blue colour appeared, and O.D. and Absorbance were calculated as follow:

Absorbance corresponds to 0.1 ml of test = x of glucose

Total free amino acids

Protein-free samples were used for the estimation of total amino acids, according to Hawk (1965). Deproteinization of samples was carried out through the following procedure:

Protein free amino acids

The 0.5 gm of finely powdered sample was added to 50 ml of distilled water. After dissolving the soluble matter, 10 ml of 10% $ZnSO_4$ solution was added followed by 10 ml of 0.5 N-NaOH drop by drop with continuous stirring, and filtered immediately. The filtrate was protein-free extract. The experiment was conducted in the following manner.

Nine flasks of 25 ml were taken, of which one was for standard solution and the other seven for the solution of the sample. The last was blank. The 5 ml of protein free filtrate and standard solution were placed into separate tubes. The blank was maintained with distilled water in another tube. One drop of 0.25% alcoholic solution of phenolphthalein was added followed by 0.1 N-NaOH solution, drop by drop, until a pink colour was obtained. Later, 1 ml of borax solution and 1 ml of freshly prepared naphthoquinone

solution was added and the contents were stirred and placed in a boiling water bath for 10 min, and then for 5 min in an ice water bath. After cooling, 1 ml of acid formaldehyde and 1 ml of 0.1 N sodium thiosulphate solution were mixed and the volume was made up to 15 ml with distilled water.

After 30 min, optical density was determined with the help of a Systronics Colorimeter at 490 nm.

Amino acid nitrogen = /100 ml (in mg) $\times 0.03 \times 100 / 0.5$

Proline content

Proline content was determined by following the method of Sadasivam & Manikam (1992). For this purpose, 5 gm of root sample was homogenized in 10 ml of 3% aqueous sulphosalicylic acid. Homogenate was filtered through Whatmann's Filter Paper No. 2, 2 ml of filtrate was taken in a test tube, and Glacial acetic acid and Acid nin-hydrin (2 ml of each) were added. Test tubes were kept in boiling water for 1 h and the reaction was terminated by placing the tubes in an ice bath. Then, 4 ml of toluene was added and stirred for 20–30 s, following which the toluene layer was separated out. The colour intensity was read at 520 nm and the total proline content was calculated as follows:

$$\mu moles / gm\ tissues = \frac{proline / ml\ x\ ml\ toluene \times 5}{115.5 x\ gm\ sample}$$

Results and Discussion

Total carbohydrate content

A decreased amount of total carbohydrate was recorded in the diseased roots of both the bottle gourd and sponge gourd plants. Although the total carbohydrate increased with advancing age of both the infected and healthy plants, its concentration was less in infected roots over healthy ones. A maximum (45.49%) decrease in total carbohydrate content was observed in 45 d infected roots of bottle gourd, followed by 25.74% and 20.31% decrease after inoculation of 30 d and 60 d, respectively. In the case of sponge gourd, the maximum (33.64%) decrease of total carbohydrate was recorded after the 45th d of inoculation. However, in 30

d and 60 d old plants, 31.90% and 22.39% reductions in total carbohydrate were observed in root-knot nematode-infected roots over healthy roots of sponge gourd (see Table 13.1 below)

Table 13.1. Alterations in total carbohydrate content induced by *M. incognita* in bottle gourd and sponge gourd roots

Plant	30 d	45 d	60 d
Sponge gourd infected	36.72	55.38	61.81
Sponge gourd healthy	53.92	83.46	79.64
Bottle gourd infected	30.94	42.26	54.33
Bottle gourd healthy	41.67	77.53.	68.48

Sponge gourd roots showed greater percentage reductions when compared to bottle gourd roots of 30 d, 45 d and 60 d after inoculation. A lower concentration of total carbohydrates in infected roots showed a possible utilization of simple sugars by parasites during the process of parasitization. Metabolic leakage of carbohydrates during the post-infection period, particularly in the early stages of gall formation due to the hyperactivity of giant cells, may be the second possible reason for the quantitative reduction of total carbohydrates in infected roots.

The reports of Sharma & Sethi (1976), Basu & Sukul (1983) on okra, Tayal & Agrawal (1982) on tomato and the final report of ICAR Ad-hoc Research Project (1997) on tomato and eggplant infected with *Meloidogyne incognita* were also in accordance with the present findings. However, the observations of Farooqui et al. (1980) on the increased amount of carbohydrate in root galls of aubergine and tomato were almost parallel to the present observations. Kannan & Chandraguru (1981) reported greater sugar depletion in galls on the 30th d when compared to the 60th d in cowpea and Dolichos. Mohanty et al. (1997) also reported increased (31.52%) sugar content in root-knot nematode-inoculated roots, possibly due to the movement of various metabolites towards the infection site from other parts of the plant.

Reducing sugar content

Reducing sugar increased the infected roots of bottle gourd and sponge gourd plants over the corresponding healthy roots. Their magnitude was also more pronounced at 45 d after inoculation in the bottle gourd resulting in a 67.06% reduction of sugar content. However, in the case of

sponge gourd, it was highest (30.30%) at 30 d and 45 d, as observed in bottle gourd after inoculation. After 60 d of inoculation reducing, sugars showed a 17.45% increase in sponge gourd in comparison to bottle gourd, which showed a 18.33% increase of reducing sugars in diseased roots over normal sugars, showing a decreasing trend (7.50%) in infected roots after 60 d (see Table 13.2 below).

Table 13.2. Alterations in reducing sugar content induced by *M. incognita* in bottle gourd and sponge gourd roots

Plant	30 d	45 d	60 d
Bottle gourd healthy	39.73	57.14	94.17
Bottle gourd infected	47.21	95.46	87.01
Sponge gourd healthy	56.16	68.81	77.55
Sponge gourd infected	73.18	88.98	91.08

According to Owens & Specht (1966), the development of a disease syndrome on the biochemical reactions taking place between substances secreted by the pathogens and those already present are produced by the host as a response to the infection. Roy (1979) suggested high invertase activity resulting in changed carbohydrate metabolism during the course of host parasite interaction. Increased amylase activity resulted in a decrease of total sugar level and an increase in the amount of reducing sugars due to the enhanced hydrolysis of starch (Tayal & Agrawal 1982).

According to Sharma & Trivedi (1996) and ICAR Ad-hoc Research Project-Final report (1997), a 4.44%–49.44% increase in reducing sugars had been observed in root-knot nematode-infected tomato, aubergine and okra roots. The reports of Pandey & Trivedi (1991) on *Capsicum annum* infected by *M. incognita* were also in accordance with the present study. However, the findings of Singh et al. (1978) and Sarna & Trivedi (1987) were totally different, as they found a reduction in reducing sugars in the diseased tissue of aubergine and *Cicer arietenum* infected with *M. incognita*.

Total free amino acid content

The amount of free amino acids was found to increase in healthy as well as infected tissues with the advancing age of plants, except in 45 d old healthy and infected sponge gourd plants. Bottle gourd infected roots

showed 57.26%, 47.74% and 59.07% increases in free amino acids after 30 d, 45 d and 60 d of inoculation, respectively.

A gradual percentage increase of amino acids with increasing time of inoculation had been observed in the diseased roots of sponge gourd, as it accounted for 5.22%, 19.10% and 30.72% increases at 30 d, 45 d, and 60 d after inoculation, respectively. The total free amino acid content was greater quantitatively in bottle gourd when compared with sponge gourd (see Table 13.3 below).

Table 13.3. Alterations in total free amino acid content induced by *M. incognita* in bottle gourd and sponge gourd roots

Plant	30 d	45 d	60 d
Bottle gourd healthy	28.69	32.63	34.38
Bottle gourd infected	45.12	47.81	54.69
Sponge gourd healthy	28.55	26.38	29.3
Sponge gourd infected	30.04	31.42	38.3

Tayal & Agrawal (1982) suggested that the increased amount of free amino acids was due to the enhanced turnover for the benefit of the nematode into easily assimilable forms of amino acids. Similarly, increased levels of free amino acids in diseased plants were observed by several other workers (Singh et al. 1978; Khan et al. 1980; Farooqui et al. 1980). Matsubra & Feder (1970) and Tayal & Agrawal (1982) correlated the increased level of soluble proteins and amino acids with high protease activity in infected tissues. The proteases are secreted by the nematode into the host tissue for such a proteolytic degradation.

Sharma & Trivedi (1996) also observed greater amounts of free amino acid in root-knot nematode-infected okra roots of 15.42% to 31.81% over healthy roots, whereas, in the present study, it ranged between 5.22% to 59.07%. Mohanty et al. (1997) reported the intensity of ten free amino acids in nodules and *M. incognita* infected roots of green gram. Their study also showed a higher concentration of amino acids in nematode-inoculated roots as compared to their healthy counterparts and suggested this increase was probably due to the enzymatic breakdown of host components.

Proline content

Proline showed 14.28%, 18.57% and 42.40% increases in infected samples over healthy samples in 30 d, 45 d and 60 d inoculated infected roots of bottle gourd, respectively. However, in the case of sponge gourd, it was 23.89%, 79.59% and 36.81% in 30 d, 45 d and 60 d inoculated roots, respectively (see Table 13.4 below).

Table 13.4. Alterations in proline content infused by *M. incognite* in bottle gourd and sponge gourd roots

Plant	30 d	45 d	60 d
Bottle gourd healthy	94.5	98.25	102
Bottle gourd infected	108	116.5	145.25
Sponge gourd healthy	68	73.5	91
Sponge gourd infected	84.25	132	124

Proline accumulation in inoculated samples is considered to be important, because this amino acid is always associated with the water stress condition (Stewart et al. 1966) following the disruption of the xylem and the translocation of water from roots in response to infection, resulting in wilting of the aboveground parts. The therapeutic property of proline as a defence mechanism against infection, affecting the rate of nematode reproduction, was pastulated by Epstein (1972). To confirm this, many people studied the effect of different amino acids against plant parasitic nematodes (Prasad & Webster 1967; Reddy et al. 1975), reaching very similar conclusions.

Sharma & Trivedi (1996) reported 76.85% and 23.8% increases in proline content in two cultivars of okra. However, 14.77%, 25.28% and 68.5% increases in proline in okra, tomato and aubergine, respectively, were recorded by Jagpal (1997). In the present study it ranged from 14.28% to 79.59% in infected roots over healthy ones. Mohanty et al. (1997) also observed an enhanced amount of proline in nematode-inoculated green gram roots. According to them, this may be due to the conversion of L arginine to L proline through the ornithine cycle.

Table 13.5. Biochemical metabolites in normal and root-knot nematode-infected roots of bottle gourd and sponge gourd

Metabolites	Host plants	30 *days after inoculation*			45 *days after inoculation*			60 *days after inoculation*		
		Healthy	*Infected*	*%I / D*	*Healthy*	*Infected*	*%I / D*	*Healthy*	*Infected*	*%I / D*
Total carbohydrates (µg/mg)	Bottle gourd	41.67	30.94	-25.74	77.53	42.26	-45.49	68.48	54.33	-20.31
	Sponge gourd	53.92	36.72	-31.90	83.46	55.38	-33.64	79.64	61.81	-22.39
Reducing sugars (µg/mg)	Bottle gourd	39.73	47.21	18.83	57.14	95.46	67.06	94.17	87.10	-07.50
	Sponge gourd	56.16	73.18	30.30	68.81	88.98	29.31	77.55	91.08	17.45
Total Free amino acids	Bottle gourd	28.69	45.12	57.26	32.36	47.81	47.74	34.38	54.69	59.07
	Sponge gourd	28.55	30.04	05.22	26.38	31.42	19.10	29.30	38.30	30.72
Proline (µg/mg)	Bottle gourd	94.50	108.00	14.28	98.25	116.50	18.57	102.00	145.25	42.40
	Sponge gourd	68.00	84.25	23.89	73.50	132.00	79.59	91.00	124.50	36.81

From the foregoing account it can thus be concluded that altered biochemical reactions in diseased tissue perhaps result in enhanced metabolism after the infection of a parasite (Table 13.5). The hydrolysis of reserved chemical metabolites is carried out by their respective enzymes to cater to the needs of growth and proliferation of the pathogen, providing susceptibility to the host and resulting in the ultimately reduced growth and low yield of the plant product

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CHAPTER FOURTEEN

IMPACT OF PLANT EXTRACTS ON FECUNDITY, LONGEVITY AND FEEDING OF *CALLOSOBRUCHUS MACULATUS* AND BIOCHEMICAL ALTERATION IN INFECTED/TREATED CHICKPEA SEEDS

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Abstract

Plant extracts of *Piper nigrum* (black pepper) seed, *Azadirachta indica* (neem) kernel, and *Allium sativum* (garlic) bulb were evaluated using petroleum ether as solvent and tested on *C. maculatus* infesting chickpea seeds. Observations on fecundity and longevity of test insect and feeding deterrent activity of extracts were recorded and analysed statistically. The nutritional changes of chickpea seeds treated with a 2% concentration of these plant extracts and infested with test insects were evaluated after one month. Nutritional changes in one month and six month old infected chickpea seeds, taken from the culture, were also evaluated. Extract of black pepper showed the highest efficacy over neem and garlic and these extracts showed mortality in descending order of effectiveness, being found to be effective for antiovipositional activity and in reducing the percentage of adult emergence. Extracts of black pepper and neem exhibited complete feeding deterrent activity at all concentrations. Only 1–2 g of chickpea seed powder soaked with garlic extract were eaten by larvae of *C. maculatus*. Along with the increased weevilisation of chickpea seeds, an increase in total ash and protein content and a decrease in crude fat and carbohydrate were recorded. Little difference in the nutritional value of normal chickpea seeds, as well as those treated with

these extracts, was noticed. However, when seeds taken from the culture were compared with normal seeds for their nutritional value, lots of changes occurred. The nutritional change of six month infected seeds showed significant differences from normal seeds.

Introduction

Bruchids are the most degraded stored grain pest, breeding on a variety of pulses, with a short life span and a high degree of reproductive potential. The pest develops during storage and adult beetles come from seeds. The use of ecofriendly and biodegradable natural insecticides of plant origins are used to control this insect-pest. Some plants have shown the potential of inhibiting the growth and development of *Callosobruchus maculatus.* The repellent effect of edible and medicinal plant oils against *C. chinensis* were evaluated by Kaushik & Rathi (2010). Kiradoo & Srivastava (2010) studied the ovipositional deterrent activity of *Mentha spicata* against the pulse beetle *C. chinensis.*

In the present investigation, botanical pesticides were used to protect the chickpea seeds infested with *Callosobruchus maculatus.* These botanical pesticides exhibited their impact on the growth and development of *C. maculatus* under laboratory conditions. The total ash, protein, crude fat and carbohydrate content of treated, normal and infected chickpea seeds was also analysed.

Materials and Methods

Neem kernel, garlic bulbs and black pepper seeds were shade dried and powdered. Extract was prepared in petroleum ether solvent in a 1:5 ratio and treated as stock (Ahmad et al. 1991). From the stock solution, three concentrations of each extract were prepared at 1%, 1.5% and 2%. The 20 g of healthy chickpea seeds were treated with these three concentrations of plant extracts @ 20 ml/kg seeds. Three pairs of 3 d old adults were released into each jar. These jars were kept in B.O.D. at 27 ± 1ºC and 75% relative humidity. The test insects were taken out after three days and the jars were kept for one month. The percentage mortality of the test insects and number of eggs laid were recorded. The percentage reproductive success (R.S.) and growth index (GI) were calculated with the following formula:

$$RS = \frac{\text{No. of adults emerged}}{\text{No. of eggs}} \times 100$$

$$GI = \frac{\text{percent reproductive success}}{\text{Developmental period}} \times 100$$

For the evaluation of nutritional changes, the seeds from the continuous culture of *C. maculatus* were taken after one month and six months of infestation. Seeds treated with 2% of plant extracts and normal seeds were also taken for biochemical analysis. All the seeds were dissected for the removal of excrement material and dried at 60ºC for an hour to bring the moisture level to equilibrium, and a powder was made from the grains. The observation on the qualitative losses such as crude protein, crude fat, total carbohydrate and ash content were analysed according to the Association of Official Analytical Chemists (1985).

Results and Discussion

The extract of black pepper showed 100% mortality of test insects at all concentrations, while 2% garlic bulb and neem kernel showed 55.6% and 61.1% mortality of test insects, respectively. Egg laying on treated seeds was found to be nil with 2% concentration of black pepper seed, while at 1.5% and 1% concentrations it was 3.7 and 4.3 respectively (see Table 14.1 below). Neem kernel and garlic bulb were also very effective in reducing egg deposition when compared with the control. All the conc. of neem kernel and black pepper were found to have ovicidal activity against *C. maculatus* eggs under laboratory conditions and the percentage reproductive success was found to be nil. However, with all concentrations of garlic bulb extracts, reduction in percentage of adult emergence (20.1% to 26.1%) has been reported in comparison to the control (91.9%) (see Table 14.1 below).

The treated seeds were totally protected from insect attack and extent of feeding and percentage of weight loss of seeds were found to be nil with all concentrations of neem kernel and black pepper seed.

Table 14.1. Effect of petroleum ether extract on fecundity and longevity of *C. maculates*

Plant extr-act	Percentage mortality of test insect				Eggs laid				Percentage emergence			adult	Growth index				Developmental period			
	1%	1.5 %	2.0 %	p>.0 1	1%	1.5 %	2.0 %	p>.0 1	1%	1.5 %	2.0 %	p>.0 1	1 %	1.5 %	2.0 %	p>.0 1	1%	1.5 %	2.0 %	p>.0 1
Neem kernal	27.8	50	61.1	0.16	21.6	15.0	5.7	0.43	-	-	-	-	-	-	-	-	-	-	-	-
Garlic bulb	27.8	38.9	55.6	0.15	32.7	26.7	25.3	0.23	26.1	25.1	20.0	0.03	0.6	0.6	0.5	0.01	41.2	42.8	43.5	0.001
Black pep-per seed	100	100	100	-	4.3	3.7	-	0.92	-	-	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	123	-	-	-	91.9				3.6				25.3			

There was a significant reduction in the extent of feeding and percentage weight loss of seeds treated with garlic bulb extract as compared to control (see Table 14.2 below). Nutritional changes in chickpea seeds treated with these extracts were not analysed as they were found to be effective against *C. maculatus*. However, the infected control showed nutritional changes in comparison with normal and treated seeds. Significant differences in the biochemical components of six month infected seeds and normal seeds have been evaluated. Increase in protein content has been reported in more weeviled seeds, i.e. it was 29.87% and 42.72% for one month and six month infected seeds, respectively. There is no significant difference in the percentage of crude fat in treated and normal seeds, while it decreased in infected seeds. The total carbohydrates decreased in infected seeds and it was observed to be 68.84% for normal seeds, and a more or less similar percentage of carbohydrate was observed for seeds treated with extracts. The total ash of infected chickpea seeds increased significantly during storage, totalling 7.83% and 8.96% for one month and six month infected seeds respectively, in comparison with normal seeds (4.02%). Thus, it was concluded that total ash and protein content increased while total carbohydrate and crude fat decreased with more weevilisation (see Table 14.3 below).

The seed extract of black pepper was found to be an effective grain protectant by significantly reducing the population of the test insect and ultimately minimizing weight loss of chick pea seeds, which confirmed the report given by Khanna (1995). Kumar et al. (2010) reported that black pepper powder reduced the percentage of damage and weight loss of wheat grain due to *S. oryzae.* Jood et al. (1993) also reported the effect of powder of garlic bulb and neem kernel in reducing damage of wheat grain caused by *T. castaneum*. Similarly, in the present investigation, extracts of garlic bulb and neem kernel reduced the damage of chickpea seeds caused by *C. maculatus*. Upadhyay & Jaiswal (2007) also evaluated the biological activities of *P. nigrum* oil against T. *castaneum*. Kiradoo & Srivastava (2010) also found that botanical insecticides are effective against bruchids and showed that leaf formulations of *Mentha* significantly reduced oviposition by *C. chinensis*. Gupta et al. (1984), Srivastava et al. (1988) and Modgil & Mehta (1997) evaluated nutritional changes in stored grain in relation to bruchid damage, and concluded that total ash and protein increased in infected seeds in accordance with the present study.

Table 14.2. Effect of petroleum ether extract on feeding of *C. maculates*

Plant extract	Extent of feeding (in gm)				% weight loss of seed			
	1%	1.5%	2.0%	p>.01	1%	1.5%	2.0%	p>.01
Neem kernal	-	-	-	-	-	-	-	-
Garlic bulb	1.87	1.45	1.13	0.09	9.4	7.2	5.7	0.09
Black pepper seed	-	-	-	-	-	-	-	-
Control	9.16	-	-	-	45.8	-	-	-

Table 14.3. Nutritional changes in treated and infected chickpea seeds in comparison to normal seeds

	Percent total Ash	Percent total fat	Percent total nitrogen	Percent crude protein	Percent total carbohydrate
Normal seed	4.02	5.40	3.48	21.74	68.84
Seeds treated with extract					
Neem kernal	4.25	5.13	3.55	22.19	68.44
Black pepper seed	4.10	5.21	3.53	22.08	68.61
Garlic bulb	4.50	5.00	4.02	25.12	65.38
Infected control	5.03	4.77	4.53	28.33	61.87
Seeds taken from culture					
One month infected	8.83	4.48	4.78	28.87	57.82
Six month infected	8.96	3.93	6.84	42.72	44.39
Sem ±	0.26	0.07	0.02	0.10	0.28
CD at 5%	0.87	0.23	0.07	0.33	0.93

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CHAPTER FIFTEEN

HISTOLOGY AND SCANNING THE ELECTRON MICROSCOPY OF AMPHISTOMID PARASITE *GASTROTHYLAX CRUMENIFER* FROM CHANDIGARH, INDIA

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Abstract

Of the parasitic amphistomes harboured in the rumen of cattle slaughtered in abattoirs in Chandigarh, *Gastrothylax crumenifer* was selected for study through histological studies supplemented by scanning the electron microscopy. This work emphasises the morphological differences of *Gastrothylax crumenifer* compared to other species.

Introduction

Amongst digenetic trematodes, amphistomes constitute one of the important groups of parasites. They are parasitic in the digestive tract of vertebrates of many animals, fishes to mammals. In ruminants they are conspicuously present in the small intestine of the immature. They have attracted a lot of attention since they cause a disease called paramphistomiasis, responsible for debility and the reduction of milk yields in cows and buffalo. The amphistomes look so much alike morphologically that it is difficult to identify them from a systematic point of view. A considerable amount of work has been done on the morphology and systematic distribution of these trematodes. Over the last few decades the electron microscope has enabled us to sufficiently study the structures at higher magnifications, to identify the surface morphologies of amphistomes as being different from species to species, and the use of the scanning electron microscope gives

better results in identifying various genera and species. The amphistomid parasites are grouped into fifteen subfamilies under the family *Paramphistomidae*. The various genera of amphistomes are difficult to identify because of their close resemblance to one another. Earlier, they were identified from their morphological characters only, and consequently many of them were mistaken for other forms or were wrongly identified and classified. Nasmark (1937) realized all those difficulties of identification and tried to solve them by histological examination of median saggital sections, with particular reference to the structures like acetabulam, pharynx and genital atrium. The scanning electron microscope (SEM) together with the currently used criteria for grouping species may be helpful in the demarcation of the minute morphological differences between them (Kuntz et al. 1979). The arrangement of papillae on the adult may be useful in providing a basis for diagnosing closely related species.

Materials and Methods

The material for the present work was collected from the cattle and goat slaughtered in abattoirs at Chandigarh. These Amphistomes were picked up from the rumen with the help of a pair of forceps and then gently washed several times in 0.9% sodium chloride solution. The entire collection comprised amphistome *Gastrothylax crumenifer*.

Fixation

For the preparation of whole mounts (w.m.), the specimens were flattened by placing them between two glass slides and by tying a piece of thread around them so they could straighten out. These were then kept in this condition in 70% alcohol overnight. The following day they were released from the slides and preserved in 70% fresh alcohol.

For section cutting

For section cutting, unflattened specimens were fixed in Bouin's fluid for 12–24 h. The Bouin's fluid was prepared by mixing the components, which were then dissolved by heating and filtered. A crystal of Thymol was added to prevent fungal growth.

Methods of staining

Specimens preserved in 70% alcohol were first downgraded and then washed in distilled water. After thorough washing for about 2–3 h, depending on the thickness, they were put in Gower's carmine stain overnight. Then they were washed in distilled water. Differentiation was done in acid water. The specimens were upgraded to 70% alcohol, through 30% and 50% alcohol, by keeping them in 90% alcohol for 1 h and in Cedar wood oil or Clove oil, before mounting them in Canada Balsam.

For staining with Borax Carmine, the specimen preserved in 70% alcohol was put in Borax Carmine stain directly (as stain is prepared in 70% alcohol) for 10 min. It was washed in 70% alcohol thoroughly and differentiation was done in 70% acid alcohol. It was then upgraded to 90% alcohol and to absolute alcohol for 1 h each, kept in Clove oil overnight, and mounted on Canada balsam on the next day.

Staining of sections

Staining of sections was done by the double staining method using haematoxylin and eosin.

Fixation for SEM

For the SEM study, amphistomes were taken and fixed in 4% glutraldehyde for one and half hours followed by 2–3 washings in phosphate buffer prior to dehydration.

Histological studies

Body wall

Histologically, the body wall of *Gastrothylax crumenifer* consists of tegument, sub tegument, muscle layer and tegumental cells. The body of *Gastrothylax crumenifer* is completely covered by a thick continuous tegument on both dorsal and ventral surfaces. At the anterior end, the tegument is invaginated into the pharynx and ventral pouch, and at the posterior end this invaginates into the acetabulum. The tegument on the dorsal surface is smooth, whereas on the ventral side it is thrown into projections giving it a wavy appearance.

Below the tegument on the dorsal and ventral surfaces an inert layer known as the sub-tegument is present, which is much thicker than the tegument. Its thickness also varies on the ventral and dorsal surfaces.

Beneath the subtegument layer muscles are present in bands. These are easily distinguishable into longitudinal and circular muscles. Thickness of muscle layer also varies on the ventral and dorsal surfaces. Tegumental cells are also present below the longitudinal muscle fibres.

Scanning electron microscopy (SEM)

A papillate or beaded tegumental surface, as observed in *G. crumenifer*, has also been reported for *Posthodiplostomum minimum* (Mitchell & Crang 1976), *Gorgoderina attenuata* (Nadakavukaren & Nollen 1975). It has been suggested that these raised protuberances increase the absorptive surface of the trematode (Nadakavukaren & Nollen 1975). A similar function has also been assigned to the spineless micritriches in the posterior part of strobilla of the Caryophyllidean cestode, *Hunterella nodulosa* (Hyunga & Mackiewicz 1975) and in the pseudophyllidean, *Diphyllobothrium* sp. (Anderson 1975).

The papillate elevations on the general tegument of the amphistomes studied herein must be primarily involved in food absorption, since anchorage seems to be entirely by the acetabulum and the anterior muscular thickening, i.e. the pharynx. This is suggested because the tegument lacks spines observed in many trematode species, e.g. in *Fasciola hepatica* by Bennett (1975ab), *S. japonicum* by Sakamoto & Ishii (1977), and *S. bovis* by Kuntz et al. (1979).

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CHAPTER SIXTEEN

POTENTIAL OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON SOIL HEALTH AND THEIR INTERACTION WITH CHEMICAL FERTILIZERS

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Abstract

Soil is an important ecological niche for the microbial community. The excessive use of chemical fertilizers not only adversely affects the soil health and soil physiochemical properties, but also pollutes the ecosystem. The present investigation deals with the role of PGPR and chemical fertilizers, alone or in combination, on important soil enzymes, e.g. urease, invertase phosphatase, MDA contents, and the general impact on seedling of safflower cvv. Thori and Saif-32 differ in their response to moisture contents. The PGPR were applied at 10^6 cells/mL as seed inoculation prior to sowing. Chemical fertilizers were applied at full (Urea 60 Kg/ha and DAP 30 Kg/ha), half (Urea 30 Kg/ha and DAP 15 Kg/ha), and quarter doses (Urea 15 Kg/ha and DAP 7.5 Kg/ha) during sowing. The chemical fertilizers and PGPR induced urease and invertase activities of soil were further augmented when used in combination (PGPR augmented the CF-induced urease and invertase activities of soil at quarter and half doses of chemical fertilizers). The cv. Thori exhibited greater response to PGPR and CFF for soil phosphatase activity. The response of the PGPR strain and safflower variety were discussed for the decrease in leaf MDA content and increase in the growth of seedling. It is inferred that 50% CF can be supplemented with PGPR for the better health of soil and fertility status.

Introduction

Plant Growth Promoting Rhizobacteria (PGPR) comprise a group of bacteria that colonize root surfaces and improve plant growth and development (Wu et al. 2005). As the excess use of chemical fertilizers adversely affects not only the soil but also the ecosystem by causing soil pollution and reducing water holding capacity, as well as being costly, efforts are being made to replace chemical fertilizers with more sustainable, environmentally friendly and cost effective measures such as PGPR. Previous studies demonstrate the positive role of PGPR inoculation on crop production (Egamberdiyeva 2007).

PGPRs have been reported to boost plant growth by bringing positive improvements to the soil quality, measured by the capability of a soil to sustain biological production inside the ecosystem. Soil enzymes are essential for organic matter turnover and the metabolic activity of soil microorganisms (Nannipieri et al. 2002). The most active enzymes in soil include protease, urease, pectinase, cellulase, dehydrogenase, catalase, amylase and phosphatase. Ureases catalyze the hydrolysis of urea to CO_2 and NH_3, which is a vital process in the regulation of N supply to plants after urea fertilization. In soil, ureases are tightly bound to organic matter and minerals in soil, and were demonstrated to correlate with soil nutrients (Li et al. 2006). Invertase is a hydrolase, cleaving sucrose into two monosaccharides and hence providing energy for germination. Phosphatases have been detected on root surfaces and in rhizosphere soil. The hydrolytic cleavage of P, by extracellular phosphatases of microbial or root origin, is one mechanism of such mineralization.

Malondialdehyde (MDA) is a major cytotoxic product of lipid peroxidation and has been widely used as an indicator of free radical production (Mohammdkhani & Heidari 2007), and the concentration of MDA in the cell or tissue shows the degree of cell macromolecule destruction resulting in the loss of the cell function and ultimately cell death (Stoparic & Maksimovic 2008).

Safflower is an important oil seed crop and is tolerant to drought and salt stress, being used as a source of dye, medicine and food. The presence of linoleic acid in safflower oil imparts its medicinal value. It can also be grown on soil with poor fertility, and safflower is currently being used as a source of alternative fuel (Ullah & Bano 2011).

Keeping in view the importance of PGPR in improving plant growth and those of soil enzymes in maintaining soil fertility, the current investigation is aimed at assessing the role of PGPR, both alone and in combination with commercial fertilizers on safflower growth, and their

impact on some biologically important soil enzymes in order to economize the commercial fertilizers.

Materials and Methods

Plant material and growing conditions

Certified seeds of Safflower cv. Thori (spineless) and cv. Saif-32 (spiny) were obtained from the National Agriculture Research Centre (NARC), Islamabad, where the surface was sterilized with 10% chlorox solution for 5 min and subsequently washed three times with sterilized distilled water. The seeds were sown in plastic pots (11 × 8 cm^2) filled with soil and sand (1:1) in the greenhouse under controlled environmental conditions. The pots were arranged in a completely randomized design (CRD).

The PGPR were applied as seed inoculation @10^6 cells/mL, and the number of bacterial cells/seed were measured as 4×10^5. The chemical fertilizers were applied as an aqueous solution at the time of sowing.

The following treatments were made.

Treatments	Abbreviation
Control (Without inoculation and without chemical fertilizers)	C
Chemical fertilizers full dose (Urea 60 Kg/ha and DAP 30 Kg/ha)	CFF
Chemical fertilizers half dose (Urea 30 Kg/ha and DAP 15 Kg/ha)	CFH
Chemical fertilizers quarter dose (Urea 15 Kg/ha and DAP 7.5 Kg/ha)	CFQ
Seed soaking with *Azospirillumbrasilense* (accession no. GQ255949)	SP
Azospirillumbrasilense+full dose of chemical fertilizers	SPF
Azospirillumbrasilense+half dose of chemical fertilizers	SPH
Azospirillumbrasilense+quarter dose of chemical fertilizers	SPQ
Seed soaking with *Azotobactervinelandii* (accession no. GQ849485)	BT
Azotobactervinelandii +full dose of chemical fertilizers	BTF
Azotobactervinelandii+half dose of chemical fertilizers	BTH
Azotobactervinelandii+quarter dose of chemical fertilizers	BTQ
Seed soaking with consortium of *A. brasilense*+*A. vinelandii* (coinoculation)	SPBT

The pots were separated into two sets, each with three replicas for all treatments. From one set, melondialdehyde (MDA) contents were measured in emerging cotyledonary leaves at post germination stage (emerging 72 h after sowing). The MDA was estimated according to Hernandez & Almansa (2002). Emerging cotyledonary leaves (0.2 g) were

homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 15,000 g for 10 min, and 0.5 mL of the obtained supernatant was added to 1.5 mL of thiobarbituric acid in 20% (w/v) TCA. The mixture was incubated at 90°C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice water bath. The samples were centrifuged at 10,000 g for 5 min, and the light absorbance of the supernatant was read at 532 nm.

The plants in another set were harvested 30 d after sowing when the plants were fully established. The soluble protein content of leaves was determined following the method of Lowry et al. (1951) using BSA as the standard. The chlorophyll and carotenoid estimation of leaves was made following the method of Arnon (1949), as modified by Kirk (1968). Leaf area was measured according to Ahmed & Morsy (1999).

The effect of PGPRs on soil enzymes in the rhizosphere was determined after harvesting the plants (30 d after sowing). Soil invertase activity was measured by the method of Zhou & Zhang (1980). The 8% sucrose was used as a substrate. Five mL of phosphoric acid buffer (pH 5.5) and 15 mL of substrate were mixed with 5 g of soil and incubated at 37°C for 2 h. Next, 3 mL of 3, 5-dinitrosalicylic acid were added to 1 mL of the soil filtrate and heated for 5 min at 95°C in a water bath. The amount of 3-amino-5-nitrosalicylic acid formed was determined based on the absorbance at 508 nm using a spectrophotometer (Hitachi U-1500). Invertase activity was expressed as glu. g (g/h).

The soil urease was measured according to the method of Douglas & Bremner (1970). A soil sample (5 g) was placed in a universal bottle and 4 mL of 0.5 M sodium acetate buffer (pH 6.5), 1 mL of toluene and 10 mL of the substrate (5 mM urea-N) solution were added followed by the incubation of the mixture for 8 h at 25°C. After completion of the incubation period, the reaction was stopped by the addition of 2 M potassium chloride-phenyl mercuric acetate reagent (20 mL). Further shaking of the flasks was carried out for 30 min, filtered through Whatman No. 1 filter paper and the urea concentration of the soil extract was analyzed at 525 nm using a spectrophotometer (Hitachi U-1500). Urea concentration was calculated from a urea-N standard curve (0--10 ug ml^{-1}) prepared on the day of analysis. Soil phosphatase activity was determined by the method of Tabatabai & Bremner (1969) using disodium paranitrophenyl phosphate as substrate.

Statistics

The data was analyzed statistically by factorial ANOVA using Statistix software version 8.1 techniques, and comparison among the mean values of treatments was made by Duncan's Multiple Range Test (Duncan 1955).

Results

The soil used for the cultivation of Safflower was sandy loam, having EC 0.52 dS/m, pH 7.3, soil organic matter content 4.2%, available phosphorus 3.5 mg/kg, total nitrogen 0.021%, available potassium 100 mg/kg, and sodium 1138 ppm.

The urease activity of soil was enhanced in both the varieties in all treatments with chemical fertilizers (CF) and PGPR either alone or in combination (see Table 16.1 below). The maximum increase (82%) was due to *Azotobacter*in combination with a quarter dose of chemical fertilizers in cv. Thori. The stimulatory effects of *Azospirillum* (SP) on soil urease activity in rhizosphere of cv. Thori were more pronounced (26%) than *Azotobacter* (BT). Treatments SPQ and SPF showed 66% and 57% increases in urease activity over control. In cv. Saif-32, the urease activity was 74%, 72% and 69% higher in treatment SPH followed by BTQ and BTH, respectively, as compared with the untreated control. The CFF and CFH increased soil urease activity significantly by 46% and 47% as compared with the control. The SPH and BTH had 51% and 42% higher urease activity over CFH, while BTQ exhibited a 61% higher urease activity as compared with CFQ.

The soil invertase activity was greater in the rhizospheric soil of cv. Saif-32 as compared with cv. Thori. All the treatments except CFF and CFQ significantly enhanced the invertase activity in the rhizosphere of cv. Thori (see Table 16.1 below). The effect of SP on invertase activity was higher (27%) than BT. The treatments SPF and BTF were highly effective in improving the invertase activity by 67% and 66% when compared with untreated control. The greatest increase (84%) was recorded in the rhizosphere of plants inoculated with BT in combination with a quarter dose of chemical fertilizers. The treatment SPH increased invertase activity by 79% and 57% as compared with uninoculated control and CFH, respectively. The magnitude of increase by BTF was recorded as 71% and 29% higher over untreated control and a single inoculation of BT.

Table 16.1. Effects of PGPR and chemical fertilizers on Soil Urease (ug urea/g/h) and Invertase $(g/gh)^{-1}$ activity of Safflower. The experiment was carried out in pots with three replicates

Treatments	Soil Urease Activity (ug urea/g/h)		Soil Invertase Activity (gh^{-1})	
	CV. Thori	CV. Saif-32	CV. Thori	CV. Saif-32
C	53.78 ^{y}	55.56 ^{w}	36.33 ^{m}	24.00 ^{p}
CFF	79.44 ^{p}	103.33 ^{j}	36.33 ^{m}	33.67 ^{n}
CFH	70.56 ^{t}	104.44 ^{i}	70.00 ^{g}	48.33 ^{k}
CFQ	72.78 ^{s}	76.33 ^{r}	28.00 ^{o}	48.33 ^{k}
SP	114.44 ^{h}	70.00 ^{u}	85.00 ^{d}	20.00 ^{q}
SPF	126.67 ^{g}	92.22 ^{m}	113.00 ^{b}	75.00 ^{e}
SPH	76.67 ^{r}	215.56 ^{b}	54.00 ^{j}	114.00 ^{b}
SPQ	161.67 ^{e}	97.78 ^{l}	47.33 ^{k}	32.67 ^{n}
BT	84.67 ^{o}	77.22 ^{q}	62.00 ^{h}	59.33 ^{i}
BTF	56.11 ^{v}	87.22 ^{n}	109.00 ^{c}	84.00 ^{d}
BTH	150.56 ^{f}	182.78 ^{d}	39.33 ^{l}	62.33 ^{h}
BTQ	312.78 $_{a}$	198.89 ^{c}	48.00 ^{k}	147.33 ^{a}
SPBT	101.11 ^{k}	55.00 ^{x}	71.33 ^{f}	61.33 ^{h}
LSD	0.4641		1.0970	

All such means which share a common English letter are similar; otherwise, they differ significantly at $P < 0.05$.
C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A.vinelandii*, BTF: *A.vinelandii* + full dose of chemical fertilizers, BTH *A.vinelandii* + half dose of chemical fertilizers, BTQ: *A.vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A.vinelandii*, LSD: Least Significant Difference.

The results show that all the treatments increased soil phosphatase activity in the rhizosphere of both the varieties. In cv. Thori, the greatest phosphatase activity (17%) was recorded in CFF, followed by BTH (15%), as compared with untreated control (see Table 16.2 below).

Similarly, both the treatments SPF and BTF exhibited a 13% increase in soil phosphatase activity than control. The seed inoculation with SP and BT markedly increased (13% and 14%) the phosphatase activity in the rhizospheric soil as compared with respective controls. In cv. Saif-32, a maximum increase in soil phosphatase activity was observed in BT (10%), followed by SP (9%).

The greatest increase (42%) in MDA content was due to the full dose of chemical fertilizers, followed by BTF (40%), as compared with the uninoculated control in cv. Thori. In cv. Saif-32, the treatment CFQ exhibited a significant increase (28%) in MDA content as compared with the untreated control. The treatment with SP supplemented with a full dose of chemical fertilizers (SPF) showed a decreased MDA as compared with treatment CFF. Similarly, the coinoculation treatment SPBT showed significantly lower MDA content (62%) as compared with the respective uninoculated control (see Table 16.2 below) in cv. Thori, whereas in cv. Saif-32 the consortium of both microbes increased the leaf MDA contents.

Table 16.2. Effect of PGPR and chemical fertilizers on soil Phosphatase (ug p-Np/g/min) activity and leaf MDA (nmol/gFW) contents of Safflower. The experiment was carried out in pots with three replicates

Treatments	Soil Phosphatase Activity (ug p-Np/g/min)		Leaf MDA (nmol/gFW)	
	Thori	Saif-32	Thori	Saif-32
C	5.44 ^{jk}	5.40 ^{k}	0.460 ^{efghi}	0.350 ^{ij}
CFF	6.58 ^{a}	5.85 ^{fghi}	0.807 ^{a}	0.362 ^{hij}
CFH	6.36 ^{abc}	5.45 ^{jk}	0.444 ^{efghi}	0.428 ^{fghi}
CFQ	6.05 ^{cdefg}	5.69 ^{hijk}	0.486 ^{defghi}	0.498 ^{cdefgh}
SP	6.26 ^{abcd}	5.93 ^{efgh}	0.416 ^{ghi}	0.560 ^{cdef}
SPF	6.22 ^{bcde}	5.58 ^{ijk}	0.545 ^{cdefg}	0.414 ^{ghij}
SPH	6.29 ^{abcd}	5.75 ^{ghij}	0.550 ^{cdefg}	0.480 ^{efghi}
SPQ	6.10 ^{cdef}	5.59 ^{ijk}	0.476 ^{efghi}	0.623 ^{cd}
BT	6.32 ^{abcd}	6.00 ^{defgh}	0.580 ^{cde}	0.514 ^{cdefg}
BTF	6.29 ^{abcd}	5.75 ^{ghij}	0.770 ^{ab}	0.434 ^{fghi}
BTH	6.44 ^{ab}	5.68 ^{hijk}	0.560 ^{cdef}	0.353 ^{ij}

BTQ	5.69 ^{hijk}	5.57 ^{ijk}	0.272 ^{jk}	0.483 ^{defghi}
SPBT	5.79 ^{fghi}	5.72 ^{hijk}	0.132 ^{k}	0.637 ^{bc}
LSD	0.3239		0.1426	

All such means which share a common English letter are similar; otherwise, they differ significantly at $P < 0.05$.

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A.vinelandii*, BTF: *A.vinelandii* + full dose of chemical fertilizers, BTH *A.vinelandii* + half dose of chemical fertilizers, BTQ: *A.vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A.vinelandii*, LSD: Least Significant Difference.

Root length was significantly increased in all treatments in both the safflower cultivars. The maximum significant increase was due to *Azospirillum* in combination with a quarter dose of chemical fertilizers, followed by *Azotobacter* in combination with a quarter dose of chemical fertilizers in cv. Thori. Also similar was the case with cv. Saif-32; however, the percentage increase was higher in cv. Thori. The treatments varied in the following order with respect to their effect on root length SPQ > BTQ > SPH > BTH > SP > BT. Among chemical fertilizer treatments, CFQ and CFH showed significantly higher root lengths than CFF at $P < 0.05$ (see Table 16.3 below).

The treatments CFH and CFQ possessed the highest shoot lengths, followed by CFF, SPH, BTH and BTQ, respectively (see Table 16.3 below). The PGPR had a positive and significant effect on the shoot length, such that both cvv. Thori and Saif-32 exhibited 10% increases in shoot length in response to SP inoculation as compared to the respective uninocualted controls.

Table 16.3. Effect of PGPR and chemical fertilizers on on root length (cm) and shoot length (cm) of safflower. The experiment was carried out in pots with three replicates

Treatments	Root Length (cm)		Shoot Length (cm)	
	CV. Thori	CV. Saif-32	CV. Thori	CV. Saif-32
C	17.33 ^{t}	20.83 ^{rs}	12.66 ^{l}	13.03 ^{k}
CFF	22.34 ^{q}	26.33 ^{m}	14.66 ^{fg}	17.16 ^{b}
CFH	23.33 ^{p}	33.33 ^{ij}	14.60 ^{g}	18.36 ^{a}
CFQ	34.00 ^{i}	37.33 ^{h}	17.33 ^{b}	18.66 ^{a}
SP	28.33 ^{l}	38.66 ^{g}	14.06 ^{i}	14.83 ^{efg}
SPF	33.00 ^{j}	23.00 ^{pq}	16.20 ^{c}	14.60 ^{g}
SPH	37.33 ^{h}	44.33 ^{c}	14.16 ^{hi}	17.50 ^{b}
SPQ	51.33 ^{a}	42.33 ^{d}	13.30 ^{jk}	14.86 ^{efg}
BT	25.00 ^{n}	23.66 ^{op}	12.66 ^{l}	13.60 ^{j}
BTF	30.00 ^{k}	37.83 ^{gh}	15.0 ^{ef}	14.50 ^{gh}
BTH	40.00 ^{f}	41.33 ^{e}	13.66 ^{j}	16.50 ^{c}
BTQ	48.00 ^{b}	24.33 ^{no}	15.53 ^{d}	16.16 ^{c}
SPBT	20.00 ^{s}	21.00 ^{r}	15.16 ^{e}	10.83 ^{m}
LSD	0.9631		0.3605	

All such means which share a common English letter are similar; otherwise, they differ significantly at $P < 0.05$.

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A.vinelandii*, BTF: *A.vinelandii* + full dose of chemical fertilizers, BTH *A.vinelandii* + half dose of chemical fertilizers, BTQ: *A.vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A.vinelandii*, LSD: Least Significant Difference.

The seeds inoculated with PGPR and supplemented with half and quarter doses of chemical fertilizers caused a significant increase in the leaf area as compared with the uninoculated control. The maximum increase (63%) was recorded in *Azotobacter* in combination with half and full doses of chemical fertilizers. Among the chemical fertilizers, CFH showed maximum and significant increase in leaf area over control. In both cvv. Thori and Saif, inoculataion with BT increased the leaf area by 32% and 27% over respective uninoculated controls. In cv. Thori, the co-inoculation treatment SPBT resulted in a significant increase (51%) in leaf area over uninoculated control (see Table 16.4 below).

Results show that leaf chlorophyll resulting from treatments with PGPR combined with half doses of chemical fertilizer were statistically similar to the chlorophyll content with a full dose of chemical fertilizers without PGPR. The maximum increase (45%) was recorded in *Azotobacter* in combination with a quarter dose of chemical fertilizer. In cv. Thori, the seed inoculation with *Azospirillum* (SP) showed 12% higher chlorophyll content as compared with BT. In cv. Saif, the SPBT co-inoculation was highly effective (36% and 60%) in improving the leaf chlorophyll content compared to single inoculations of SP and BT, respectively (see Table 16.4 below).

Table 16.4. Effect of PGPR and chemical fertilizers on Leaf Area (cm^2) and Chlorophyll contents (mg/g) of safflower. The experiment was carried out in pots with three replicates

Treatments	Leaf Area (cm^2)		Leaf Chlorophyll (mg/g)	
	CV. Thori	CV. Saif-32	CV. Thori	CV. Saif-32
C	10.67 ^{r}	10.13 ^{s}	0.054 ^{cdef}	0.042 ^{efgh}
CFF	17.40 ^{j}	16.14 ^{n}	0.062 ^{bcde}	0.077 ^{ab}
CFH	20.100 ^{e}	16.70 ^{lm}	0.035 ^{fgh}	0.042 ^{efgh}
CFQ	18.48 ^{fg}	14.08 ^{p}	0.0713 ^{bc}	0.036 ^{fgh}
SP	13.64 ^{p}	11.60 ^{q}	0.049 ^{cdefg}	0.035 ^{fgh}
SPF	18.00 ^{ghi}	17.60 ^{ij}	0.048 ^{defg}	0.051 ^{cdefg}
SPH	21.32 ^{d}	18.13 ^{gh}	0.070 ^{bcd}	0.036 ^{fgh}
SPQ	18.70 ^{f}	17.07 ^{kl}	0.038 ^{fgh}	0.034 ^{fgh}

BT	15.88 ^{n}	15.00 ^{o}	0.0433 ^{efgh}	0.0223 ^{h}
BTF	26.47 ^{b}	18.73 ^{f}	0.040 ^{efgh}	0.039 ^{fgh}
BTH	29.17 ^{a}	16.18 ^{n}	0.099 ^{a}	0.047 ^{efg}
BTQ	16.25 ^{mn}	17.78 ^{hij}	0.030 ^{gh}	0.036 ^{fgh}
SPBT	22.06 ^{c}	8.54 ^{t}	0.035 ^{fgh}	0.055 ^{cdef}
LSD	0.4995		0.0224	

All such means which share a common English letter are similar; otherwise differ significantly at $P < 0.05$.
C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A.vinelandii*, BTF: *A.vinelandii* + full dose of chemical fertilizers, BTH *A.vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*, LSD: Least Significant Difference.

Comparison of means for the carotenoid contents in leaves of cv. Thori showed that half doses of chemical fertilizers plus PGPR (SP and BT) were comparable to full doses of chemical fertilizers without inoculants. In cv. Saif, for the treatment of PGPR plus chemical fertilizers at all doses, only the inoculants SP and BT supplemented with half doses of chemical fertilizers exhibited the same carotenoids content as full doses of chemical fertilizers (see Table 16.5 below).

The leaf protein contents were markedly increased by different doses of chemical fertilizers as well as with SP and BT when supplemented with chemical fertilizers in both the cultivars (see Table 16.5 below). However, in cv. Thori a significantly higher increase (41%) in leaf proteins was recorded in treatment CFQ followed by SP. The effect of BT was more pronounced (13%) in the presence of half doses (BTH) of chemical fertilizers. In cv. Saif, a maximum increase (36%) in leaf soluble proteins was recorded in BTH as compared with the uninoculated control.

Table 16.5. Effect of PGPR and chemical fertilizers on leaf carotenoid (ug/ml) and protein contents (mg/g) of safflower. The experiment was carried out in pots with three replicates

Treatments	Leaf Carotenoid (ug/ml)		Leaf Protein (mg/g)	
	CV. Thori	CV. Saif-32	CV. Thori	CV. Saif-32
C	2.59 ^{cdef}	2.23 ^{defgh}	239.39 ^{efg}	242.48 ^{efg}
CFF	2.80 ^{bcde}	3.55 ^{ab}	263.85 ^{cdef}	296.56 ^{cde}
CFH	1.70 ^{ghi}	2.18 ^{defghi}	242.36 ^{efg}	273.31 ^{cdef}
CFQ	3.33 ^{bc}	1.96 ^{efghi}	403.08 ^{a}	289.31 ^{cde}
SP	2.23 ^{defgh}	1.86 ^{fghi}	315.83 ^{bcd}	267.94 ^{cdef}
SPF	2.21 ^{defghi}	2.49 ^{cdefg}	299.59 ^{cde}	325.17 ^{bc}
SPH	2.88 ^{bcd}	1.80 ^{fghi}	295.50 ^{cde}	252.05 ^{def}
SPQ	1.85 ^{fghi}	1.85 ^{fghi}	258.36 ^{cdef}	256.84 ^{def}
BT	2.13 ^{defghi}	1.34 ^{i}	231.96 ^{efg}	270.39 ^{cdef}
BTF	1.86 ^{fghi}	1.95 ^{efghi}	261.87 ^{cdef}	317.11 ^{bcd}
BTH	4.37 ^{a}	2.32 ^{efgh}	277.17 ^{cdef}	384.04 ^{ab}
BTQ	1.47 ^{hi}	2.02 ^{defghi}	271.44 ^{cdef}	249.25 ^{defg}
SPBT	1.64 ^{ghi}	1.91 ^{fghi}	205.10 ^{fg}	178.94 ^{g}
LSD	0.8823		72.482	

All such means which share a common English letter are similar; otherwise, they differ significantly at $P < 0.05$.

C: Control, CFF: Chemical fertilizers full dose, CFH: Chemical fertilizers half dose, CFQ: Chemical fertilizers quarter dose, SP: *A. brasilense*, SPF: *A. brasilense* + full dose of chemical fertilizers, SPH: *A. brasilense* + half dose of chemical fertilizers, SPQ: *A. brasilense* + quarter dose of chemical fertilizers, BT: *A.vinelandii*, BTF: *A. vinelandii* + full dose of chemical fertilizers, BTH *A. vinelandii* + half dose of chemical fertilizers, BTQ: *A. vinelandii* + quarter dose of chemical fertilizers, SPBT: *A. brasilense* + *A. vinelandii*, LSD: Least Significant Difference.

Discussion

Soil enzymes play a crucial role in the fertility of soil. Urease has been involved in urea hydrolysis and increases the utilization rate of nitrogen fertilizer (Klose & Tabatabai 1999). These results are in concurrence with those of Tian-cai et al. (2001) that show urease activity increases with nitrogen fertilizer treatment and reaches its peak at maximum dose of fertilizer. Madhaiyan et al. (2010) reported that co-inoculation of *A. brasilense* CW903 and *Methylobacteriumoryzae* CBMB20 significantly increased the soil urease activity. Previous studies indicate that the urease activity of *A. vinelandii* spp. is very closely connected with N inputs (Mikanová et al. 2009). It is evident that fertilizers assist the PGPR in stimulating the urease activity. The root length of cv. Thori was positively and significantly correlated (r = 0.529) with soil urease activity. Soil invertase is an important indicator of soil quality and varies with land type. Invertase cleaves sucrose into hexoses to supply cells with fuel for respiration and with carbon and energy for the synthesis of several diverse compounds. PGPR markedly increased the invertase activity in the rhizospheres of both cvv. Thori and Saif-32, and its activity was further augmented in the presence of CF in cv. Thori or with a half dose of CF in cv. Saif-32, but *Azotobacter* requires a full dose of CF for maximum response. Hui et al. (2004) reported that inorganic fertilizers increased the soil invertase and urease activities, being positively correlated with increased microbial activities in the rhizosphere. The coinoculation of *A. brasilense* and *A. vinelandii* (SPBT) was more effective than the single inoculation of either of the microbes. Previous studies show that *Azotobacter chroococcum* secreted invertase into the medium (Vega et al. 1991). The soil invertase activity of cv. Saif-32 exhibited a significant negative correlation (r = -0.496***) with MDA content. Interestingly, the soil invertase activity in the rhizpsphere of cv. Thori showed a significant negative correlation (r = -0.474***) with urease activity.

Soil phosphatase activity has often been anticipated as an indicator of the soil potential for organic phosphorus mineralization and biological activity. The soil phosphatase activity was significantly increased in cv. Thori in all the treatments with PGPR and CF except BTQ (*Azotobacter* with a quarter dose of chemical fertilizers). However, in cv. Saif-32 phosphatase the activity of soil was significantly increased by a full dose of chemical fertilizers alone, and also with SP and BT alone or in combination. The higher phosphatase activity in cv. Thori, even in the presence of a greater dose of CF, is worth mentioning.

It is noteworthy that in cv. Thori, CF at all doses, and even with a single application of SP and BT alone, effectively enhanced phosphatase activity. The effect of PGPR was similar to that of CF. A lack of a significant effect of half and quarter doses of CF alone or with SP may be attributed to P-fixation and decrease in activity. Previous studies show that agrochemicals significantly inhibit the population of PGPR (Balamurugan et al. 2010).

Malonyldialdehyde (MDA) is a cytotoxic product and indicates the degree of lipid peroxidation. The *Azotobacter* alone (BT) and in combination with a half dose of chemical fertilizers (BTH) were stimulatory to MDA contents. The increased MDA caused by a full dose of chemical fertilizers at post germinating stage was augmented by *Azospirillum*. PGPR inoculation has been observed to decrease the MDA content under stressful conditions which indicates its positive role in preventing lipid peroxidation (Habibi et al. 2010). This attribute of PGPR is important in oxidative stress and other stresses leading to ROS generation.

A. brasilense has the potential to synthesize plant hormone indole acetic acid (IAA) to stimulate root growth in soybean (Molla et al. 2001). The present data demonstrate that the stimulatory effects of PGPR were higher in the presence of half and quarter doses of chemical fertilizers. This might be because PGPR enhance the effect of organic and chemical fertilizers on agricultural production by increasing the activity of microbial biomass (Shata et al. 2007). Mia et al. (2010) reported a substantial increase in root length following PGPR inoculation. The beneficial effects of PGPR were also higher on shoot length in the presence of half and quarter doses of chemical fertilizers. This may be due to the fact that these bacteria directly affect the growth of the plants by improving the nitrogen absorption, the synthesis of phytohormones and the dissolving of minerals (Herman et al. 2008). Similarly, Ilyas & Bano (2010) reported increased shoot length in wheat when inoculated with *Azospirillum brasilense*.

Malik et al. (1997) found that *Azospirillum* inoculation could contribute about 70% of the total N requirement of the host plant which plays an important role in its protein build up. PGPR supplemented with a half dose of chemical fertilizers was found to be significantly stimulatory for chlorophyll production, and this was also true for *Azotobacter* sp. in cv. Thori. The half dose of chemical fertilizers supplemented with *Azospirillum* (SPH) was on the same level as a full dose of chemical fertilizers. It is evident from the results that lower doses of CF are required to augment the stimulatory effects of PGPR. The growth parameters, like shoot length and leaf area and contents of pigment fractions in plant seedlings, have been reported by bacterial inoculation (Karakurt &

Aslantas 2010). *Azotobacter* appeared more effective in cv. Saif-32 and responded better to chemical fertilizers, whereas *Azospirillum* was effective for cv. Thori. It is notable that the half and quarter doses of CF where most effective when applied alone, the effect of which was further enhanced with *Azospirillum* and *Azotobacter* inoculation.

Conclusion

The chemical fertilizers can be supplemented with PGPR to improve plant growth and soil health. The response of PGPR to an applied dose of chemical fertilizer depends on the variety as well as the type of PGPR and the parameter studied. The *Azospirillum* and *Azotobacter* can be supplemented with a quarter dose of chemical fertilizers for better plant growth. The performance of microbes as a consortium depends on the types of microbes combined. It is inferred that 50%–75% of chemical fertilizers can be saved by the application of PGPR. Therefore, the application of these PGPR in crop fields may be beneficial for the agriculturist and can be recommended as biofertilizing agents in the sustainable and environmentally friendly management of agricultural practices.

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PART IV.

MECHANISMS OF PGPR AND OTHER MICROBIALS

CHAPTER SEVENTEEN

INDIGENOUS PGPR AND BELOWGROUND MICROBIAL COMMUNITIES OF AN ORGANICALLY MANAGED DESERT AGRO-ECOSYSTEM

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Abstract

Microbial inoculants such as plant growth promoting rhizobacteria (PGPR) with biocontrol activity provide an environmentally friendly and promising strategy for plant and stress protection. For application in desert ecosystems, specific, drought-resistant strains are required. Therefore, we selected promising candidates from one of the most popular organic desert farms, Sekem in Egypt, using a hierarchical screening procedure. Isolates were characterised by their anti-phytopathogenic potential towards fungi (*Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium culmorum*), bacteria (*Ralstonia solanacearum*), and nematodes (*Meloidogyne incognita*). In general, the indigenous antagonistic potential was highly dominated by Gram-positive, spore-forming bacteria. The three most promising strains (*Streptomyces subrutilus* Wb2n-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14) were selected for *ad planta* field applications on German chamomile (*Matricaria chamomilla* L.) in comparison to three Gram-negative strains (*Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas*

rhizophila P69, *Serratia plymuthia* 3Re4-18), which were already evaluated under humid conditions. The influence of bacterial inoculants on plant growth promoting effects was evaluated based on blossom harvest yield. Secondary metabolites of chamomile blossoms, as well as structures of rhizosphere microbial communities, were compared between different treatments. In parallel to the selection and evaluation of strains, bacterial and fungal communities of the target habitat—the rhizosphere and endorhiza of medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L., *Solanum distichum* Schumach. & Thonn.)—were characterised in comparison to the surrounding field and desert soil. To get an insight into the community of the nitrogen-fixing PGPR, the *nifH* gene communities were deeply assessed with a pyrosequencing-based approach. Specificity could be observed for all investigated communities of each medicinal plant.

Introduction

The organically managed Sekem farms extend over an area of 6,000 ha in the north-eastern desert region of Egypt and have the largest market for organic products outside Europe and North America. The Egyptian fair trade company produces organic food, herbal teas and phyto-pharmaceuticals as well as non-edible products such as cotton textiles. However, in recent years soil-borne phytopathogens have caused significant yield losses. These pathogens comprise different taxonomic groups, e.g. fungi (*Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn, *Fusarium culmorum* [Wm. G. Sm.] Sacc.), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita* [Kofoid and White] Chitwood). Therefore, the objective of this project in general is to develop a biological control strategy against these soil-borne pathogens suitable for the arid conditions of desert farming (Köberl et al. 2013b).

A polyphasic ecological study revealed a high abundance of Gram-positive, spore-forming bacteria under arid conditions in general and an overwhelming dominance among the indigenous antagonistic potential (Köberl et al. 2011). Based on genomic fingerprints and antagonistic potential, 45 unique strains were selected of which 89% belonged to the *Bacillus/Paenibacillus* cluster. *Bacillus subtilis* was the main cultivated species from farm samples. In contrast, efficient antagonists from the surrounding desert soil belong mainly to *Streptomyces*. Details about their antagonistic activities against soil-borne phytopathogens were described in Köberl et al. (2013a). From this hierarchical screening, three promising antagonists were selected for evaluation in the field: *Streptomyces*

subrutilus Wb2n-11 isolated from desert soil from Sinai, *Bacillus subtilis* subsp. *subtilis* Co1-6 obtained from the rhizosphere of *Calendula officinalis*, and *Paenibacillus polymyxa* Mc5Re-14 isolated from the endorhiza of *Matricaria chamomilla*. These Gram-positive strains were tested for their plant growth promoting effect *ad planta* in comparison to three Gram-negative strains, which are already known for their beneficial plant-microbe interactions in humid soils (Lottmann & Berg 2001; Wolf et al. 2002; Kai et al. 2007; Zachow et al. 2010): *Pseudomonas fluorescens* L13-6-12 isolated from the rhizosphere of potato (*Solanum tuberosum*), *Stenotrophomonas rhizophila* P69 from the oilseed rape (*Brassica napus*) rhizosphere, and *Serratia plymuthia* 3Re4-18 from the endorhiza of potato. First, results on the impact of applied BCAs on the indigenous bacterial communities associated with roots of chamomile plants grown under organic management in Egypt were demonstrated in Köberl et al. (2013b).

Within the scope of this chapter we present the direct and indirect *in vitro* plant growth promoting abilities of all 45 genotypically different broad-spectrum antagonists isolated from arid areas and their tolerance to the abiotic stresses of desert habitats.

Materials and Methods

Belowground communities were studied at the organic desert farm Sekem (www.sekem.com) in Egypt (30°22’88"N, 31°39’41"E) in comparison to the surrounding desert soil (30°35'01"N, 32°25'49"E; 35°59'0"N, 41°2'0"E). The physico-chemical data of the soil is provided in Luske & van der Kamp (2009). The sampling strategy and isolation of bacteria are described in detail in Köberl et al. (2011). Based on *in vitro* antifungal potential and genomic diversity, 45 strains were selected (Köberl et al. 2011) and screened for their plant growth promoting abilities and tolerance to abiotic stress. Their affiliation to bacterial genera and isolation sources are summarised in Table 17.1 below.

Table 17.1. Genotypically unique antifungal isolates and their isolation sources

Phylum	**Genus**	**Rhizosphere**			**Endorhiza**			**Soil**		
		Mc	Co	Sd	Mc	Co	Sd	Sekem	Desert	
Firmicutes	*Bacillus*	4	3	4	8		4	7	2	32
	Paenibacillus	2			2		1	1	1	7
	Brevibacillus	1								1
Actinobacteria	*Streptomyces*								4	4
Proteobacteria	*Lysobacter*	1								1
		8	3	4	10	0	5	8	7	45

Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*.

Protease activity (casein degradation) was determined from clearing zones on skim milk agar (Berg et al. 2002). β-1,3-glucanase activity was tested by chromogenic azurine-dyed, cross-linked (AZCL) substrates (Megazyme, Bray, Ireland). Formation of blue halos was recorded until 5 d after incubation at 20°C. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested on chitin minimal medium (Berg et al. 2002). Clearing zones were detected 7 d after incubation at 20°C. The production of siderophores under Fe^{3+}-limited conditions was analysed using the plate assay developed by Schwyn & Neilands (1987). The sizes of orange haloes formed around the streaks were measured after 3 d of incubation at 20°C. Phosphate solubilisation was tested on the National Botanical Research Institute's phosphate growth agar (NBRIP) (Fürnkranz et al. 2009). The formation of clear halos was recorded 5 d after incubation at 20°C. Tolerance to abiotic stress was tested according to Marasco et al. (2012). Salt resistance was assessed by growth on nutrient agar (NA) containing 5%, 8% and 10% of NaCl. Tolerance to osmotic stress was evaluated by adding 10%, 15% and 20% of Poly-Ethylen-Glycol (PEG) to nutrient broth (Sifin, Berlin, Germany).

Results and Discussion

The isolate collection was screened for direct and indirect plant growth promoting abilities and resistance to abiotic stresses occurring in arid soils (see Table 17.2 below). Because of the promising antifungal properties (Köberl et al. 2011; 2013a), a special focus was given to the fungal cell wall degrading enzymes. Production of chitinases could be detected for 18% of the total antagonist collection, and *Lysobacter enzymogenes* Mc1-3 especially showed a high chitinolytic activity, as well as all *Streptomyces* isolates. Glucanase activity was shown for almost all antagonists (93%), and only the isolates identified as *Bacillus endophyticus* (Wb1-13 and Mc4-18) and *Brevibacillus limnophilus* Mc6-4 were unable to degrade β-1,3-glucan. Casein degradation by proteases could be shown for 69% of the strains (all isolates of the *Bacillus subtilis* group and *Lysobacter enzymogenes*). The production of siderophores was shown for almost all antagonists (89%) except three isolates of *Paenibacillus* spp. (Wb2-3, Mc5-5, and Mc2Re-16), *Bacillus endophyticus* Wb1-13 and *Streptomyces subrutilus* Wb2n-11. No isolate of the selected antagonists was able to solubilise inorganic phosphate. Concerning the tolerance to abiotic stress, 71% of the antagonist collection exhibited a high resistance to salt stress and 68% showed a remarkable tolerance to low water availability. Isolates

of *Bacillus* revealed the predominant role in halotolerance and together with *Brevibacillus* in resistance to drought stress.

While none of the antifungal strains showed all the assayed PGP activities, because no isolate was able to solubilise phosphate, 9% of the antagonists presented four of them. By rating all PGP properties, *Bacillus subtilis* Sb3-24 and *Bacillus atrophaeus* Sb3-13, both isolated from bulk agricultural soil, and *Bacillus subtilis* Mc3Re-13, isolated from the endorhiza of the chamomile, were identified as the most promising PGP antagonists. *Lysobacter enzymogenes* Mc1-3, isolated from the rhizosphere of the chamomile, was also positively tested for four PGP abilities, but in comparison to the *Bacillus* strains it revealed *Lysobacter* to have a low tolerance to the abiotic stresses of desert ecosystems.

The three strains selected for field application based on their outstanding antagonistic potential *in vitro* also revealed promising PGP activities and stress tolerances. *Bacillus subtilis* Co1-6 also exhibited, besides high drought and salt resistance, the production of siderophores as well as protease and glucanase activity. *Paenibacillus polymyxa* Mc5Re-14 had a lower tolerance to abiotic stresses in comparison to the *Bacillus* strain, but also tested positively for siderophores and glucanase activity. *Streptomyces subrutilus* Wb2n-11 showed the hydrolytic degradation of chitin and glucan.

Table 17.2. Distribution of plant growth promoting potential according to bacterial genera

Genus	Isolates	Direct and indirect PGP activities and tolerance to abiotic stress (%)										
		Prot	Gluc	Chit	Sid	P sol	5% NaCl	8% NaCl	10% NaCl	10% PEG	15% PEG	20% PEG
Bacillus	32	94	94	9	97	0	100	100	100	100	97	78
Paenibacillus	7	0	100	0	57	0	0	0	0	86	57	29
Brevibacillus	1	0	0	0	100	0	0	0	0	100	100	100
Streptomyces	4	0	100	100	75	0	50	0	0	nd	nd	Nd
Lysobacter	1	100	100	100	100	0	0	0	0	0	0	0
Total	45	69	93	18	89	0	76	71	71	95	88	68

Prot = protease activity; Gluc = glucanase activity; Chit = chitinase activity; Sid = siderophore production; P sol = phosphate solubilisation; nd = not determined.

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PART V.

NEW TECHNOLOGIES AND EXTENDED APPLICATIONS OF PGPR

CHAPTER EIGHTEEN

APPLICATION OF PCR-DGGE IN EXAMINATIONS OF THE MICROBIAL COMMUNITY ASSOCIATED WITH THE RHIZOSPHERE OF RICE AS AFFECTED BY SALINITY

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Abstract

The microbial communities associated with the rhizosphere of rice varieties as affected by salinity were analyzed using a molecular approach. The total community DNA was obtained from the rhizosphere samples using a commercially available soil DNA extraction kit, while the cultivable microbial community DNA from culture enrichments was extracted using the CTAB method. The V6 to V8 region of the Bacterial 16s rRNA gene was amplified through Polymerase Chain Reaction (PCR) and the amplicons were separated using the Denaturing Gradient Gel Electrophoresis (DGGE). The DGGE profiles from total community DNA revealed a higher number of species, while the DGGE profiles from

cultivable microbial communities revealed the dominance of a few microbial species due to selective enrichment. The DGGE profiles from total community DNA were analyzed and the banding pattern and intensity values obtained were used to calculate the Shannon index of diversity and the Simpson index of dominance. For all rice varieties, bacterial diversity is higher under saline conditions compared to normal conditions. In addition, the difference in bacterial diversity is greater at the reproductive stage compared with the vegetative stage. More importantly, the increase in bacterial diversity is greater for varieties that are tolerant to salinity compared to the salt-sensitive varieties. A database search of the sequenced bands revealed that the majority of the sequences from enriched samples have a high level of similarity to the well-studied members of the Gammaproteobacteria family, while the matches from total soil DNA include members of the Deltaproteobacteria family.

Introduction

Salinity is considered to be one of the most severe environmental factors limiting the productivity of agricultural crops. In fact, many researches have been done and are still being pursued to mitigate this complicated abiotic stress. Successful plant breeders in the International Rice Research Institute (IRRI) have developed rice varieties that can withstand salinity. These salt-tolerant lines were tested in different locations and those which survived under salt-stress and still retained desirable grain qualities were either released directly or bred into widely grown and popular local varieties. Aside from coming up with salt-tolerant lines, researchers also aim to uncover the underlying mechanisms of salt-tolerance. Some of these mechanisms, including the synthesis of osmoprotectants, transcription factors, and reactive oxygen species (ROS), have been discussed by crop physiologists in detail. One viewpoint that has not been fully explored is the fact that plants have the ability to interact with various groups of microorganisms that could possibly contribute to promoting tolerance to salinity. It has long been reported that plants have the ability to interact with specific groups of microorganism that exert beneficial effects on plant growth and are thus termed plant growth-promoting rhizobacteria (PGPR).

The rice rhizosphere represents the soil area under the direct influence of roots and serves as a dynamic circumjacent site with intense microbial activity. It also represents a favourable aerobic-anaerobic interface suitable for diverse groups of microorganisms. On the other hand, salinity was reported to be a major environmental determinant of microbial community

composition (Lozupone 2007). Unfortunately, very few researches have worked on the combined effects of salinity and plant type on the microbial community, and the effect of salinity on the microbial community associated with rice rhizosphere has not yet been established. There is also limited information on the microbial community associated with the rhizosphere of salt-tolerant and susceptible rice varieties.

Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates PCR-generated DNA products based on sequence differences of the DNA present in the sample (Hovig et al. 1991, as cited by Nakatsu 2007). In DGGE, a linear gradient of denaturant is used to separate PCR products which have the same size, but have different sequence compositions. Differential migration occurs due to the differences in the number of H bonds between complementary nucleotides holding DNA strands together. There are three H bonds between guanine and cytosine, and only two between adenine and thymine. This property makes A-T rich DNA denature faster compared with G-C rich DNA. As DNA strands separate, their migration becomes retarded in the gel. They will not migrate farther in the gel even with increasing electrophoresis time. With the aid of molecular techniques such as PCR-DGGE, several researches have been conducted to trace changes in the soil microbial communities.

This study aims to provide first-hand data on the microbial community associated with different rice varieties under normal and saline conditions using molecular techniques. The information generated from this study can help scientists determine if soil microorganisms can definitely contribute to the resistance and productivity of crops grown on salt-affected soils. This can also serve as a guide in the selection and utilization of beneficial soil microorganisms that can be applied in such conditions.

Methodology

Field experiment

Four rice varieties with various responses to salinity were used for this study. IR29 is an improved *indica* cultivar that is currently used as a salt-sensitive standard for salinity tolerance experiments, while PSB Rc 82, a popular variety among farmers, is categorized as moderately tolerant to salt stress. FL478, a recombinant inbred line derived from a population developed for salinity tolerance studies, has high tolerance to salinity stress, particularly at the vegetative stage of growth (Gregorio 1997). Salinas1 is a salt-tolerant rice variety released by IRRI in the Philippines

in 2010. The rice varieties were transplanted in concrete plots specifically designed for experiments on salinity tolerance. All plots were initially flooded with normal irrigation water prior to transplanting. Two weeks after transplanting, three plots were salinized using sea water with three plots remaining as control. Electrical conductivity (EC) was initially maintained at 6 to 7 dS/m during the vegetative stage and was raised to 9 dS/m during the reproductive stage.

Sample collection

Sample collection was performed during the vegetative and reproductive stages of rice. Three replicated rice plant samples were randomly collected for each variety on each plot. The plants were uprooted using a spade to ensure that the root system was still intact. The samples were placed in sterile polypropylene bags and sealed in a container half-filled with ice while being transported to the laboratory. To obtain the rhizosphere, the tillers at the side of each rice plant were pulled away from the central tiller while still inside the polypropylene plastic. The central tiller containing the rhizosphere soil was placed in a sterile flask containing 100 ml sterile saline solution (0.85% NaCl). Since three rice plants were uprooted for every treatment, the three tillers were placed in a single flask to generate a homogenized sample. To separate the rhizosphere soil from the roots, the flask was attached to a wrist action shaker and shaken for 30 min. After shaking, the roots were removed from the flask. The remaining soil suspension containing the rhizosphere sample was transferred to sterile 50 mL tubes and served as the source of samples for direct DNA extraction and culture enrichments from the rhizosphere. To obtain samples for direct DNA extraction, 2 mL of the soil suspension was transferred to a collection tube and centrifuged at 10,000 rpm for 1 min. The supernatant was removed and the soil pellet was washed with TE Buffer and stored at -20° prior to DNA Extraction. To prepare culture enrichment from the soil suspension, serial dilutions of up to 10^{-6} were prepared using sterile water as diluents. One hundred microlitre samples from dilutions 10^{-4} to 10^{-6} were inoculated in 10 mL vials of Nutrient Broth (NB) and Tryptic Soy Broth (TSB). The cultures were placed in a rotary shaker for 5 d at ambient temperature. After 5 d, 2 mL of cultures were transferred to a collection tube and centrifuged at 10,000 rpm for 1 min. The supernatant was removed and the cell pellet was washed with TE Buffer then stored at -20° prior to DNA extraction.

DNA extraction

A direct DNA extraction procedure from the rhizosphere was performed using a DNA Isolation Kit (MoBio Ultraclean™) following the manufacturer's instructions with some modifications. The CTAB method was used to extract DNA from the pellets obtained from culture enrichments (Wilson 1997). The quality and quantity of extracted DNA was assessed by both agarose gel electrophoresis and nanodrop.

PCR amplification

A 17-mer forward primer, designated 968f (5'AA CGC GAA GAA CCT TAC 3'), to which a 40-mer GC clamp (5'-CGCCCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G 3') was attached at the 5' end was combined with 1378r (5'GCG TGT GTA CAA GGC CCG GGA ACG 3') to amplify the bacterial 16S rRNA gene fragments (Brons & van Elsas 2008). In all PCR reactions, a total volume of 30 uL was used. PCR mixtures were composed as follows: 3 ul of 10x PCR buffer (iNtRON Biotechnology), 2 ul of dNTP mixture (2.5 mM each), 10 pmol of each primer, and 1 U of i-Taq^{TM} DNA polymerase. About 30 ng of template were used for each reaction. The PCR mixture was incubated in a G-STORM GS1 Thermal Cycler programmed as follows: initial denaturation of double-stranded DNA for 5 min at 94°C; 10 (touchdown) cycles consisting of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C with a decrease in the annealing temperature of 0.5°C per cycle; 25 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and extension for 30 min at 72°C. All amplification products were analyzed by electrophoresis in 1.0% (wt/vol) agarose gels, followed by staining with SYBR® Safe DNA Gel Stain for 20 min and viewing under the AlphaImager™ gel documentation system. A negative control (sterile deionized water) was always included in every PCR run to ensure that the PCR products formed from the samples and not due to contamination.

Denaturing gradient gel electrophoresis

DGGE was performed using a DCode™ (Bio-Rad, Hercules, Calif., USA) universal mutation detection system using 6% polyacrylamide gels with a gradient of 30% to 60% denaturing conditions. Electrophoresis was initially started at 100 V for 10 min and was then lowered to 60 V and allowed to run for 15 h. The gel was then stained with ethidium bromide for 5 min and was destained with deionized water for 20 min. The gel was

then viewed under (Bio-Rad, Hercules, California, USA) and photographed using QuantityOne™ 1-D Gel Analysis Software (Bio-Rad). Dominant bands were excised using sterile plastic forceps through the guidance of the image produced by the QuantityOne software. Excised bands were then transferred to carefully labelled Eppendorf tubes containing 50 μL HPLC water. It was then macerated using a sterile yellow tip and centrifuged for 30 s, and then incubated at 37°C for 30 min in a water bath. The resulting DNA was stored at below-freezing temperatures prior to re-amplification. The bands were re-amplified under the same conditions described using the reverse primers.

DGGE profile analysis

The indices of diversity and dominance of bacterial populations were calculated using the images of DGGE profiles. To determine the diversity and evenness of the bacterial communities, the Shannon index of diversity (H') and Simpson Index of Dominance (D) were calculated for each of the gel lanes using the trace quantities generated by Quantity One™ 1-D Gel Analysis Software Ver. 4.6.8 (Bio-Rad, Hercules, California, USA).

16S rDNA sequence analysis

DGGE bacterial DNA fragments were submitted to Macrogen, Inc. in Korea for further purification and sequencing using the 1378r primer. The quality of the sequences obtained was then assessed using Finch TV Version 1.4.0 (Geospiza Inc.) Chimera check with Decipher was the program used to check for chimeric sequences (http://decipher.cee.wisc.edu/index.html). Processed sequences were compared to those available in GenBank using the BLAST tool to determine the putative identities of the excised bands and to determine their evolutionary hierarchy. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura et al. 2011). Multiple alignment and refinement of sequences were performed using ClustalW built in the MEGA software. The phylogenetic tree was constructed based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using Maximum Composite Likelihood as the nucleotide substitution model. *Methanobacterium oryzae* was used as the out-group and the tree topology was evaluated by 1,000 replications of bootstrap analysis.

Results and Discussion

Field performance

A few days after salinization, symptoms of salt stress were already seen on IR29 and PSB Rc82 plants sown on salinized plots. The plants were smaller compared to those planted in normal plots. The tips of the leaves initially turned white, and later progressed as tip burns. While the first two varieties showed a serious negative response to salinity at the vegetative stage, FL478 and Salinas 1 remained green and healthy just like the plants under normal conditions. During reproductive stage, all plants showed symptoms of salt stress such as reduced tillering and spikelet sterility. It can be noticed, however, that Salinas 1 performed better than the other varieties (see Fig. 18.1 below).

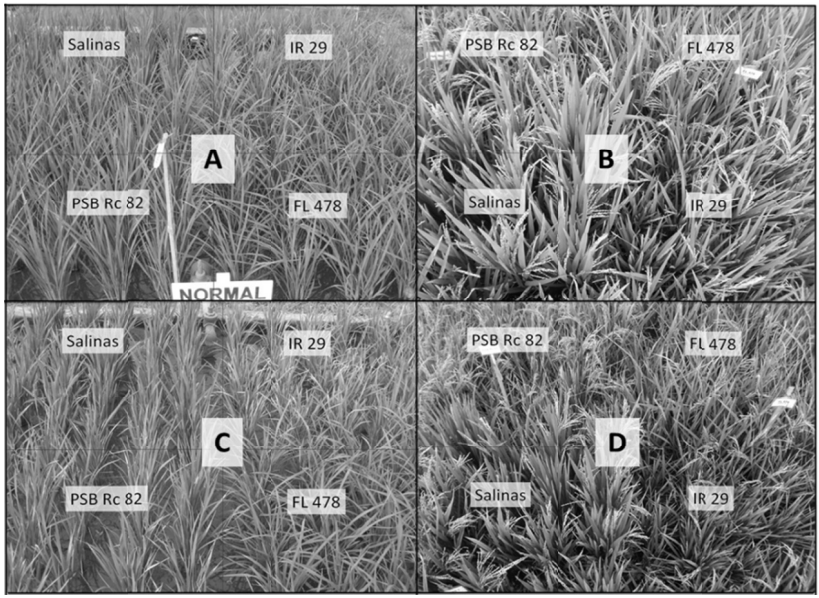

Fig. 18.1. Field performance of the four rice varieties under normal and saline conditions during the vegetative and reproductive stage. (a) vegetative stage under normal condition; (b) reproductive stage under normal condition; (c) vegetative stage under saline condition; (d) reproductive stage under saline condition.

Salinity affects rice growth at all stages of its development but to variable extents. Rice is tolerant during germination, becomes very sensitive during the early seedling stage, gains tolerance during tillering growth, and again becomes sensitive during pollination and fertilization, and becomes more tolerant during grain filling and maturity (Ponnamperuma 1984, as cited by Flores 2004). IR29 is known to be highly sensitive to salinity and will not survive if salinization happens during the early seedling stage. Thus, to ensure that all varieties will survive, transplanting was done using 21 day old rice seedlings, and salinization was done after 1 week. The set of samples under the vegetative stage was taken during the active tillering phase of the rice plant. Although all the varieties survived an electrical conductivity of 7 dS/m, the sensitive varieties showed signs of salt-stress unlike the salt-tolerant varieties. During the reproductive stage, however, all the varieties showed signs of salt-stress such as spikelet infertility, but Salinas1 performed better than the other varieties. The results of the field experiment agree with the expected results based on the known properties of the rice varieties and are thus acceptable sources of samples for the microbial community analysis.

PCR-DGGE banding pattern analysis

A great difference in the banding patterns between the DGGE profile from the total rhizosphere DNA and from sample enrichments was observed (see Fig. 18.2 below). The DGGE profile generated from sample enrichments has few but very distinct bands, an indication of the abundance of some species of soil microorganisms due to selective enrichment. In addition, the banding patterns among samples based on soil salinity level, rice variety and growth stages are very different from one another. On the other hand, the DGGE profiles generated from direct DNA extraction have multiple bands, indicating great diversity. The bands are also not as intense as the samples from culture enrichments and seem to have similar banding patterns across salinity level and rice variety during the vegetative stage. Looking at the reproductive stage, however, it is evident that the DGGE profiles from salinized samples differ from the normal samples. The presence of an intense band in the rhizosphere sample from FL478 under saline conditions during the reproductive stage is very noticeable, in addition to some intense bands from IR29, FL478 and Salinas1. The Shannon index (H') of diversity values and the Simpson index of dominance (D) values obtained are plotted. For all rice varieties, the Shannon index of diversity is higher under saline conditions compared to normal conditions. It is also very noticeable that, when comparing

normal and saline conditions for the same rice variety, the difference in bacterial diversity is greater during the reproductive stage. More importantly, the increase in bacterial diversity is greater for varieties that are tolerant to salinity. Interestingly, the Simpson index of dominance is lower during saline conditions as compared with that of the normal condition, an indication of greater evenness in the microbial community as the plants are subjected to salinity.

BLASTn search and phylogenetic analysis

A total of 34 bands were excised and sequenced. Thirteen bands were from NB culture enrichments, fourteen from the TSB culture enrichment and seven from the total rhizosphere DNA. The obtained sequences were compared to those available in GenBank using the BLASTn tool and the 16s ribosomal DNA database for bacteria and archaea to determine the putative identities of the excised bands and to determine their evolutionary hierarchy. Identification at the genus level was defined as a 16S rDNA sequence similarity of $\geq$ 97% with that of the prototype strain sequence in GenBank (May 1999). According to Tajima et al. (1999), phylogenetic clustering of bacterial groups, rather than similarity value, should be used as a guide for defining bacterial taxa. A total of 30 sequences were considered for phylogenetic analysis to clarify their taxonomic position based on neighbour-joining methods. Eighteen conserved 16S ribosomal DNA sequences of known microorganisms were downloaded using BLAST to perform sequence comparisons. Multiple sequence alignment of the known microorganisms and the 30 sequences were carried out using the ClustalW alignment function of MEGA and a phylogenetic tree was constructed using the neighbour-joining function of the same software.

The BLASTn search and the phylogenetic analysis gave the same results. Out of the 27 sequences from the DGGE profiles from culture enrichments, 26 (96%) are members of the Gammaproteobacteria family and the remaining sequences belong to Firmicutes. On the other hand, 4 out of the 7 sequenced bands (57.14%) from the DGGE profile of the DNA directly extracted from the rhizosphere belong to Deltaproteobacteria, and only 14% belongs to Gammaproteobacteria.

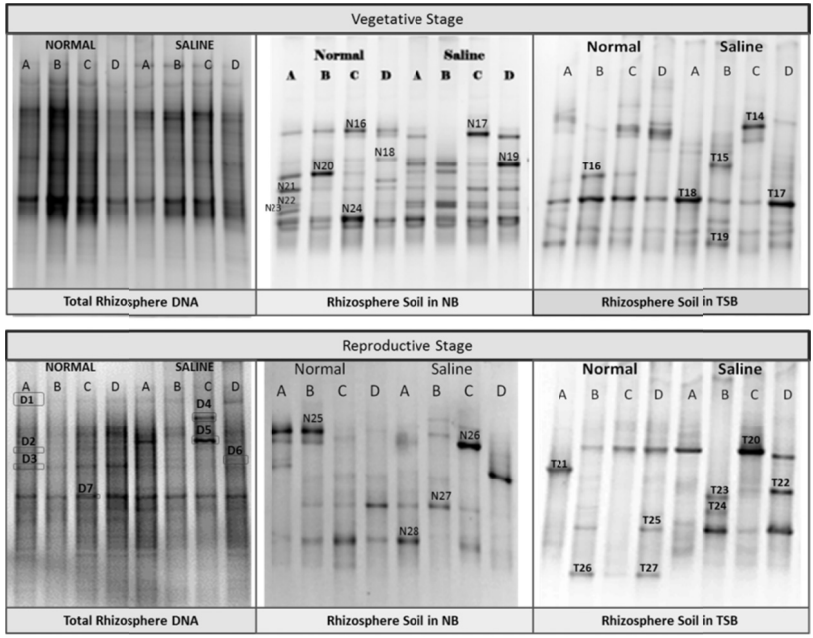

Fig. 18.2. DGGE profiles generated from total rhizosphere DNA and culture enrichments using the primer pair 968fGC-1401r under normal and saline conditions for the vegetative and reproductive rice growth stages. (A) IR29; (B) PSB Rc 82; (C) FL478; (D) Salinas 1

Table 18.1. Putative identities of the excised DGGE bands based on the 16s ribosomal DNA database for bacteria and archaea using the Basic Local Alignment Search Tool for nucleotides (BLASTn)

Code	Length (bases)	Closest Match under 16s rDNA Seq for Bacteria and Archaea	Genbank Accession #	Query Coverage	Percent Similarity
N16	**285**	*Shewanella japonica*	NR_025012	100%	98%
N17	**366**	*Several Aeromonas sp*		100%	100%
N18	**198**	*Klebsiella variicola*	NR_025635	98%	94%
N19	**365**	*Aeromonas hydrophila*	NR_042155	100%	96%
N20	**365**	*Several Providencia sp*		100%	99%
N21	**361**	*Aeromonas hydrophila*	NR_042155	100%	98%
N22	**364**	*Aeromonas hydrophila*	NR_042155	100%	99%

N23	**367**	*Aeromonas hydrophila*	NR_042155	100%	99%
N24	**368**	*Aeromonas salmonicida*	NR_043324	100%	99%
N25	**367**	*Serratia marcescens*	NR_041980	100%	99%
N26	**236**	*Providencia vermicola*	NR_042415	100%	100%
N27	**256**	*Several Aeromonas sp*		100%	99%
N28	**366**	*Several Aeromonas sp*		100%	99%
T14	**364**	*Serratia nematodiphila*	NR_044385	100%	100%
T15	**363**	*Bacillus thuringiensis*	NR_043403	100%	100%
T16	**367**	*Aeromonas punctata*	NR_029252	100%	99%
T17	**237**	*Aeromonas jandaei*	NR_037013	99%	99%
T18	**366**	*Aeromonas hydrophila*	NR_042155	100%	100%
T19	**324**	*Aeromonas jandaei*	NR_037013	100%	100%
T20	**323**	*Providencia stuartii*	NR_024848	100%	99%
T21	**324**	*Morganella morganii*	NR_028938	100%	100%
T22	**195**	*Cedecea davisae*	NR_025243	100%	93%
T23	**367**	*Aeromonas hydrophila*	NR_042155	100%	96%
T24	**367**	*Several Aeromonas sp*		100%	100%
T25	**238**	*Several Aeromonas sp*		100%	100%
T26	**297**	*Several Aeromonas sp*		100%	99%
T27	**304**	*Aeromonas jandaei*	NR_037013	100%	99%
D1	**365**	*Tolumonas auensis*	NR_026283	100%	92%
D2	**264**	*Geobacter argillaceus*	NR_041019	99%	92%
D3	**210**	*Desulfuromonas alkaliphilus*	NR_043575	100%	96%
D4	**379**	*Geobacter pickeringii*	NR_043709	99%	86%
D5	**370**	*Caloramator coolhaasii*	NR_043576	100%	85%
D6	**192**	*Desulfuromonas acetexigens*	NR_024955	100%	96%
D7	**202**	*Thermolithobacter carboxydivorans*	NR_044770	100%	89%

Conclusion

The results of this study suggest the interplay of salinity level and rice variety as a factor affecting the characteristics of the microbial community in the rhizosphere. A detailed physiological analysis of the four rice

varieties is highly recommended. An analysis of the exudates that the rice varieties release under salt stress can provide a great insight into how the microbial community in the rhizosphere is affected by salinity. According to Sung et al. (2006), plants are capable of increasing soil microbial population through root exudates, which are used by microorganisms as a nutrient source for their growth. The chemical compositions of root exudates vary, not only among different plant species, but also among cultivars of the same plant species (Grayer et al. 2004). Besides, the chemical compositions of root exudates are a result of different factor interactions, such as nutritional status, age, stress, diseases and environmental factors that affect the microbial community associated to the rhizosphere (Mahaffee & Kloepper 1997). In the rice rhizosphere, the Shannon index of diversity is noticeably higher under saline conditions compared to normal conditions, which is inconsistent with previous reports that salinity reduces bacterial diversity as it favours the more tolerant bacterial species. In addition, when comparing normal and saline conditions for the same rice variety, the difference in bacterial diversity is greater during the reproductive stage. More importantly, the increase in bacterial diversity is greater for varieties that are tolerant to salinity. Based on the results of this study, it is possible that under salt stress, the plants may exude substances which tend to enhance the bacterial diversity. Salt-tolerant varieties may also have a greater capability to produce more varieties of root exudates that can promote the growth of more diverse and, possibly, beneficial microorganisms.

The dominance of several Deltaproteobacterial bacteria in the rhizosphere is also interesting. Geobacter species specialize in making electrical contacts with extracellular electron acceptors and other organisms. The proficiency of Geobacter species in transferring electrons to insoluble minerals, electrodes and possibly other microorganisms can be attributed to their unique "microbial nanowires," pili that conduct electrons along their length with metallic-like conductivity (Lovley 2011). Since salinity is associated with increased amounts of solutes and ions in the rhizosphere, it appears that the Geobacter species plays an important role in saline environments. It is therefore necessary to further analyze the community and possibly focus on the Deltaproteobacterial species. Analyzing the archaeal community in the rhizosphere is also recommended.

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CHAPTER NINETEEN

POTENTIAL OF PGPR FOR THE BIOREMEDIATION OF PB IN MAIZE *(ZEA MAYS L.)*

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Abstract

The efficiency of phytoremediation can be enhanced by the application of plant growth promoting rhizobacteria (PGPR) which can interact, sequester the heavy metal and detoxify. This study is an attempt to evaluate the metal pollution index of the rhizophere soil of maize collected from a waste dumping site of the Attock oil refinery and to determine the relative accumulation of metals in the plant part of maize. In addition, the role of PGPR isolated from the rhizosphere of maize was studied on the uptake and accumulation of metals under induced Pb stress. The soil was polluted with Pb, Cr, Zn and Ni, but only the Pb content was higher in the leaves of maize grown in polluted areas. The PGPR isolates were able to tolerate and survive well both at 100 as well as 500 ppm Pb added to culture media. The Pb treatment altered the uptake and translocation of Pb but inhibited the uptake and accumulation of Cr, Zn, Co and Ni, and induced the accumulation of Ni, Mn and Cu in leaves. The inoculation with both the PGPR showed the complete inhibition of Pb accumulation in leaves and also reduced the Pb-induced accumulation of Zn, Mn, Ni, Co and Cu. Both the PGPR also overcame the Pb-induced inhibition in Cr accumulation. The PGPR2 was more efficient. The Pb treatment decreased the accumulation of Ca, Mg, Fe and K in roots but not in leaves. The PGPR1 overcame the Pb-induced inhibition in the accumulation of Ca, Mg and Fe in roots and leaves. The change in the morphology of PGPR in response to Pb stress has been discussed. The PGPR can be used as bio inoculants of the plant for bioremediation purposes.

Introduction

The use of plants to decontaminate soil with heavy metals through biological processes has been reported as safe, economically feasible and effective technology (Alkorta & Garbisu 2001). In maize crops, roots and shoots are major sinks for Pb ions since these parts are not consumed as human food, but because of its higher biomass and faster growth, maize can be used for phytoremediation. The present investigation evaluates the metal pollution index of the rhizophere soil of maize collected from a waste dumping site of the Attock oil refinery and determines the relative accumulation of metals in the plant part of maize. Further, it evaluates the performance of two PGPR isolates from the rhizosphere of maize grown in polluted areas on the uptake and translocation of metals under induced Pb stress and compares this with IAA applied under the Pb stress.

Materials and Methods

Determination of plant nutrients

The extraction of the elements in plant samples was done by Perchloric-acid digestion as described by Allen (1974) and their concentrations were measured using an Atomic Absorption Spectrophotometer (AA-670 Shimadzu, Japan).

Enrichment factor (EF)

The EF for heavy metals in soil was determined using the modified formula of Loska et al. (2004) and Buat-Menerd et al. (1979).

Geo-accumulation index

The Geo-accumulation index (Igeo) was calculated according to Muller (1969).

Metal pollution index (MPI)

The Metal Pollution Index and pollution load index were determined according to Usero et al. (2000) and Linson et al. (1980).

Isolation of PGPR from rhizosphere

Rhizosphere soil (10 g) from 6 cm depth of the upper soil surface was mixed with 9 mL of autoclaved distilled water, and stirred with a magnetic stirrer. The suspension was centrifuged at 3,000 rpm for 10 min and the supernatant was used for making decimal dilutions. An aliquot of the dilution was spread on selective media to produce a bacterial colony. The calculation for colony-forming units of PGPR was made according to James (1978). For inoculation, maize seeds were soaked for 3–4 h in the broth culture (24 h old) of PGPR isolates with 109 colonies/ml.

Treatments made

A pot experiment was conducted to compare the effects of Pb stress alone and in combination with plant growth promoting rhizobacteria (PGPR) and with Indole acetic acid used as a seed soaking treatment prior to sowing. Plants were harvested after 40 d of sowing. The following treatments were made—C = Un-inoculated unstressed control; T1 = Plants treated with Pb (500 mg/L), T2 = PGPR Isolate 1 + Pb (500 mg/L), T3 = PGPR Isolate 2 + Pb (500 mg/L), T4 = Seeds soaking with IAA(10^{-5} M)+Pb (500 mg/L).

Results

The results presented in Figs. 19.1 and 19.2 below reveal that Na, K, Pb, Cr, Zn and Ni were significantly higher in the polluted soil as compared to non-polluted soil/control, but the Fe and Mn were significantly lower. The roots of plants from polluted areas showed higher Na, K, Mg, Pb, Cr, Zn, Ni, Fe and Mn, but the Ca contents have no significant differences to the control. The magnitude of increase was higher for Cr, Zn and K. Less Na was translocated to the leaves of polluted plants and the Ca content was consistent with the control. The Mg and K contents were much higher but the Pb, Fe and Mn were 3-6X higher, the Cr was 3x less with no marked increase in Zn, Co, and the Ni was consistent with the control.

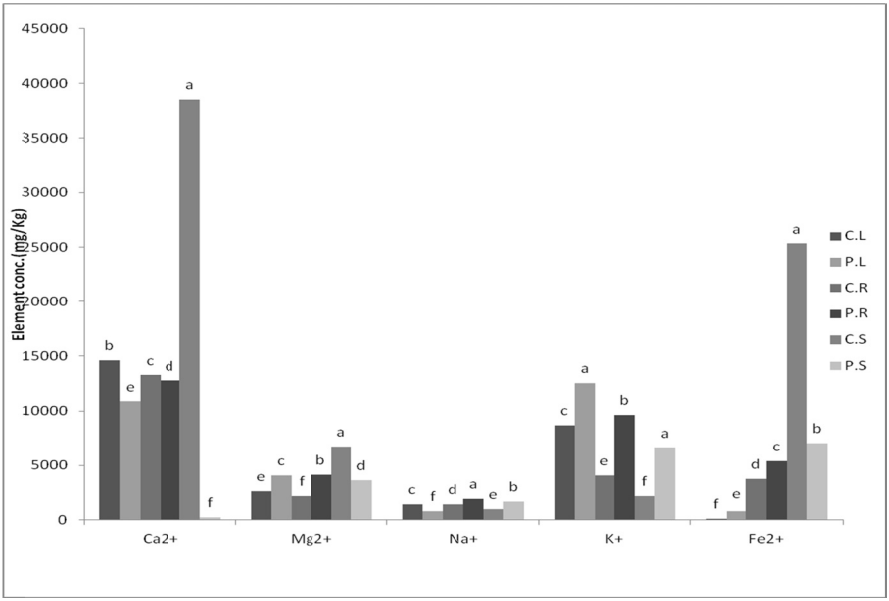

Fig. 19.1. Comparison of Nutrient composition (mg/kg) of rhizospheric soil, roots and leaves of Maize collected from Polluted area with non-polluted area for the elements Ca^{+2}, Mg, Na^{1}, K , Fe
C.L. = Control Leaf, C.R.= Control Root, C.S. =control Soil, P.L.= Polluted Leaf, P.R.= Polluted Root, P.S. = Polluted soil

Analysis of variance was conducted for all the treatments with three replicates at level of significance $P<0.05$. The data represents the mean of three replicates.

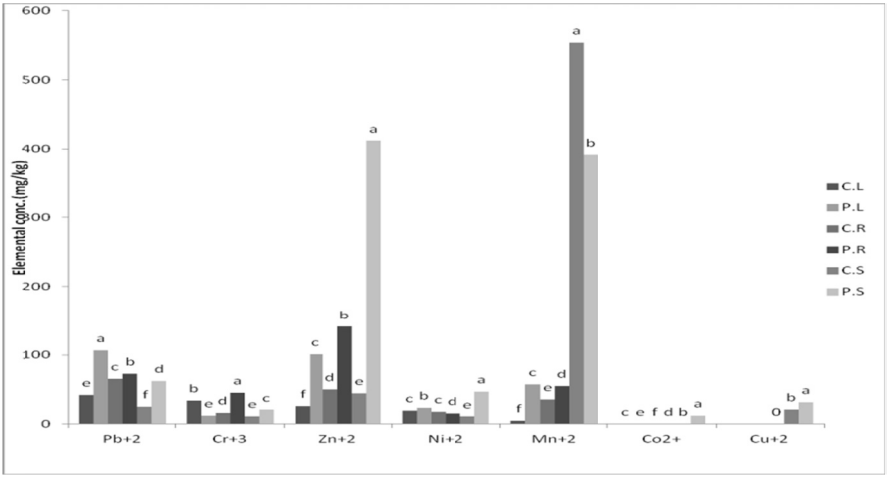

Fig. 19.2. Comparison of elemental composition (mg /kg) of rhizospheric soil, roots and leaves of Maize collected from Polluted area with non-polluted area Leaves for the elements Pb, Cr, Zn, Ni, Co, Cu, Mn.
C.L. = Control Leaf, C.R.= Control Root, C.S. = Control Soil, P.L.= Polluted Leaf, P.R.= Polluted Root, P.S.= Polluted soil

Analysis of variance was conducted for all the treatments with three replicates at level of significance $P<0.05$. The data represents the mean of three replicates.

In the leaves of *Zea mays* plants (see Figs. 19.3 and 19.4 below), the Pb treatment increased the accumulation of Pb by 32% of the control with concomitant inhibition in the accumulation of Cr and Co, which became undetectable, and Zn showed a further 49% decline. The Ni, Mn and Cu were significantly higher. The PGPR inoculation reduced the Pb accumulation in leaves and ameliorated the Pb-induced inhibition of Cr accumulation, Ni and Co were undetectable, and Cu was reduced significantly over the Pb treatment alone. The application of PGPR2 further increased the Cr accumulation such that the value was similar to that of control, whereas the response of other elements were similar to that of PGPR1. The response of Pb stressed plants to IAA was similar to that of PGPR1, except that Ni accumulated at a similar magnitude comparable to untreated unstressed control, and Cr accumulation was further enhanced as compared to that of PGPR1.

The roots of Pb-treated plants had taken up and retained Pb 100% higher than control, the PGPR1 decreased the Pb uptake, and accumulation

by 50% of the untreated control and PGPR2 augmented the decrease further by 50% of the PGPR1. 1AA treatment has a similar magnitude of reduction in Pb uptake as PGPR1.

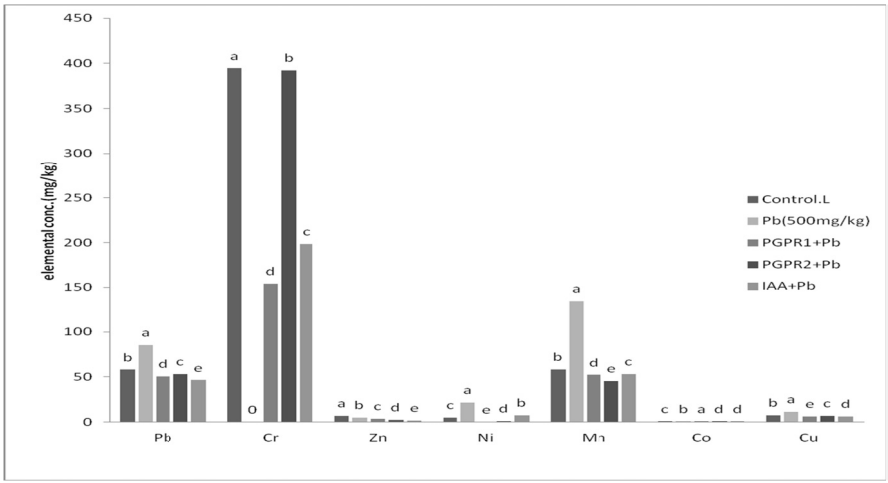

Fig. 19.3. Effect of different treatments on elemental composition of the elements Pb, Cr, Zn, Ni, Co, Cu, Mn in *Zea mays*.L Leaves

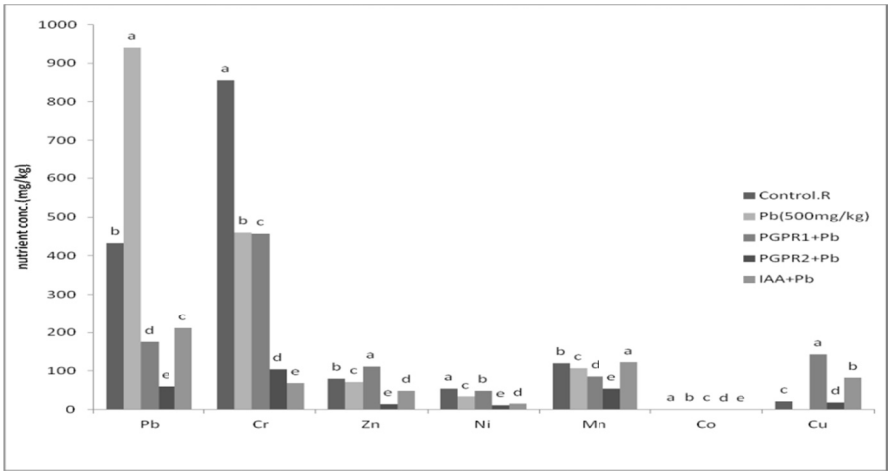

Fig. 19.4. The effect of different treatments on nutrient composition of the elements Pb, Cr, Zn, Ni, Co, Cu, Mn in *Zea mays*.L Roots

The Cr uptake was reduced by 50% in Pb treatment. PGPR1 was ineffective in ameliorating the adverse effects of Pb on Cr accumulation; however, PGPR2 decreased the Cr accumulation by 10% of the control and IAA was an inhibitor. The Zn accumulation was less in roots of Pb treated plants, but PGPR1 stimulated the uptake of Zn in roots and decreased the Zn uptake by root. IAA performed better than PGPR2 but was less stimulatory when compared to PGPR1. A similar pattern was followed for Ni accumulation. The uptake of Ni was less in Pb treated plant roots, and PGPR2 and IAA significantly inhibited Ni accumulation greater than 50% of the polluted plant. The Mn accumulation was decreased by Pb treatment and both the PGPR decreased the accumulating further, PGPR2 being the most effective. IAA had no significant effect over control. The accumulation of Cu was completely inhibited by Pb treatment while PGPR1 and IAA showed a manifold increase in Cu accumulation, while PGPR2 accumulated Cu similar to control.

The PGPR1 and PGPR2 overcame the Pb induced decline (see Figs. 19.5 and 19.6 below) of K accumulation in leaves, but both have similar magnitude of increase for Mg. The effect of 1AA was less pronounced. Higher Na was accumulated in the leaves of Pb treated plants. All other treatments had a decreased Na accumulation, such that Na was not detectable in PGPR inoculated plants exposed to Pb stress. The Fe content of Pb treated leaves was higher than 50% of control, and the PGPR1 and PGPR2 exhibited equal magnitude of inhibition for Fe^{++} content. IAA was stimulatory. In the roots of Pb treated plants the Ca, Mg, Fe and K accumulation were decreased. The greatest decrease was in Ca accumulation while K was undetectable in Pb treatment. The PGPR1 and IAA partially ameliorated the Pb induced inhibition in Ca, Mg, Fe and K contents. The PGPR2 was less effective.

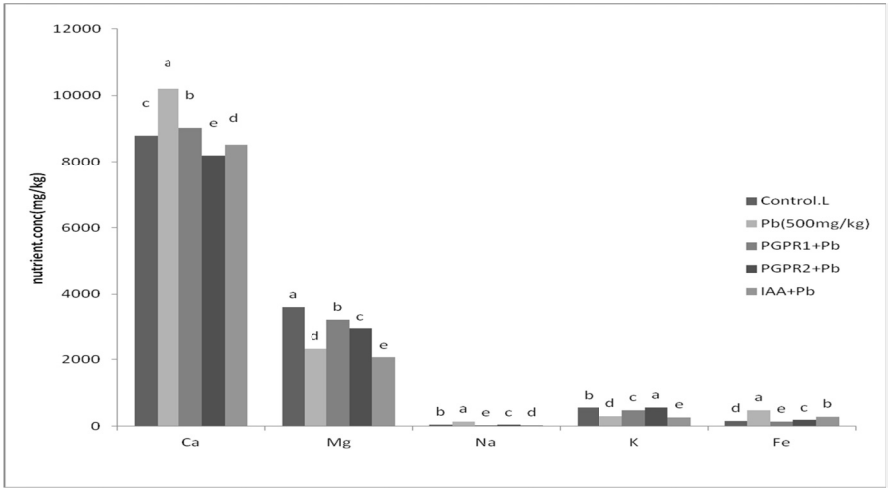

Fig. 19.5. Effect of different treatments on Leaves Nutrient composition of the elements Ca, Mg, Na, K, Fe in *Zea mays*.L

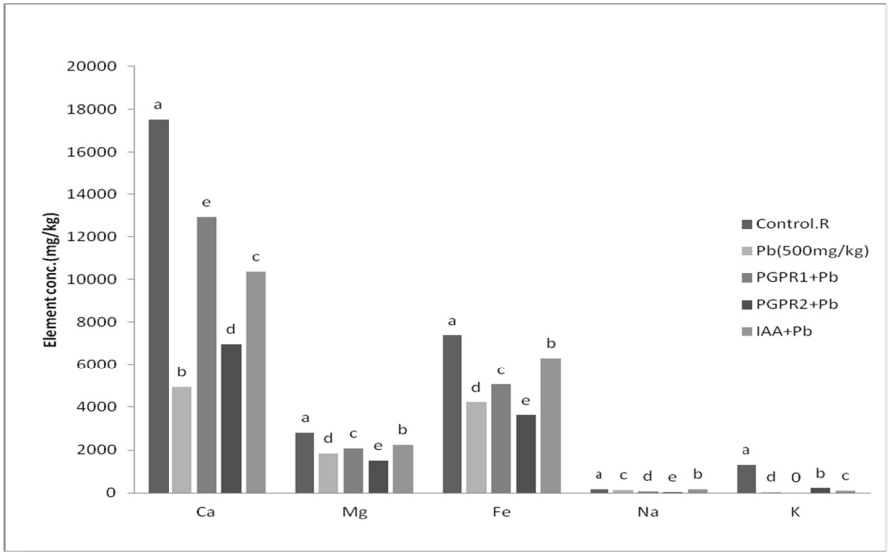

Fig. 19.6. Effect of different treatments on Root Nutrient composition of the elements Ca, Mg, Na, K, Fe in *Zea mays*.L

Discussion

Metal Pollution Index (see Fig. 19.7 below) calculations showed that the polluted area soil has a value below 2 so it comes under the category of moderately polluted. The PLI for the sampled soils indicated that polluted area soil is moderately polluted as according to a standards value below 2. Similarly, MPI values are also very low, indicating that there is negligible metal pollution while the Risk Indices (RI) values are below 50, which falls under the category of less ecological risk by Zhu et al. (2008).

The Risk Indices (R1) values of sampled soil were below 50, falling under the category of less ecological risk. The RI of sediment samples from the three zones of the Yangtze River catchment of Wuhan, China were also lower than 300, suggesting that more sediment samples exhibited low and moderate ecological risk of heavy metals (Hakanson 1980).

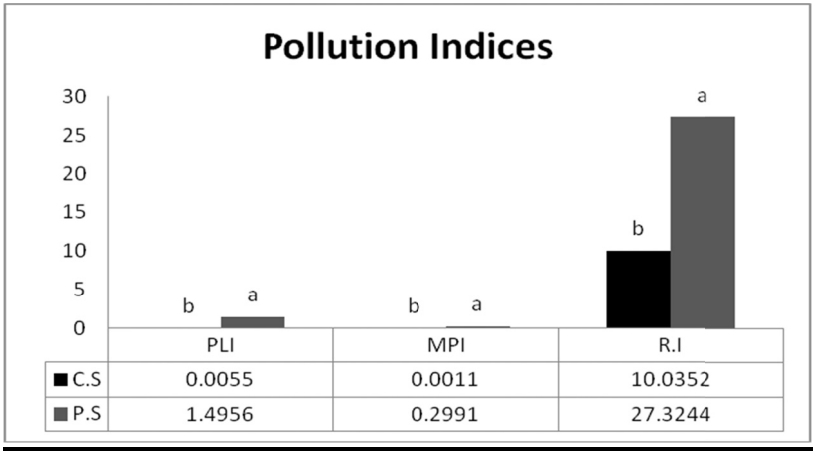

Fig. 19.7. Indicates the pollution indices calculated from the maize soil collected from control and polluted sites. PLI stand for Pollution Load Index, MPI = Metal Pollution Index, R.I. = Ecological Risk Assessment index

The PLI for the sampled soils indicated that polluted area soil is moderately polluted as according to standards value below 2. Similarly, MPI values are also very low, indicating that there is negligible metal pollution, while the RI values are below 50 which falls under the category of less ecological risk (Zhu et al. 2008).

The results showed values of Cr, Ni and Cu for control soil as below zero, which means there was leaching of these metals, and Cr had a value above 1, indicating that it is found in considerable amounts. The control soil for Pb and polluted area soil for Pb, Zn, Ni and Cu had values falling under the category of less polluted for other metals, and more polluted for Pb and Zn.

The data demonstrate that leaves of maize plants from the polluted soil of the Attock Oil Refinary accumulated higher concentrations of Pb, Mn and Fe. The PGPR reduced the Pb induced accumulation of Pb, Ni, Co and Mn, Ca, Mg, and enhanced the accumulation of K.

Pb treatment enhanced Pb uptake in roots and its translocation to leaves, as well as modulating the uptake and translocation pattern of other elements, such as Cr and Ni.

Inoculation treatment with isolates PGPR1 and PGPR2 reduced the absorption by roots and translocation to leaves, with PGPR2 being more effective. Seed soaking treatment with IAA demonstrates the significant inhibition on translocation of Pb to leaves. An et al. (2004) demonstrated that certain metals have a composition for the update of other metals and also organise the action of others, and subsequently the accumulation of the first metal can either be stimulated or decreased when present in mixture (Peralta-Videa et al. 2002). The Pb stress also decreased the uptake of Ni and Co in roots but significantly increased its translocation to leaves. IAA reduced the translocation of Pb but retained higher Pb in roots than that of PGPR inoculations. The uptake and translocation of other heavy metals Cr, Zn, Ni and Co was significantly affected.

The two isolates differed in their efficiency. The isolate PGPR1 has no significant effect on Cr uptake over that of Pb stressed plants but reduced its transport to leaves, while the isolate PGPR 2 decreased its uptake in roots but increased its translocation to leaves. The total IAA reduced the total uptake of all the elements. The PGPR1 isolate decreased translocation to the leaves but increased retention in roots. In the case of heavy metals, isolate PGPR1 completely inhibited transport of Ni to leaves, whereas the PGPR2 isolate greatly reduced the uptake as well as transport to leaves.

The Fe and Mn uptake and translocation were increased following IAA treatment under Pb stress as compared to Pb treated plants. Both the total uptake and translocation for Ni and Co were reduced following PGPR2 isolate inoculation. As reported by other workers, the level of Ca and Mn were decreased with Pb treatment in maize roots, as in Norway spruce needles, and the levels of Ca and Mn decrease with Pb treatment, which could be a result of a decrease in the number of root tips and sites for

apoplastic solute flux through the endodermis. Pb physically blocks the access of many ions from the absorption sites of the roots (Godbold & Kettner 1991).

Accumulation of Zn was significantly decreased by 50% Pb stress. Inoculation treatments further reduced the Zn accumulation in leaves. The IAA treatment had the lowest Zn accumulation and roots showed similar Zn content at basal level as that of leaves. The Pb treatment decreased the Zn content whereas the PGPR1 significantly increased the Zn content over the control.

The isolate PGPR2 showed a marked decrease in Zn whereas 2x Pb accumulated in root 3x less in isolate PGPR1, and 15x less in isolate PGPR2. Indole Acetic Acid (IAA) has more Pb than isolate M1. Cr was absent or non-detectable in the Pb treatment. The isolate PGPR1 decreased the Pb accumulation by 50%, whereas isolate PGPR2 has no significant effect over that of Pb stressed plants. The IAA treatment also reduced the Cr accumulation as compared to control, but the Cr content was higher than that of M1 isolate treatment. In roots, the Cr content was reduced by half in plants treated with Pb only or with M1 isolates and Pb. When the isolate PGPR2 was decreased the Cr accumulation was 4% higher and the IAA treatment had 85% less Cr accumulation. Yildirim (2011) demonstrated that the Bacterial inoculations with manure significantly increased uptake of macronutrients and micronutrients.

The enrichment factor for sampled soils for Pb and Zn present EF ranging from 1 to 3 was similar as for the International Bridge border between Spain and Portugal, with a mean value of 1.8 for Pb and 2.5 for Zn. Higher EF ($2 < EF < 4$) for Pb were determined in zones near the bridge downstreams (See Fig. 19.8 below). This phenomenon may be related to the Pb pollution (leaded gasoline) associated with traffic on the bridge. Upstream, the EF maintain values in the same range followed by a dilution effect of seawater reaching values even lower than 1 at the river mouth.

The geo accumulation index for the sampled soils showed that Cr and Ni were in a lower proportion than Cu, Pb and Zn, which (1 geo) means that the soils were moderately contaminated (see Fig. 19.9 below). As indicated by other workers, Cr and Ni appear to be the least contaminated elements by the geo-accumulation index in all cities of China, while Cu, Pb, Zn and Cd show the highest I geo values for most cities (Binggan et al. 2010).

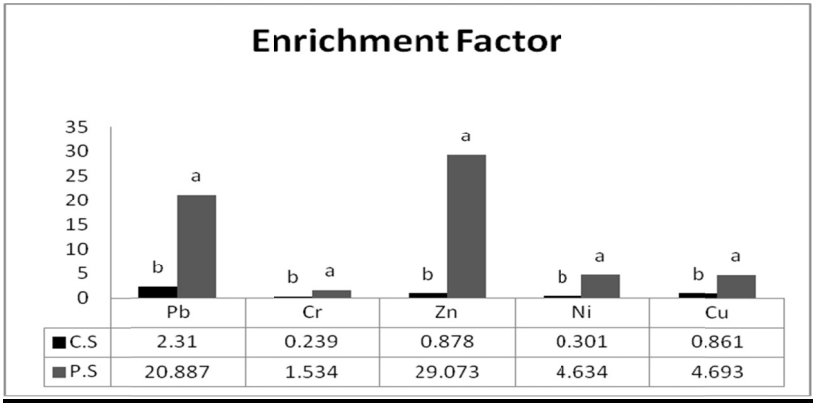

Fig. 19.8. Indicates the Enrichment factor for sampled soils. The results show values of Cr, Ni and Cu for control soil as below zero which means there was leaching of these metals and Cr had a value above 1, indicating that it is found in a considerable amount. The control soil for Pb and polluted area soil for Pb, Zn, Ni and Cu had the values which fall under the category of less polluted for other metals and more polluted for Pb and Zn

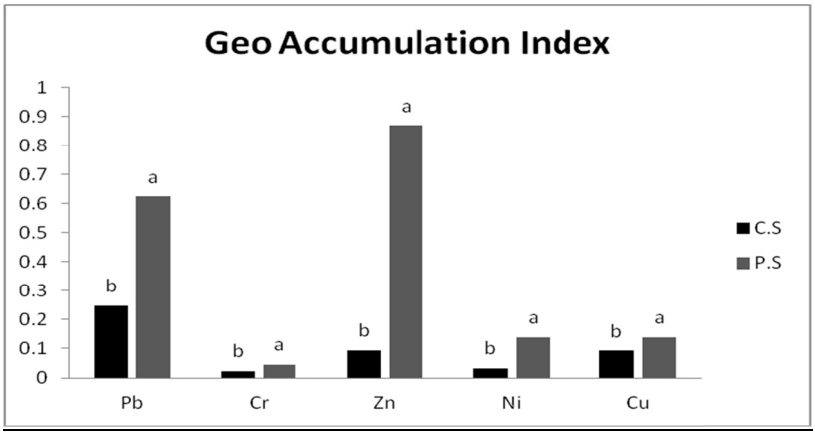

Fig 19.9. Ihe Geo accumulation Index for the sampled soils was less than 1, meaning the soils are uncontaminated to moderately contaminated (Muller 1969)

In general Cr and Ni appear to be the least contaminated elements by Geo-accumulation index in all the cities, while Cu, Pb, Zn and Cd show the highest I geo values for most cities (Binggan 2010).

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CHAPTER TWENTY

INDUCTION OF PLANT IMMUNE RESPONSES BY GASEOUS COMPOUNDS AGAINST PLANT PATHOGENS AND INSECT PESTS

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Abstract

Systemic acquired resistance (SAR) is a plant self-defence mechanism against broad-ranged pathogens and insect pests. Among chemical SAR triggers, plant and bacterial volatiles are promising candidates due to highly effective and cheap chemicals with relatively low concentration related to agrochemicals. However, for agricultural usage, high evaporation rate after application, plant growth alteration and inconsistent effectiveness are major pitfalls that need to be considered before their large-scale application to manage diseases. In this study we provide new evidence of volatile organic compound (VOC) mediated SAR against both a bacterial angular leaf spot pathogen, *Pseudononas syringae* pv. Lachrymans, and a sucking insect aphid, *Myzus persicae*, in the open field without changing plant growth until harvesting the fruit yields. Unexpectedly, the drench of two VOCs 3-pentanol and 2-butanone into cucumber seedlings caused a significant increase of the ladybird beetle that is known to be a natural enemy of aphid. The defence-related gene, *CsLOX*, was induced in volatile treatments indicating a triggering of the oxylipin pathway responding to the emission of green leaf, volatile to natural enemies. Our results demonstrate that VOCs are good resources for preventing plant diseases and insect damage by elicitation of SAR, even in the open field.

Introduction

Induced resistance is an innate resistance to enemies including insects and microbial pathogens (Schneider et al. 1996). Until now, two types of induced resistance have been indicated, including systemic acquired resistance (SAR) and induced systemic resistance (ISR). First, systemic acquired resistance (SAR) was discovered by Ross (1991), who discovered plant systemic induced resistance while studying virus-plant interactions. In addition to microbe-elicited SAR, later studies revealed that plant and microbe-derived chemical compounds also trigger induced resistance. Scientists also called this type of induced resistance SAR. Such compounds include chitin, ergosterol, glucans, lipopolysaccharides, proteins, peptides, salicylic acid and sphingolipids. Secondly, plant growth-promoting rhizobacteria (PGPR), a certain group of root-associated bacteria (rhizobacteria) conferring enhanced plant growth and yield under greenhouse and field conditions, elicited induced resistance referred to as ISR.

In the present study, we attempted to identify effective VOCs with the capacity to elicit SAR for the open field application of insect and bacterial volatiles to control plant pathogens and insects as well as minimize the negative effects of plant growth. Due to the ineffectiveness of a bacterial volatile 2,3-butanediol on induced resistance in cucumber plants, there is a demand to isolate effective VOCs from a greater number of known bacterial volatiles even under open conditions. Here, we provide new evidence on VOC-elicited SAR against both a microbial pathogen and an insect pest at the same time. The drench application of 1 mM 3-pentanol and 0.1 μM 2-butanone on cucumber seedlings consistently triggered systemic plant defence against *Pseudomonas syringae* pv. lachrymans in cucumbers. The examination of plant defence responses after VOC treatments revealed the upregulated expression of the *CsLOX1* gene. *CsLOX1*, a cucumber lypoxygenase, is a marker protein of oxylipin pathway for indirect defence that releases green leaf volatile attracting natural enemies, resulting in protection from herbivores (Dicke & Baldwin 2010). After conducting experiments, a natural outbreak of aphid population occurred. The cucumber applied with VOCs achieved a higher number of ladybird beetles and led to a significant reduction of aphid density compared to water control. Our results indicate that VOCs can be used to manage plant disease and insect pests through the elicitation of induced resistance even in the open field.

Materials and Methods

Plant and bacteria preparation and greenhouse experiment

Cucumber plants (*Cucumis sativus* L. cv. backdadagi) were cultivated in the open field under natural conditions. The seeds were directly seeded into soilless medium (Punong Co. Ltd, Gyeongju, Korea). The germinated seeds were transplanted into large pots (d = 30 cm; height = 30 cm). Chemical treatment that elicited induced resistance in cucumbers was carried out as previously described (Lee et al. 2012). Cucumber seedlings were treated by a direct drench-application of 50 mL of 1 mM and 10 μM 3-pentanol and 0.1 μM and 10 nM 2-butanone at 14 d post seeding (dps). Treatments with 0.5 mM BTH and water were used as positive and negative controls, respectively.

Assessment of angular leaf spot disease and aphid infestation

For pathogen challenge, a culture of the compatible bacterial pathogen *Pseudomonas syringae* pv. lachrymans (OD_{600} = 1 in 10 mM $MgCl_2$) was spray-challenged on the cucumber leaves until drop-out at 7 d after drench-application of two chemicals to the cucumber roots 21 d after seeding. The severity of symptoms was scored from 0 to 5 as follows: 0—no symptoms; 1—below 20% diseased area; 2—21–40 diseased area; 3—41–60 diseased area; 4—61–80 % diseased area; 5—above 81% diseased area of the whole leaf. Bacterial pathogens were cultured overnight at 28°C in King's B mediμM supplemented with the appropriate antibiotics (100 μg/ml). Chemical treatment on cucumber roots was performed as described previously (Lee et al. 2012). As a positive control, roots were treated with 0.5 mM benzothiadiazole (BTH). Intact cucumber leaves were used for non-stress treatments. Following inoculation with the pathogen, plants were returned to the growth chamber and leaf tissue was harvested 0 and 6 h post-inoculation with *P. syringae* pv. lachrymans. The experiment had a completely randomized design with 10 replications and was independently repeated four times. To investigate whether the two VOCs elicit plant immunity to aphid feeding, we counted aphid numbers that naturally occurred in 2012, Daejeon, S. Korea. 0.5 mM BTH was used as a positive control. The total number of nymph and adult aphids was counted at 34 d 34 d after seeding. The experiments were repeated with similar results.

Results and Discussion

3-Pentanol and 2-butanone-elicited SAR against *P. syringae* and aphid

Drench application of 3-pentanol and 2-butanone resulted in reduction of disease severity at 28 d post seeding (dps) 7 d after spray-challenge of *P. syringae* pv. lachrymans. Disease severity of cucumber plants treated with 1 mM 3-pentanol, 0.1 μM 2-butanone, and 10 nM 2-butanone caused 24%, 26% and 17% respectively, and fewer symptoms than water control. The 10 μM M 3-pentanol application did not show any statistical ($P = 0.05$) difference. BTH as a positive control displayed a similar level to that of 0.1 μM 2-butanone.

Among many bacterial metabolites, bacterial volatiles have recently reported candidates for induced resistance against diverse plant pathogens. The representative example is a bacterial volatile 2,3-butanediol. Previous work demonstrated that 2,3-butanediol produced *Bacillus* spp. and *P. chlororaphis* induced systemic plant defences against *Pectobacterium carotovorum* subsp. *carotovorum* in Arabidopsis and tobacco (Ryu et al. 2004; Han et al. 2006). *P. chlororaphis* failed to elicit ISR against *P. syringae* pv. tabaci in tobacco (Han et al. 2006). Thus, the authors concluded that 2,3-butanediol was not effective against biotrophic pathogens but was against necrtrophic pathogens. However, recent reports provided controversial evidences on the effectiveness of 2,3-butanediol protected Arabidopsis seedlings against a biotrophic pathogen *P. syringae* pv. tomato (Rudrappa et al. 2010). The 2,3-butanediol may mediate induced resistance depending on plant species dependency.

In applying a large-scale trial of VOCs in the open field, the weak point was the inconsistent results depending on plant species and rapid evaporation rate after treatments. The field trials with 2,3-butanediol on tobacco revealed a successful reduction of symptom development (Cortes-Barco et al. 2010a; 2010b). Our preliminary study obtained that 2,3-butanediol was rarely effective on cucumber plants regarding biotrophic pathogen *P. syringae* pv. lachrymans (data not shown). The further screening of VOC-mediated SAR on the same pathogen allowed for the selection of two new candidates, 3-pentanol and 2-butanone. The two volatiles have not reported SAR capacity on any plant species. In our laboratory, root application of 3-pentanol also elicited SAR against *Xanthomonas axonopodis* pv. vesicatoria in pepper (unpublished data). For 3-pentanol-mediated SAR, the concentration (1 mM) was relatively higher than that of 2-butanone but a similar level to that of BTH.

Unexpectedly, in mid-summer 2011 an outbreak of aphids occurred in cucumbers in the Daejeon area of South Korea, resulting in the plants being severely infested, especially on the newly developing leaf tissues. We measured aphid damage by counting aphids (individual nymph and adult) number per leaf. At 34 dps, the number significantly decreased in all treatments compared to control treatment, which recorded 361 nymphs and 19 adults per leaf. Soil drench of 1 mM and 10 μM 3-pentanol and 0.1 μM and 10 nM 2-butanone exhibited 21, 34, 25 and 112 nymphs and 1.0, 3.0, 0.1 and 2.7 adults respectively (see Fig. 20.1).

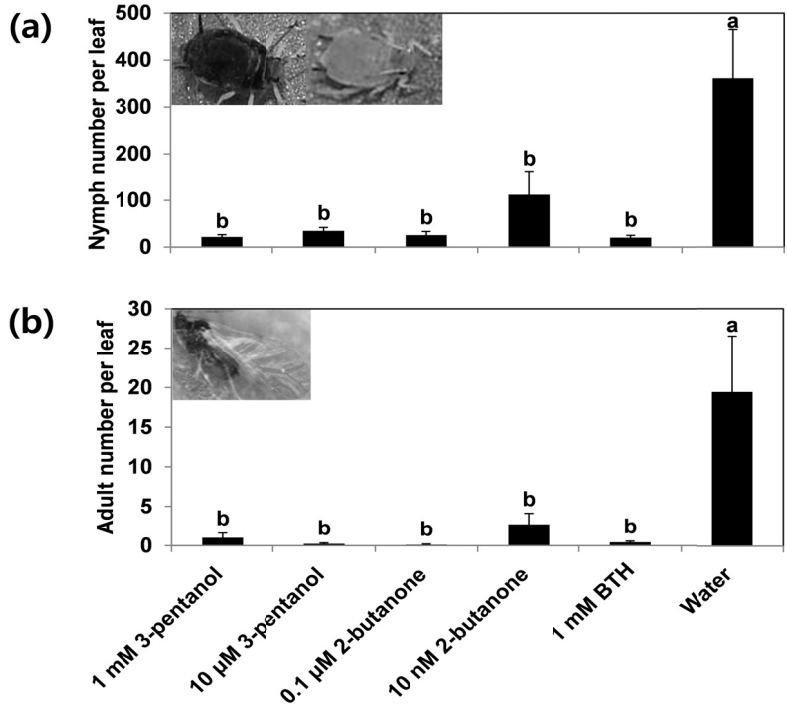

Figure 20.1. 3-Pentanol and 2-butanone confer induced resistance against aphids in cucumber: a, Nymph number; b, Adult number

3-Pentanol and 2-butanone cause increase in number of ladybirds

When we counted aphid number on the cucumber leaves in each time point, the seven spotted ladybird beetles were differently detected depending on each treatment. The mean number of cucumber leaves treated with 1 mM 3-pentanol, 0.1 μM 2-butanone and 10 nM 2-butanone were 7.2, 7.1 and 7.0, respectively, while the control plant was detected at 2.8 (see Fig. 20.2). The two VOC and BTH treatments are of lower numbers compared to the control treatment. Only 10 μM 3-pentanol application did not show (P = 0.05) any different treatments. BTH was ineffective on the reduction of ladybird numbers compared to the control. The ladybird beetle is the best-known natural enemy of insect pests, including the aphid. In the closed system such as greenhouse conditions, and sometimes in the open field, the natural enemy has often been used to control insect pests in crop plants (van Lenteren 2000). For instance, the predatory generalist ladybird beetle has provided biological control of aphids (*Marcosiphulm euphorbiae*) on roses grown in greenhouse (Snyder et al. 2004). In this system, ladybird beetles successfully reduced aphid populations during outbreaks without altering the density of a specialist parasitoid ratio, indicating the minimization of ecological disruption by affecting intraguild predation.

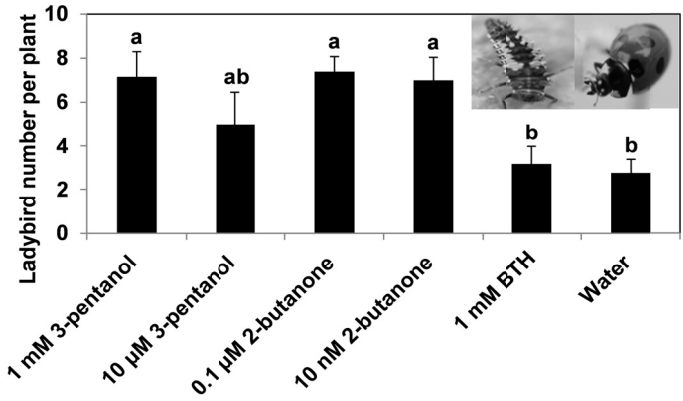

Figure 20.3. 3-Pentanol and 2-butanone treatments increase the number of ladybird beetles

Acknowledgements

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CHAPTER TWENTY-ONE

EFFICACY OF *PSEUDOMONAS FLUORESCENS* STRAINS IN ENHANCING DROUGHT TOLERANCE AND YIELD IN PEANUTS

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and provide benefits through growth promotion. Inoculation of crop plants with certain strains of PGPR at an early stage of development improves biomass production through direct effects on root and shoot growth. *Pseudomonas fluorescens* are gram negative PGPR that have wide applicability due to various mechanisms of action. The present study focuses on the evaluation of various *P. fluorescens* strains on growth promotion, drought tolerance and yield enhancement in peanuts. Eleven *P. fluorescens* strains were isolated from peanut rhizosphere and evaluated on peanut cultivar "Narayani." A greenhouse study was conducted in a RCBD with 11 treatments and four replications per treatment during the kharif season of 2011. Prior to seeding, the seeds were treated with PGPR strains at a concentration of $1X10^9$ cfu/ml, and control seeds were treated with water. Water Use

Efficiency (WUE) traits and biometric characteristics, such as root growth and pod yields, were increased with one strain (IFT 30) over control. The strain enhanced WUE in terms of a high SPAD Chlorophyll Meter Reading (SCMR) and low specific leaf area (SLA) values and specially, increasing the pod yield by 10.7% compared to control. During the rabi season in 2011–12, the strain (IFT-30) was tested for its efficacy under imposed end-of -season moisture stress conditions along with foliar sprays of 2% urea, and Kaoline 2%. Our results validated the efficacy of the *P. fluorescens* strain in improving drought tolerant traits in terms of higher WUE, total chlorophyll content, photosynthetic activity, plant water status, and oxidative stress tolerance over the control, as well as 2% urea spray under stress imposed field conditions. Similarly, higher biometric traits such as plant height, shelling percentage, pod yield, and harvest index were also recorded compared to other treatments. Such ameliorating effects of *P. fluorescens* strain on the peanut crop can be attributed to altered concentrations of plant growth hormones, cell protecting enzymes and improved nutrient uptake capabilities. Experiments are in progress at field level to confirm the growth promotion, yield enhancement and drought tolerance activity of the *P. fluorescens* strain in peanuts.

Introduction

Peanut (*Arachis hypogaea* L.) is the fourth most important oil seed in the world, cultivated mainly in tropical, subtropical and warm temperate climates. It adapts to a wide range of environments and is cultivated over about eight million hectares in India. It is predominantly grown as a rainfed crop (70%), hence average productivity is low due to the regular occurrence of drought. Due to climate change, the shift in the rainfall pattern further shortens the available soil moisture especially under rainfed areas.

Drought is one of the most important abiotic stresses that cause adverse effects on the productivity of crops. Recent physiological studies provide hints that peanut is a relatively drought tolerant crop having improved water use efficiency mechanisms (Nautiyal et al. 2002). The plants with water saving or mining abilities mainly refer to the effective use of water resources in the process of growth and development. There is every need to explore methodologies to sustain cell water status and growth under drought conditions.

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and directly regulate plant physiology by mimicking synthesis of phytohormones such as IAA,

cytokinins, gibberellins and inhibitors of ethylene. *Pseudomonas* spp. is a potential PGPR identified in improving the growth of agricultural crops. They can also survive under dry conditions, and hence are highly useful for rainfed agriculture (Saharan & Nehra 2011).

Materials and Methods

Rhizospheric soil samples were collected from peanut crops and *P. fluorescens* were isolated using standard protocols. Confirmation of PGPR strains was carried out using standard procedures. These PGPR were further screened for their efficacy in containing major soil borne pathogens in peanuts under *in-vitro* conditions. Superior strains with high antagonism were selected for growth promotion studies under greenhouse conditions. Growth promoting characterization of these superior strains was also carried out using standard procedures for traits such as production of Indole Acetic Acid (IAA), siderophores, cellulase and phosphate solubilization capacity.

A greenhouse study was conducted during kharif season 2011 in an RCBD fashion with twelve treatments and four replications to evaluate these superior PGPR for enhancement of growth and yields. A popular peanut cultivar "Narayani" was used in the present study. Prior to seeding, seeds were treated with PGPR strains at a concentration of 1×10^9 cfu/ml. Eleven PGPR strains were screened in this experiment. Control seeds were treated with SDW. The seeds were later sown and seedlings were allowed to grow under GH conditions in plastic pots using standard procedures. At 6O DAS surrogate methods of water-use efficiency (WUE), *viz*., SPAD Chlorphyll meter readings (unit less) and specific leaf area (cm^2/g) were recorded using standard protocols. Biometric characteristics such as plant height, and root traits such as root length, root dry, and number of pod yields/plants, were recorded at harvest.

The selected potential strain was further validated under field conditions under imposed end-of-season moisture stress conditions (50–80 DAS) during Rabi (December 22, 2011) along eight treatments. The treatments were sown in an RBD design and replicated thrice. Two check plots were maintained as one plot was irrigated and the other was unirrigated. The data on drought tolerance traits such as SCMR, SLA, percentage relative water content (RWC), total leaf chlorophyll content (mg/g), and oxidative stress enzyme super oxide dismutase (SOD) was recorded as per standard protocols after imposing moisture stress. After harvest, biometric characters such as pod yield and harvest index were recorded.

Results and Discussion

The root traits of any crop play a pivotal role in drought tolerance in terms of water mining abilities. Among the root traits, root length and root dry weight are reported to be directly correlated with water mining abilities as well as drought tolerance in crops. The data revealed significant variability among *P. fluorescens* strains in root length and root dry weights. Highest root length was recorded with Pf strain IFT-30 (33.8 cm) followed by IFT-24, IFT-34, IFT-17, IFT-14, IFT-12, IFT-32 and are significantly superior over control. Similarly, root dry weight was also highest with IFT-30 (7.1 g/plant) followed by strains IFT-12, IFT-14, IFT-24 and are significantly superior than control (see Table 21.1 below).

Table 21.1. Screening of various *Pseudomonas fluorescens* isolates in promoting root, shoot growth and water-use efficiency traits in peanut seeds (CV: Narayani) bacterized with Pf strains at 1 × 10^9 cfu/ml

PGPR Identity	Root Length(cm)	Root Dry Weight (g/plant)	SLA (cm^2/g)	SCMR
IFT-1	21.6	2.3	226.9	36.5
IFT-2	23.6	2.6	205.4	36.0
IFT-11	24.6	2.0	186.2	35.8
IFT-12	28.8	4.1	205.2	38.9
IFT-14	30.0	3.8	165.5	38.6
IFT-16	28.5	2.5	206.1	39.3
IFT-17	29.6	2.7	190.3	38.2
IFT-24	30.8	4.0	219.6	39.8
IFT-30	33.8	7.1	202.5	40.6
IFT-32	25.8	1.9	176.9	38.8
IFT-34	30.3	2.3	185.6	39.9
Control	27.0	2.1	200.4	36.8
S.E.M	1.12	0.44	4.06	0.41
CD(5%)	3.21	1.42	12.36	1.04

SCMR and SLA are simple and reliable surrogate methods of measuring WUE, and low SLA and high SCMR are established to have direct correlation with higher crop yields under moisture stress conditions in many crops including peanuts (Wright et al. 1994). The variability among the Pf strains for SCMR is 35.8–40.6 and SLA is 185.6–226.9 (cm^2/g). Among the eleven Pf strains IFT-30 recorded a lower SLA (202.5 cm^2/g) and the highest SCMR (40.6) values compared to all other strains and the control (see Table 21.1 above). Based on the pot culture data, it was concluded that it promotes drought tolerance in peanuts in terms of higher root-mining abilities and water-use efficiency. Hence, the strain IFT-30 was selected and tested for its efficacy under field conditions.

The results of field evaluation also validated the efficacy of the IFT-30 strain in improving the drought tolerance in peanuts under imposed moisture stress conditions. WUE traits—SCMR and SLA values—were significantly higher with IFT-30, followed by kaoline 2% and urea 2% sprays compared to other treatments and stress control. Similar results were recorded with other drought tolerance traits such as RWC and Leaf chlorophyll content, which plays an important role in maintaining cell water content and photosynthetic activity for sustaining crop growth under drought conditions. Oxidative stress relieving phyto-enzymes SOD activity was also higher in the IFT-30 Pf strain inoculated treatment compared to other treatments and the control.

The pod yield/plant was highest in the treatment of seeds bacterized with IFT-30 (847.8 Kg/acre), and was significantly superior over others. This is followed by *Acalifa Indica* 0.5 % + Urea 1% and kaoline 2% sprays. Another important physiological trait harvest index was highest with urea 2% followed by *Acalifa Indica* 1% spray and IFT-30 bacterized seed treatment. Control plants registered the lowest pod yield of 579 kg/acre and a lower harvest index of 29.8 (see Table 21.2 below). The study further reveals the existence of crop specificity among Pf strains in the physiological intervention of crop growth. The efficacy of Pf strain-30 in enhancing drought tolerance and yield is well demonstrated under field conditions. Such ameliorating effects of the *P. fluorescens* strain on peanut crops can be attributed to improved drought tolerance, raising the regulation of cell protecting oxidative enzymes, and could be due to improved nutrient uptake capabilities. A similar enhancement of physiological and biochemical activities under stress conditions was also reported by Tripati et al. (1998) in sorghum and Han & Lee (2005) in lettuce.

Table 21.2. Efficacy of various *Pseudomonas fluorescens* isolates as seed treatment in promoting drought tolerance traits, oxidative stress tolerance, and yield in peanuts under field conditions

Treatments	SCMR	SLA (cm^2/g)	RWC (%)	Chl. (mg/g)	SOD (OD/ min/g)	Pod yield/ ac.(kg)	Harvest index
Acalifa Indica leaf extract 1%	43.2	125.5	76.8	1.88	1.06	641.7	34.3
Kaoline 2%	44.6	109.3	75.2	2.09	0.60	744.5	29.3
Urea 2%	44.1	120.4	75.6	2.01	1.20	728.3	34.6
Potassium Chloride 1%	43.2	126.5	76.5	1.38	0.68	713.3	32.9
Acalifa Indica 0.5% + Urea 1%	43.7	130.2	80.6	1.71	0.68	744.5	33.3
seed bacterized with IFT-30	44.9	119.1	82.1	2.07	1.05	847.8	34.1
Water spray only	43.7	126.1	75.1	1.32	0.79	691.7	23.7
Stress control	43.7	133.1	66.5	1.48	0.59	579.0	28.9
S.E.M	0.24	3.18	0.81	0.19	0.03	31.2	0.69
CD (5%)	0.64	9.44	2.93	0.56	0.11	84.5	2.3

Peanut seeds (CV: Narayani) were bacterized with Pf strains at 1 × 109 cfu/ml.

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PART VI.

COMMERCIAL POTENTIAL, TRADE AND REGULATORY ISSUES AMONG ASIAN COUNTRIES

CHAPTER TWENTY-TWO

IMPACT OF INADEQUATE REGULATORY FRAMEWORKS ON THE ADOPTION OF BIO-FERTILIZER (E.G. PGPR) TECHNOLOGIES: A CASE STUDY OF SUB-SAHARAN AFRICA

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Abstract

Recently, there has been a lot of interest in promoting bio-fertilizers for eco-efficient intensification of agricultural systems in sub-Saharan Africa (SSA). Bio-fertilizers are considered to be cost-effective and environmentally-friendly. In SSA, bio-fertilizers have not been sufficiently evaluated for quality and efficacy because of weaknesses or absence of regulatory frameworks. Consequently, a proliferation of low quality and inefficient bio-fertilizer products has been reported. Based on a stepwise assessment of 66 bio-fertilizer products found in Ethiopia, Kenya and Nigeria in 2009–11, in more than 90% of cases product composition didn't match indications on the product labels, or label claims related to product benefits were not supported by our research results. A few products were, however, found to be very promising; for instance, Legumefix (a rhizobial inoculant for soybean) showed a benefit cost analysis > 2.5. There was an obvious need to discriminate high quality products from poor ones. A five year study (i.e. 2012–2017) has started to address that gap and to scale the most promising bio-fertilizer products. One of the key outcomes of the

new project is therefore the institutionalization of quality control and efficacy testing of bio-fertilizer products to virtually eliminate the proliferation of poor quality and inefficient ones. This will increase the confidence of smallholder farmers, with high risk aversion, in the bio-fertilizer technologies. Adoption of bio-fertilizers by the resource-poor smallholder farmers in SSA, comprising the majority of the population, will certainly result in improved crop yields, food security and consequently better livelihoods.

Introduction

Farmers in Africa must have access to improved seeds, fertilizers, knowledge and technologies for eco-efficient intensification of crop productivity and consequently improved food security. Ironically, more than 70% of farming in Africa is carried out by resource-poor smallholder farmers (*The Economist* 2012) who, in most cases, cannot afford technologies easily accessible to farmers in other countries. Thus, there is a crucial need to identify alternative technologies which are cost-effective and environmentally-friendly to improve crop productivity in sub-Saharan Africa (SSA).

In India, the government has been trying to promote bio-fertilizers as cost-effective and environmentally-friendly agricultural inputs with beneficial impacts on soil fertility. Similar products have been promoted in SSA as alternatives or complements to conventional fertilizers. Bio-fertilizers, more commonly known as microbiological inoculants, are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity (Ghosh 2004) at relatively low cost and with minimum negative impacts to the environment.

In a recent study, Jefwa et al. (2013) found that most bio-fertilizers in the marketplace in selected countries (i.e. Ethiopia, Kenya and Nigeria) were of poor-quality and ineffectual. The proliferation of inefficient products may be related to the weakness, or simply the absence, of an appropriate regulatory framework. Hence, in most cases bio-fertilizer products may have been introduced into a specific country without an adequate level of scientific scrutiny to verify (i) the guaranteed analysis (i.e. strains listed on the product label), (ii) the level of contaminants, as well as (iii) the efficacy. Such a situation undermines the confidence of the resource-poor smallholder farmers and the majority of the population in SSA (who therefore have a risk aversion) in the bio-fertilizer technologies.

To improve things, adequate regulatory frameworks seem to be mostly required. For instance, in countries like Argentina, Canada and South

Africa, fertilizer legislation, encompassing bio-fertilizers or bio-fertilizer-like products, has been put in place at the national or federal levels for consumer protection. Some states in Australia and the USA have implemented the same. Discussions are ongoing in the European Union to amend the current European Commission (EC) Fertilizers Regulations with regulations that will be more inclusive, including conventional fertilizers, liming materials, soil improvers, growing media, and bio-stimulants (European Commission 2012). Bio-fertilizer performance may vary based on not only agro-ecological zones, but also agricultural practices such as integrated soil fertility management (Carvajal-Muñoz & Carmona-Garcia 2012). Hence, the bio-fertilizers' directions for use should be based on scientific evidence generated on appropriate soils and in relevant agro-climatic zones. Utilization of bio-fertilizers in the context of integrated soil fertility management (ISFM), developed based on local conditions (Vanlauwe et al. 2010), may result in better performance and consequently increase the rate of adoption by smallholder farmers.

To build on lessons learned from the study by Jefwa et al. (2013), and addressing the legislative gap to facilitate the adoption of bio-fertilizers by smallholder farmers, a five-year study (2012–17) is ongoing in selected SSA countries, namely Ethiopia, Ghana, Kenya, Nigeria, Tanzania and Uganda. One of the key objectives of the study is to establish and implement institutionalized quality control and efficacy testing of bio-fertilizer products through providing the national regulatory-bodies with the necessary facilitation and expertise as applicable. Another key objective is to identify ISFM frameworks in which bio-fertilizers should be applied to optimize their efficacy, and consequently improve crop yields as well as the likelihood of bio-fertilizer adoption by smallholder farmers.

Issues and challenges related to the current regulatory frameworks

In the study by Jefwa et al. (2013), 66 bio-fertilizer products including plant growth promoting rhizobacteria (PGPR) were evaluated to verify the expected strains as indicated on product labels. Products that contained the adequate strains were experimented on in greenhouse conditions to verify their performance based on their labels' claims. Promising products were finally tested in field conditions, when recommended for field use. As shown in Table 22.1 below, some of the products either did not contain the expected strains or contained additional strains considered as contaminants. Some of the contaminants were potential pathogens. Similarly, some of

the products when used as directed were not efficacious enough to be profitable to smallholder farmers based on the benefit-cost analysis (BCR), i.e. BCR < 2.5. Bhattacharyya & Tandon (2012) indicated that a BCR > 2.5 should be considered as satisfactory for the adoption of a specific bio-fertilizer technology. As mentioned above, most smallholder farmers in SSA have a financial-risk aversion (Boughton et al. 1990). Any loss due to the utilization of an inefficacious bio-fertilizer or contamination of crop yields will represent a disastrous situation for them; it may also seriously undermine trust in the whole bio-fertilizer industry. The quality of bio-fertilizer products is thus of utmost importance to protect farmers, retailers, wholesalers and the bio-fertilizer industry, and to minimize health and environmental hazards. An appropriate regulatory framework is hence required to minimize the risk. The findings of Jefwa et al. (2013), as also mentioned by Carvajal-Muñoz & Carmona-Garcia (2012), clearly show the importance of testing bio-fertilizer products in the applicable agro-ecological zones prior to their extensive utilization in specific agro-climatic conditions. Blind recommendations may result in poor performance in some regions and consequently hamper the adoption of bio-fertilizer technologies. In Bangladesh, Jahiruddin & Satter (2010) recommended the consideration of bio-fertilizer in the context of ISFM for sustainable agriculture. Vanlauwe et al. (2010) defined ISFM as a set of soil fertility management practices that necessarily include the use of fertilizer, organic inputs and improved germplasm, combined with the knowledge of how to adapt these practices to local conditions, aimed at maximizing agronomic use efficiency of the applied nutrients (including bio-fertilizers) and improving crop productivity.

Similar to the selected bio-fertilizers presented in Table 22.1 above, the quality and profitability of the 66 bio-fertilizers evaluated were variable. Even for bio-fertilizers containing the same strain (e.g. products 2 & 5), the profitability was variable; an indication of bio-fertilizer formulation and use pattern also matter. Bio-fertilizer efficacy, labelling and safety requirements therefore have to be imbedded in adequate regulatory frameworks not only to ensure consumer protection, but also to create conducive conditions for the adoption of these technologies.

A scoping exercise in the various countries was conducted in 2011 to assess the state of fertilizer legislation in general, and bio-fertilizers in particular. The information was cross-validated in November 2012, during a regulatory workshop attended by the appropriate regulatory bodies, policy makers, and scientists in the area of bio-fertilizers, from the project countries (Table 22.2).

Table 22.1. Quality and profitability of selected bio-fertilizers evaluated by Joyce et al. (2013)

Product listing	Strains listed on the product label	Strains confirmed	Strains not listed on the label	Profitability (BCR)
1	*Bradyrhizobium. japonicum*	*B. japonicum*	*Lysinibacillus sphaericus* and *Bacillus nealsonii*	ND[z]
2[y]	*B. japonicum*	*B. japonicum*	None	11.0
3	*B. japonicum*	*B. japonicum*	None	< 2.5[x]
4	*B. japonicum and Bacillus subtilis*	*B. japonicum and Bacillus subtilis*	*Microlunatus parietis* and *Rhodococcus sp.*	ND
5	*Paenibacillus azotofixans, Bacillus licheniformis, B. megaterium, B. polymyxa, B. subtilis, and B. thuringiensis*	*paenibacillus azotofixans*, and *Bacillus subtilis*	*Bacillus cereus*	ND
6	*Azotobacter, chroococcum,* and *Bacillus polymyxa*	0	*Bacillus subtilis, tenotrophomonas sp., Ochrobactrum intermedium, Cellulosimicrobium sp., Ochrobactrum* sp., and *Bacillus sp.*	< 2.5

[z] ND = Not determined; [y] Product # 2 is Legumefix; [x] A BCR > 2.5 is considered satisfactory for the adoption of a specific bio-fertilizer technology (Bhattacharyya & Tandon 2012).

Table 22.2. Critical analysis of bio-fertilizer legislation in selected SSA countries

Country	Status of regulatory Landscape	Proposed Interventions
Ghana	• A fertilizers act exists • No specific bio-fertilizers policy • Bio-fertilizers are captured in the current fertilizer definition	• Development of an adequate bio-fertilizer policy • Harmonization of mandates of all the institutions concerned with the bio-fertilizers' legislation
Kenya	• Currently using the International Standard on Phytosanitary Measures 3 (ISPM 3), https://www.ippc.int/ • Fertilizer and soil conditioners bill is being drafted • No specific bio-fertilizers policy • Bio-fertilizer standards being prepared by Kenya Bureau of Standards	• Development of an adequate bio-fertilizer policy • Establishment of clear institutional mandates • Establishment of accredited laboratories for handling bio-fertilizers • Monitoring the products on the marketplace
Nigeria	• A draft fertilizer bill is ready pending approval by the Federal Executive • No specific bio-fertilizers policy for the moment	• Development of an adequate bio-fertilizer policy • Clarification of institutional mandates
Tanzania	• A fertilizers act exists • No specific bio-fertilizers policy	• Development of an adequate bio-fertilizer policy • Clarification of institutional mandates • Build capacities for the implementation and enforcement of the bio-fertilizers' legislation
Uganda	• Uganda Fertilizer Policy 2012 still in draft form • No specific bio-fertilizers policy	• Development of an adequate bio-fertilizer policy • Setting minimum standards for the laboratories • Capacity building for in-service professionals

Adapted from Tarus et al. (2012).

Methodology to address the issues and challenges

The results obtained during phase one of this project (see Table 22.1 above) and the scoping exercise (see Table 22.2 below) were critically analyzed to determine the regulatory mechanisms that could be used to address the discrepancies. Specific activities were thereafter defined to develop those mechanisms. They include:

(1) Establishing an effective collaboration with national regulatory agencies in the target countries to implement sustainable bio-fertilizer legislation through a customer-paid certification procedure including label, efficacy and safety reviews.
(2) Holding stakeholder workshops and training seminars on quality norms and standard operating procedures.
(3) Ascertaining and participating in policy formulation for bio-fertilizers.
(4) Establishing a functional independent laboratory to guarantee objectivity and credibility, with strengthened institutional capacity to meet the accreditation requirement to analyze bio-fertilizers' quality.
(5) Developing a curriculum that incorporates quality control concepts in academic institutions to build the capacity of the national systems.
(6) Conducting an economic analysis of bio-fertilizer quality assessment to advise government regulatory agencies when developing customer-paid services.
(7) Strengthening policy linkages among stakeholders from the private and public sectors to improve trust, credibility and enforcement of bio-fertilizer legislation.

Implementation of the results of these activities will not only ensure adequate label, efficacy and safety evaluations before the commercialization of bio-fertilizer products, but also a better marketplace monitoring (see Fig. 22.1 below). Such a regulatory framework will minimize the proliferation of poor-quality and inefficacious bio-fertilizer products, and consequently improve the smallholder farmers' trust in the bio-fertilizer industry.

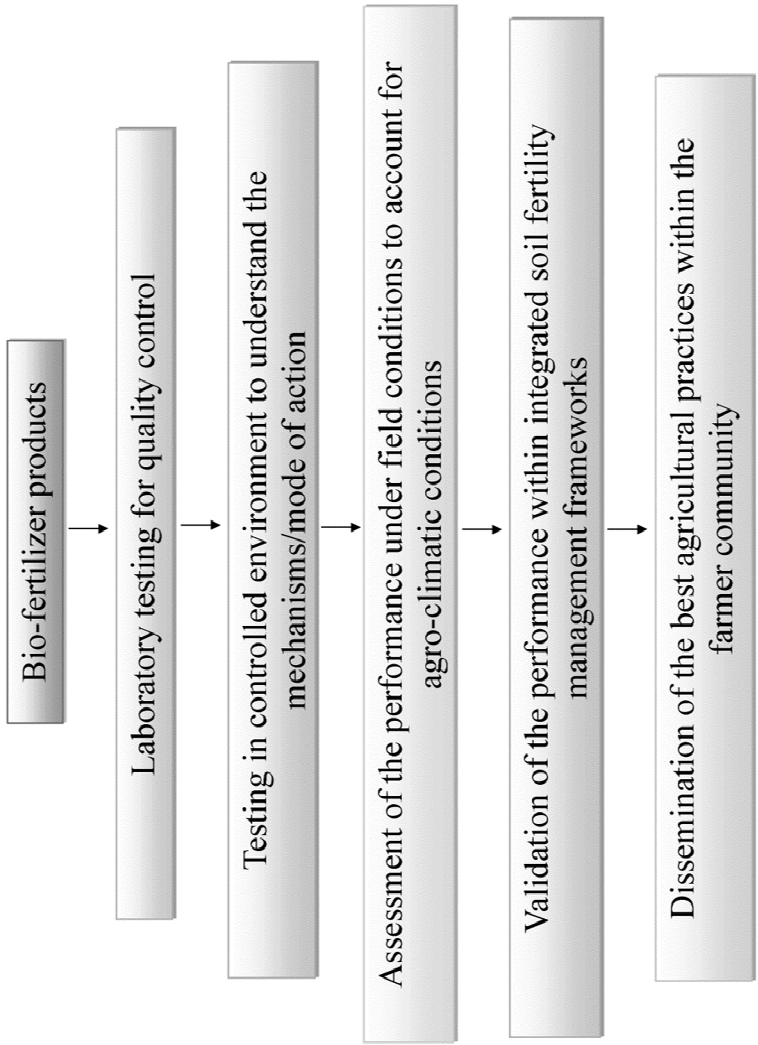

Fig. 22.1. Recommended framework for bio-fertilizer assessment for quality analysis and efficacy testing for approval, registration, certification and scale-up.

Expected improvements to the regulatory frameworks

When the recommended framework for bio-fertilizer assessment (Fig. 22.1 above) is implemented and enforced, the proliferation of poor-quality bio-fertilizer products should be virtually eliminated. The improvements below will be necessary to implement and enforce the framework:

(1) Establishing of a functional bio-fertilizer-legislation framework
(2) Institutionalization of the quality control and efficacy testing requirements
(3) Availability of knowledgeable personnel and improved laboratory capacity in the national systems
(4) Accreditation/approval of eligible laboratories to implement the recommended bio-fertilizers' assessment framework
(5) Bio-fertilizer registration based on the bio-fertilizer assessment framework
(6) Adequate service delivery standards to ensure timely registration of bio-fertilizer products not only to allow the private sector companies to develop their business plans accordingly, but also expedite farmer access to high quality and efficacious products.

Conclusion

There is enough background information to substantiate the potential benefits of bio-fertilizers for resource-poor smallholder farmers in SSA countries. However, inadequate regulatory frameworks in SSA are hampering their extensive utilization, as high-quality and efficacious products compete with poor-quality and inefficacious ones in the marketplace. Unfortunately, farmers may be attracted by the relative low-cost of poor-quality bio-fertilizers, and consequently lose their investments. Institutionalization of quality control and efficacy testing through adequate regulatory frameworks will be beneficial not only to farmers, but also to the bio-fertilizer industry, as well as other stakeholders interested in the bio-fertilizer business. Appropriate regulatory frameworks may also facilitate harmonization across SSA countries for mutual recognition, and consequently facilitate trade.

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CHAPTER TWENTY-THREE

FORMULATION AND COMPATIBILITY OF PGPR WITH PESTICIDES FOR SUPPRESSION OF INSECT PESTS

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Abstract

The use of synthetic chemicals during the last decade has raised a number of ecological problems, diverting the attention of scientists towards plant growth promoting rhizobacteria (PGPR). PGPR are easy to deliver, and they improve crop growth, activate resistant mechanisms and contribute towards increase in yields. Hence, this chapter deals with the formulation, compatibility and shelf life of a potential PGPR isolate for the management of insect pests in chilli. One potential indigenous *Pseudomonas fluorescence* isolate (Pf4) showing high efficacy in biocontrol and growth promotion was used for formulation using three carrier materials, among which talc maintained the highest population at room and refrigerated temperatures for up to 360 d of storage. Talc supported the longer shelf life with 20.66×10^7 cfu/g after a year of storage. The isolate showed compatibility with carbendazim and thiram among fungicides and imidacloprid, chlorpyriphos and carbofuran among insecticides and NSKE, and nimbicidine and eucalyptus extracts among plant products. The formulated product was used as an integral component for management of insect pests in the irrigated chilli ecosystem and scouting was attended to throughout the season by a team of entomologists and pathologists. The PGPR was intervened wherever required amongst other integrated pest management (IPM) components. The observation on number of thrips, mites and number of larvae and percentage fruit damage by fruit borer were recorded along with predatory populations. The average number of thrips was 1.77–2.20/leaf in IPM plots as against 2.59–3.18 in non-IPM plots, and 1.16–

1.61 mites/three young leaves in IPM plots as against 1.79–2.06 in non-IPM plots, with a leaf curl index of 0.71 in IPM and 1.06 in non-IPM and 0.17 *Helicoverpa armigera/*plant with fruit damage of 0.29–0.41% in IPM plots, as against 1.36 of *H. armigera*/plant with fruit damage of 5.73–5.89% in non-IPM plots. The population of predatory mite and coccinellids was also high in IPM plots as compared to non-IPM plots. Subsequently, the yield obtained was 5,000 Kg more in IPM plots compared to non-IPM plots with a net gain of Rs. 39170/ha. Thus, a PGPR mediated IPM strategy could bring down the cost of protection as well as reducing several untold ecological problems.

Introduction

Modern agriculture is highly dependent on the use of high yielding varieties, chemical fertilizers, plant growth regulators, fungicides and insecticides to fulfil the food requirement of a growing population. As a result, the natural ecology of soil and microflora and fauna are being disturbed, affecting the agricultural production. Moreover, available pesticides are often expensive and have adverse effects on human health. Therefore, the environmentally-friendly control of insect pests in plants is a pressing need for sustainable agriculture (Ermnert & Handelsman 1999). Antagonistic bacteria that are target specific, eco-friendly and capable of colonizing the inoculated environment are a requirement of effective biocontrol.

Specific strains of fluorescent pseudomonads have the potential to suppress insect pests and plant pathogens (O'Sullivan & O'Gara 1992), enhance plant growth (Glick 1995) and participate in carbon and nitrogen cycling in nature (Palleroni 1993). A large number of fluorescent pseudomonad species, such as *P. fluorescens* (Sakthivel & Gnanamanickam 1987) *P. aureofaciens* (now considered *P. chlororaphis*) (Chin-A-Woeng et al. 1998), *P. putida* 6909, and *P. fluorescens* 09906 (Yang et al. 1994) have been well documented for their antagonistic potential.

The success of any formulation depends on the suitability of the carrier materials and the shelf life of the bioagent in the formulation. After all, a carrier material should provide favourable conditions for bacteria that will sustain long-lasting survival and improve the biocontrol activity of antagonists. The efficiency of *Pseudomonas fluorescens* can be enhanced by the combined use of agrochemicals and plant products so that it will be an efficient, environmentally saf, and ecologically sound method of insect pest and plant disease control for the future.

An indigenous *P. fluorescens* isolate Pf4 had antifungal activity against *F. solani* (wilt of chilli), *C. gloeosporioides* (anthracnose of pomegranate), *A. alternata* (leaf spot of cotton), and *R. solani* (cotton root rot). It also induced defence-related enzymes peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and β-1, 3-glucanase, as well as producing different antibiotics like phenazine, HCN and salicylic acid. Thus, the Pf4 isolate emerged as the most potential bioagent (Anand et al. 2010).

Hence, the isolate of *P. fluorescens* (Pf4) was taken to evaluate shelf life in different carrier materials and its compatibility with common fungicides, insecticides and plant products for their efficient use in IPM.

Materials and Methods

Collection and preservation of *P. fluorescens* (Pf4) isolate

The Pf4 isolate was collected from the Sankeshwaral village in Raichur district, Karnataka (India) from the rhizosphere soil of chilli (var. *Byadagi kaddi*) during the winter of 2008 (Anand et al. 2010). *P. fluorescens* (Pf4) isolate was maintained in the Department of Plant Pathology, University of Agricultural Sciences, Raichur, Karnataka. It was preserved in King's Medium B Agar slants in a refrigerator at 4°C for further use.

Mass multiplication of *P. fluorescens* in carrier materials

Talc powder, vermicompost and farm yard manure (FYM) were used as carrier materials for the mass multiplication of *P. fluorescens*. The substrates were first tyndalized and then air dried and passed through 350 mesh sieves to obtain fine powders. The mass culture of *P. fluorescens* was obtained by adding sterile distilled water to its 24 h. old growth in Kings B agar, and 15 mL of such bacterial suspension is aseptically added to 1,000 mL Kings B broth and incubated at 28 ± 1°C for 24 h. The 400 mL of bacterial suspension (10^8 cfu/ml) from Kings B broth culture was added to 1 kg of substrate. Then, the inoculated substrates were mixed properly and sealed in polypropylene bags and later stored at different temperatures.

Determination of the population dynamics of *P. fluorescens* in formulations

The experiment was designed using three treatment combinations following a completely randomized design (CRD) with three replications for determination of the population dynamics of *P. fluorescens* in three bio formulations after different days of storage at two temperatures. The viable population of Pf4 in the substrates was determined at 15 d intervals by a serial dilution technique and is expressed in terms of cfu/g of substrate.

Compatibility studies with commonly used insecticides, fungicides and plant products

Determination of compatibility of the Pf4 isolate with the commonly used insecticides imidacloprid, chlorpyriphos, carbofuran, indaxocarb and endosulfon with 0.1% and 0.2% concentrations; fungicides mancozeb, carbendazim, captan, thiram and propiconazole with concentrations of 0.1% and 0.2%; and plant products neem seed kernel extrac (NSKE), nimbicidin, garlic, tulasi extract, eucalyptus leaf extract with 2.5%, 5%, 7.5% and 10% concentrations was carried out through the poisoned food technique. The colony count was done through the spread plate method.

Results and Discussion

Determination of the population dynamics of *P. fluorescens* in formulations

The mean population of the *P. fluorescens* in formulations of three carrier materials significantly increased over 135 d of storage, and thereafter showed a declining trend (see Fig. 23.1 below). The highest population of Pf4 was recovered from formulation with FYM as substrate at room temperature at 135 d (153.33×10^7). No colony was recovered from the formulation with vermicompost at refrigerator temperatures. Over all, the results depict that talc is the best carrier material to support the Pf4 for longer shelf life at both room and refrigerator temperatures, giving 20.66×10^7 and 11.33×10^7 cfu/g, respectively, at the end of a year. Overall, the talc maintained the population of bioagent uniformly even up to 135 d.

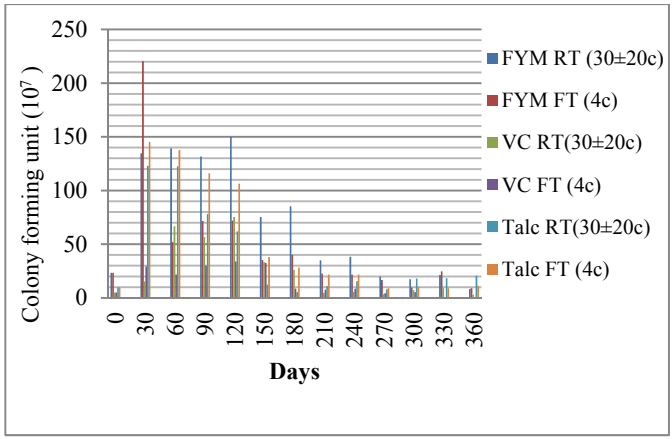

Fig. 23.1. Effect of different carriers and storage temperature on population of *Pseudomonas fluorescens* (Pf4)

The development of effective formulations for the management of insect pests depends upon the selection of an efficient strain, bacterial population over a period of time, and the method of application. The present studies clearly indicate that out of three carrier materials, studied for survival of Pf4, talc maintained the uniform population level throughout the year.

Although FYM and vermicompost could encourage better populations, mainly because of physical properties like organic matter and moisture content, maintenance of quality FYM and vermicompost throughout different locations may not be practicable. Its availability, basic materials used for preparation (plant materials, cow dung etc.) and quality standard varied to a large extent. In addition, FYM has not maintained the population of the antagonist uniformly, since the pH of FYM is towards acidic. In addition, the preparation of FYM makes it a different proposition as a package of carrier material. Talc, being an inert material and having more surface area and neutral pH, will help the *P. fluorescens* to survive for longer periods than the FYM and vermicompost. Talc-based formulations have been used and tested under field conditions against chilli wilt and sesame blight (Naik et al. 2009).

Different carrier formulations of fluorescent pseudomonads have been developed (Kloepper & Schroth 1981; Rabindran & Vidhyasekaran 1996). The application of fluorescent pseudomonads to seed (Callan et al. 1990), soil (Hebber et al. 1991), and foliage (Gnanamanickam et al. 1992) has also been attempted to control diseases. Similar results were shown by

Kloepper & Schroth (1981) regarding the potential of talc for use as a carrier for formulating rhizobacteria. The fluorescent pseudomonads did not decline in talc mixture with 20% xanthum gum after storage for two months at 4°C. *P. fluorescens* isolate Pf1 survived up to 240 d in storage. The initial population of Pf1 in talc-based formulation was 37.5×10^7 cfu/g and declined to 1.3×10^7 cfu/g after eight months of storage (Vidhyasekaran & Muthamilan 1995).

Shivakumar et al. (2000) reported the survival of *P. fluorescens* in peat and talc which maintained the highest population levels at 19.5×10^7 and 18.3×10^7 cfu/g, respectively, after four days of storage to $3.0–6.8 \times 10^7$ cfu/g in different carriers. Georgakopoulos et al. (2001) reported that the peat carrier based formulation had a good shelf life of two years. *P. fluorescens* BRG100 survived best in oat flour and the addition of 20% (wt/wt) maltose extended the shelf life of the product to more than 32 weeks (Daigle et al. 2002).

Compatibility studies with insecticides, fungicides and plant products

Table 23.1. Compatibility of *pseudomonas fluorescens* (Pf4) with different insecticides

Insecticides	**No. of colonies (10^{10})**		
(%)	**0.1**	**0.2**	**Control**
Imidacloprid	50	24	90
Chloropyriphos	29.33	0	90
Carbofuron	64.33	35.33	90
Indaxocarb	0	0	90
Endosulfon	45.33	28.33	90
S. Em±	9.19	7.12	4.11
CD at 1%	35.00	27.11	15.65

Among five insecticides, Pf4 showed compatibility with imidacloprid, chloropyriphos, carbofuron and endosulfon. Pf4 yielded 64.33 × 10^{10} and 35.33 × 10^{10} colonies with carbofuron, 50 × 10^{10} and 24 × 10^{10} colonies with imidacloprid, 45.33 × 10^{10} and 28.33 × 10^{10} colonies with endosulfon at both the concentrations, whereas 29.33 × 10^{10} colonies were obtained with chlorpyriphos at 0.1% concentration. No colonies of *P. fluorescens* were recovered with indaxocarb when compared to control having 90 × 10^{10} colonies (see Table 23.1 above).

Jayakumar et al. (2004) reported regarding the compatibility of *P. fluorescens* with avermectin and carbofuran 3G for the management of *Rotylenchulus reniformis* in okra. The application of *P. fluorescens* (2.5 Kg/ha) in combination with carbofuran 3G significantly improved plant growth (Senthilkumar et al. 2004). Moreover, the compatibility of the most efficient strain 51 with some fungicides, which are commonly sprayed at greenhouse, was investigated under *in vitro* conditions. Fungicides did not adversely affect the development of bacterial colonies at concentrations as high as 100 µg/ml *in vitro* (Gore & Altin 2006). Mahapatra et al. (2003) determined the efficacy of *P. fluorescens* against *Meloidogyne incognita* combined with 1.5 g carbofuran/m^2. *P. fluorescens* at 20 g/m^2 and at 10 g/m^2 + carbofuran caused 38.4% and 31.0% decrease in infection, respectively. The compatibility of *Pseudomonas fluorescens* with imidacloprid and carbofuron is reported by Kumar et al. (2008).

Table 23.2. Compatibility of *pseudomonas fluorescens* (Pf4) with different fungicides

Fungicides (%)	**No. of colonies (10^{10})**		
	0.1	**0.2**	**Control**
Mancozeb	0	0	82
Carbendazim	82	27.33	82
Captan	0	0	82
Thiram	68.66	32.33	82
Propiconazole	0	0	82
S.Em±	6.14	4.76	2.74
CD at 1%	23.37	18.10	10.45

Five fungicides were used to test the compatibility of *P. fluorescens*: mancozeb, carbendazim, captan, thiram and propiconazole at 0.1% and 0.2% concentrations. Among the five fungicides, *P. fluorescens* was compatible with carbendazim and thiram at both concentrations. *P. fluorescens* gave the 82×10^{10} colonies at 0.1% and 27.33×10^{10} colonies at 0.2% with carbendazim, and 68.66×10^{10} and 32.33×10^{10} colonies at 0.1% and 0.2% concentration in the case of thiram. However, *P. fluorescens* was not at all compatible with other fungicides like mancozeb, captan and propiconazole, in which no colonies were recovered at both the concentrations compared to control (82×10^{10} colonies) (see Table 23.2 above).

The study agrees with Khan & Gangopadhyay (2008) with respect to sensitivity of *P. fluorescens* towards fungicides. They reported that carboxin, chlorothalonil and carbendazim were least toxic to *P. fluorescens* strain PFBC-25, while captan was most inhibitory to this strain. Laha & Venkatraraman (2001) also reported the compatibility of *P. fluorescens* with carbendazim while studying sheath blight management in rice. Malathi et al. (2002) also reported the enhancement of growth of *P. fluorescens* by carbendazim at 100 ppm.

The *P. fluorescens* (Pf4) is compatible with NSKE (150.66×10^{10} cfu/ml) at 7.5% concentration, followed by tulasi extract with maximum nimbicidine *Eucalyptus* leaf extract and garlic extract, while the least colonies were observed in *Eucalyptus* leaf extract (2×10^{10} cfu/ml) (see Table 23.3 below). Kumar et al. (2009) tested the compatibility of *P. fluorescens* with castor cake, pongamia cake, neem cake and safflower cake and their efficacy against *Alternaria porri* and *A. alternata*. The greatest inhibition of the mycelial growth of *A. alternata* and *A. porri* was exhibited by groundnut cake, followed by castor cake.

Table 23.3. Compatibility of *pseudomonas fluorescens* (Pf4) with different plant products

Plant products	**No. of colonies (10^{10})**				
(%)	**2.5**	**5**	**7.5**	**10**	**control**
NSKE	95.33	110.6	150.66	137.33	21.66
Garlic extract	7.00	0.00	0.00	0.00	21.66

Nimbicidine	18.33	16.00	11.00	10.33	21.66
Tulasi extract	22.66	45.00	36.00	46.00	21.66
Eucalyptus extract	8.33	14.66	2.33	2.00	21.66
S. Em±	8.50		7.61		3.80
CD at 1%	31.81		28.45		14.22

Integration of PGPR in IPM demonstration

The present-day trend is to adopt an integrated pest management (IPM) schedule wherein the various components of fungicides, insecticides and bioagents are integrated and put into use in the same season. The formulation of *P. fluorescens* was used as an integral component to demonstrate the IPM trial on chilli under farming field conditions, and as a participatory approach the IPM inputs along with bioagents were distributed to famer beneficiaries, and the interventions were made throughout the season with continuous monitoring by the entomologists. The results on incidence of insect pests and yield revealed that the incidence of thrips was in the range of 1.92 per leaf, with a leaf curl index (LCI) of 0.43, and 3.3 of mites per leaf with an LCI of 0.71, 0.17 of *H. armigera*/plant with 1.36% of fruit damage in IPM plots, as compared to 3.26 thrips per plant with an LCI of 1.35, 5.2 mites per leaf with a leaf curl index of 1.06, and 0.52 of *H. armigera* per plant with fruit damage of 6.54% in non-IPM plots. In contrast, the population of coccinellids was in the range of 0.55 and predatory mite was 0.39 in IPM plots as against 0.50 of cooccinellids and 0.18 of predatory mites in non-IPM plots.

Yield and economics

Thirteen IPM interventions were made, as compared to 23 interventions in the non-IPM plot. The IPM plot recorded an average yield of 15 q/ha with a net profit of Rs. 70620/-, as against 10 q/ha of dry chilli with a net profit of Rs. 31450/- in the non-IPM plot, which is a gain of Rs 39170/- in the IPM-plot (see Table 23.4 below). Thus, a PGPR-mediated IPM strategy could bring down the cost of protection as well as reducing several ecological problems.

Table 23.4. Economics of chilli IPM and non-IPM plot

Location	Dry chilli yield (qt/ha)	Total income (Rs/ha)	Cost of cultivation (Rs/ha)		Total cost (Rs/ha)	Net Profit (Rs/ha)
			Production cost	Protection Cost		
Nelahal village						
IPM plots	15.00	96,900/-	22,000/-	4,280/-	26,280/-	70,620/-
Non-IPM plots	10.00	64,600/-	25,000/-	8,150/-	33,150/-	31,450/-

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CHAPTER TWENTY-FOUR

EFFECT OF ACC DEAMINASE PRODUCING PGPR STRAINS INOCULATION ON THE EARLY GROWTH AND NUTRIENT UPTAKE OF CROP PLANTS IN SAEMANGEUM RECLAIMED SOIL

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Abstract

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing bacteria stimulate the cleavage of ACC, the immediate ethylene precursor to ammonia and α-ketobuty rate. The halotolerant *Brevibacterium iodinum* RS16 was isolated from coastal saline soil of the Yellow Sea, Incheon, South Korea. It possesses plant growth promoting (PGP) traits like nitrogen fixation and ACC deaminsae activity. *Methylobacterium oryza* CBMB20 was isolated from rice stem and has the ability to produce ACC deaminase, which helps in the breakdown of ACC and reducing the plant ethylene level which inhibits plant growth at a high concentration. This study was conducted to examine the effect of inoculation and co-inoculation of *B. iodinum* RS16 and *M. oryzae* CBMB20 on the early growth of maize and sorghum in Saemangeum reclaimed soil. Plant growth promoting *B. iodinum* RS16 and *M. oryzae* CBMB20 were inoculated on maize (*Zea mays* L.) and sorghum-sudan grass hybrid (*Sorghum bicolor* L.) grown in Saemangeum reclaimed soil with two levels of fertilizer (70% and 100% fertilizer amendments). The impact of microbial treatment on these two crops was assessed based on plant height and dry biomass accumulation. Parameters were compared between microbial inoculation at 70% fertilizer level and non-inoculated treatment at 100% fertilizer level. Single and co-inoculation of *B. iodinum* RS16 and *M. oryzae* CBMB20 increased plant height, dry biomass accumulation of

maize, and sorghum-sudangrass hybrid at 70% and 100% fertilizer levels. Co-inoculation treatment at 70% fertilizer level increased plant height in maize by 22.4% at 30 d after sowing (DAS), shoot dry weight (82.3%), and total dry weight (62.5%) compared to non-inoculated treatment at 100% fertilizer level. In the case of sorghum-sudangrass hybrid *M. oryzae*, CBMB20 treatment at 70% fertilizer level showed a 31.1% increase in plant height compared to non-inoculated treatment at 100% fertilizer level at 30 DAS. Co-inoculation treatment at 70% fertilizer level increased root dry weight and total dry weight compared to non-inoculated treatment at 100% fertilizer level. This pot culture experiment demonstrated that *B. iodinum* RS16 and *M. oryzae* CBMB20 inoculation increased the early growth and nutrient uptake in maize and sorghum-sudangrass hybrid at reduced fertilizer level (70%). Further evaluation at field level will confirm the findings of this experiment.

Introduction

Technological advances in agriculture are helping to meet the food needs of an ever-increasing world population, for which the available land for agriculture has been shrinking. Thus, there is a need to consider using reclaimed land to increase the agriculture area suitable for cultivation. Particularly in South Korea, the land area available for cultivation has reduced drastically in the last few decades, prompting the need for more space for future food security in the country. Intensive agriculture that involves heavy and continuous use of chemical fertilizers has ensured high crop productivity, but showed negative impacts on soil. Obviously, the use of chemical fertilizers cannot be eliminated at this time without drastically decreasing food production. At the same time, the harmful environmental side-effects of fertilizer use, such as the expanding dead zones in marine systems, cannot go unabated. Hence, there is an urgent need for an integrated nutrient management that targets agricultural inputs and lowers the adverse environmental impacts of agricultural fertilizers and practices. Understanding the interactions between microbes, fertilizers and plants is very important for better nutrient use efficiency. The benefits to plants from interactions with plant growth promoting rhizobacteria (PGPR) have been shown to include increases in seed germination rate, yield, leaf area (Mahaffee & Kloepper 1994), root growth, shoot and root weights (Bashan et al. 2004; Bakker et al. 2007), nutrient uptake (Mantelin & Touraine 2004), and tolerance to abiotic stress (Yang et al. 2009). Other beneficial effects of PGPR strains include enhancing phosphorus availability (Rodriguez & Fraga 1999), fixing atmospheric nitrogen

(Bashan et al. 2004), sequestering iron for plants by production of siderophores (Bakker et al. 2007), producing plant hormones such as gibberellins, cytokinins, and auxins (Gutierrez et al. 2001), and synthesizing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels, thereby reducing environmental stress on plants (Glick et al. 2007).

Thirty percent of the land in Saemangeum has been set aside for agricultural use, of which barely 17% is under low levels of salinity and thereby sustainable for cultivation. The remaining 83% of land has salt levels ranging from 0.3%–1.0% and was deemed not suitable for crop growth. The physical and chemical properties, including organic matter (0.1%–0.5%) and major and minor nutrients levels, are very low (Choi et al. 2010). In theory, crop establishment and growth can be increased by removal of salt through mechanical or chemical methods and by the improvement of nutrient status of the soil through application of organic and inorganic fertilizers. In practice, these processes entail exorbitant costs and pose negative impacts on the environment from the use of inorganic fertilizers. A feasible and low-cost option is the use of microbial inoculants.

This study was conducted to examine the effect of inoculation and co-inoculation of *B. iodinum* RS16 and *M. oryzae* CBMB20 on the early growth of maize and sorghum in Saemangeum reclaimed soil with two levels of fertilizer application.

Methods

Bacterial strains and growth conditions

Brevibacterium iodinum RS16 isolated from saline soil is a halotolerant bacteria which exhibits nitrogen fixation (Siddikee et al. 2011), and was grown in tryptic soy broth supplemented with 5% NaCl. *Methylobacterium oryzae* strain CBMB20 was isolated from stem tissues of rice and characterized with various plant growth promotion traits (Madhaiyan et al. 2007). For inoculum preparation, *M. oryzae* CBMB20 was grown in ammonium mineral salt (AMS) media with 0.5% sodium succinate.

Soil sampling

Soil samples were collected from a low salinity level site in the Gyehwa area in Saemangeum reclaimed land. The surface soil (2 cm) was removed

and soil was collected from up to a 30 cm depth. A total of 10 samples were collected from each site and mixed together for the pot experiment.

Greenhouse experiment

Maize (*Zea mays* L.) and sorghum-sudangrass hybrid (*Sorghum bicolor* L.) were used as experimental crops which showed high salinity tolerance in Saemangeum (Baek et al. 2010; Sohn et al. 2010) and other reclaimed land (Shin et al. 2007; Sohn et al. 2009).

The experiment was done with four treatments (T1—Control, T2—RS16, T3—CBMB20 and T4—RS16 + CBMB20) using two crops (maize and sorghum-sudangrass hybrid) with two levels of fertilizer (70% and 100%) amendments. The experiment was laid out in a randomized complete block design with four replications. Plastic pots were filled with 2 Kg Saemangeum reclaimed soil. All experiments were conducted at Chungbuk National University, 410 Seongbong-ro, Heungduk-gu, Cheongju, Chungcheongbuk-do, under greenhouse conditions.

The fertilizer treatments consisted of 2 sets (70**%** and 100%). Chemical fertilizer was applied as urea (nitrogen content 46%), fused superphosphate (phosphate content 20%), and potassium chloride (potassium content 60%). Fertilizer application rate was determined based on the recommended basal chemical fertilizer application rate for maize (N : P : K, 17.4 : 3.0 : 6.9, Kg 10 a^{-1}) and sorghum-sudangrass hybrid (N : P : K, 20.0 : 15.0 : 15.0, Kg 10 a^{-1}). The fertilizer application rate at 70% was calculated based on the 100% rate. Chemical fertilizers were added 10 d after sowing (DAS). Compost prepared with 60% chicken dung, 20% bark and 20% sawdust was mixed with Saemangeum reclaimed soil at a rate of 20.0 g/kg in all treatments.

Bacterial inoculation was initially done on surface sterilized seeds (maize seeds—70% ethanol, 1 min; 6% NaOCl, 5 min; sorghum-sudangrass hybrid seeds—70% ethanol, 2 min; 1% NaOCl, 3 min), and then on the soil at weekly intervals until 27 DAS.

The inoculation effect of *B. iodinum* RS16 and *M. oryzae* CBMB20 on plant height was recorded at 10, 15, 20, 25 and 30 d after sowing. Plant biomass, in terms of root and shoot dry weight, was recorded 30 d after sowing (DAS).

Total nitrogen (N) in roots and shoots was determined by a Kjeldahl Autoanalyzer 1030. Dried powdered plant samples (200 mg) were digested with sulphuric acid and potassium sulphate on a hot plate until a clear liquid was obtained. The total N in the digested samples were then analyzed with a Kjeldahl Autoanalyzer. Phosphorus (P) was analyzed by a

UV/Vis spectrophotometer. Other macro-nutrients like potassium (K), calcium (Ca), and magnesium (Mg) absorbed by the plants were analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Optima 5300 DV, Perkin Elmer, USA). The data were expressed as uptake of nutrients by roots and shoots on a dry weight basis for individual plants.

Statistical analyses

The analysis of variance of treatments and LSD were calculated using SAS Version 9.1 [SAS 2009].

Results and Discussion

Effect of *B. iodinum* RS16 and *M. oryzae* CBMB20 on growth of maize

Significant increases over control at 30 DAS were observed with a single inoculation of M. oryzae CBMB20 (16.8%) and co-inoculation treatments (22.4%) in maize at 70% fertilizer rate. On the other hand, with 100% fertilizer rate a single inoculation of *B. iodinum* RS16, *M. oryzae* CBMB20 and co-inoculation showed significant improvement in plant height compared to non-inoculated treatment with 28.3%, 41.2% and 33.9% respectively. Single or co-inoculation of the bacterial strains at 70% fertilizer amendment considerably increased plant height compared to non-inoculated treatment plant at 100% fertilizer application level. Co-inoculation with 70% fertilizer level increased plant height by 24.1% compared to non-inoculated treatment plants at 100% fertilizer level. Total dry weight significantly differed at $P = 0.001$ between treatments with *M. oryzae* CBMB20 (1.98 g) and co-inoculation treatment (1.95 g) compared to the non-inoculated treatment. Co-inoculation treatment with 70% fertilizer amendment increased shoot dry weight (82.3%) and total dry weight (62.5%) compared to non-inoculated treatment at 100% fertilizer. A 94.7% increase in root dry weight compared to non-inoculated plants at 100% fertilizer level were observed for the treatments with *M. oryzae* CBMB20.

Effect of *B. iodinum* RS16 and *M. oryzae* CBMB20 on the growth of sorghum-sudangrass

Sorghum-sudangrass treated with *B. iodinum* RS16 and *M. oryzae* CBMB20 showed greater increase in plant height compared to non-inoculated plants in both levels of fertilizer application. Significant differences were observed between microbial treatment and non-inoculated treatment from 20 DAS at 70% fertilizer level. Higher plant height was observed in *M. oryzae* CBMB20 (32.9 cm) followed by *B. iodinum* RS16 (32.4 cm) and co-inoculation (31.4 cm) treatment at 30 DAS.

Microbial treatments at 70% fertilizer application significantly increased root, shoot and total dry weight compared to non-inoculated treatment at 100% fertilizer level. *B. iodinum* RS16 treatment at 70% fertilization level increased shoot dry weight by 54.5% compared to non-inoculated treatment at 100% fertilizer level. Likewise, increased root dry weight (87.5%) and total dry weight (63.1%) were observed for co-inoculation treatment at 70% fertilizer level, compared to non-inoculated treatment at 100% fertilizer level.

These results show that application of PGPR strains can reduce the fertilizer requirement for crop growth by enhancing the root growth essential for uptake of more nutrients from the soil. Furthermore, the use of PGPR with 70% fertilizer level is better for the improvement of plant biomass of maize, and sorghum-sudangrass can potentially reduce the negative impact of fertilizer and costs involved in crop production.

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CHAPTER TWENTY-FIVE

BIOPROSPECTING OF ENDOPHYTIC BACTERIA FROM MANGROVE, BANANAS AND SUGARCANE PLANTS FOR THEIR PGPR ACTIVITY

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Abstract

The phrase "bioprospecting" is today most frequently used to describe the collection and screening of biological materials for commercial purposes. Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate, overt negative effects. Natural products have been the traditional pathfinder compounds offering an untold diversity of chemical structures. A plethora of microbes have been discovered for a broad spectrum of applications in medicine, agriculture and industry. Endophytic bacteria could be better protected from biotic and abiotic stresses than rhizosphere bacteria. The growth stimulation of the host plant by the endophytic microbes can be a consequence of nitrogen fixation or the production of phytohormones, biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition, and induction of immunity) or by enhancing the availability of nutrients and minerals. In the present study we collected roots, stems and leaf samples from mangrove, banana, and sugarcane plants in sterile cover from Pichavaram, a mangrove forest and trial field at the Faculty of Agriculture, Annamalai University. The samples were transferred to a lab and processed immediately after surface sterilization by the standard procedure. The isolation and purification of endophytic bacteria was done by using starch casein agar with antibiotics to inhibit the growth of fungi. In total, 41 isolates were obtained from all samples, with 24 from mangrove, 7 from banana, and 10 from sugarcane. All the isolates were

taken to test the PGPR activity on: phosphate solubilisation, silicate solubilisation, starch hydrolysis, cellulose, chitinase production, salt tolerance on growth, growth on NFB medium (N_2 fixation), carbohydrate fermentation and antimicrobial activity of endophytic bacteria. Most isolates show good PGPR activity. Based on the results, 4 isolates from mangrove, 3 isolates from sugarcane and 2 isolates from banana were selected as the potential for PGPR activity in future work.

Introduction

The phrase “bioprospecting” is today most frequently used to describe the collection and screening of biological material for commercial purposes (Synnes 2007). Natural products are the traditional pathfinder compounds offering an untold diversity of chemical structures (Strobe & Daisy 2003). Since the discovery of endophytes by Darnal in 1904 (Tan & Zou 2001), various investigations have been done on endophytes in different ways, usually dependent on the prospective from which the endophytes were being isolated and subsequently examined.

Endophytic bacteria live in plant tissue without doing substantive harm or gaining any benefits other than residency. Bacterial endophytes can be isolated from surface disinfected plant tissue or extracted from internal plant tissue (Hallmann et al. 1997). Both Gram positive and Gram negative bacterial endophytes have been isolated from several tissue types in numerous plant species. Furthermore, several different bacterial species have been isolated from a single plant (Arunachalam & Gayathri 2010).

Endophytes enter plant tissue primarily through the root zone; however, aerial portions of plants such as flowers, stems and cotyledons may also be used for entry. Specifically, the bacteria enter tissues via germinating radicals, secondary roots, stomates or as a result of foliar damage. Endophytes inside a plant may either become localized at the point of entry or spread throughout the plant (Hallmann et al. 1997).

Bacon & White (2000) gave an inclusive and widely accepted definition of endophytes as "microbes that colonize living, internal tissues of plants without carrying any immediate overt negative effects." Endophytic bacteria could be better protected from biotic and abiotic stresses than rhizosphere bacteria (Hallmann et al. 1997). The growth stimulation of the host plant by the endophytic microbes can be a consequence of nitrogen fixation or the production of phytohormones, the biocontrol of phytopathogens in the root zone (through the production of antifungal or antibacterial agents, siderophore production, nutrient competition, and induction of immunity), or by enhancing the availability of nutrients and minerals (Rosenblueth &

Martinez-Romero 2006). Secondary metabolites produced by endophytes provide a variety of fitness enhancements such as increased resistant to herbivora, parasitism and growth enhancements. (Firakova et al. 2007).

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used (Strobel & Daisy 2003). Several reasonable theories govern this plant selection strategy and are as follows:

- Plants from unique environmental settings, especially those with an unusual biology
- Plants that have an ethnobotanical history
- Plants that are endemic and that have an unusual longevity
- Plants growing in areas of great biodiversity.

Mangrove are salt-tolerant plants existing at the interface between land and sea in the tropical and subtropical latitudes (Kathiresan 2000). Banana is a giant perennial herb and is one of the main fruits cultivated in subtropical and tropical regions (Cao et al. 2004). Sugarcane is an annual plant which is primarily used to produce sugar and alcohol (Mendes et al. 2007).
All the above three plants have different ethnobiological histories. In the present study we select a tree, an annual plant and a perennial herb with great biodiversity for this research.

Materials and Methods

Sample collection and transport

Samples were collected from Parangipettai (protonova) and Annamalai nagar. All samples were collected and covered by a sterile plastic, transferred to a laboratory, and processed immediately.

Sample pretreatment and endophytic bacterial isolation

For the pretreatment of samples and isolation of endophytic bacteria, the method described by Sun et al. (2006) was adopted with some modifications. All samples were excised and subjected to a three-step surface sterilization procedure. The samples were cut into pieces (of 0.5–1.0 cm), washed in running tap water, and rinsed in 70% ethanol for 30 s, and then rinsed in sodium hypochlorite (3%–5%) for 3 min, and finally thoroughly washed in sterile water 3 times. The samples were plated on starch casein agar and nutrient agar with nystatin and cycloheximide

(50µg/ml) to suppress fungal growth and incubated at 28ºC for 3 d. After incubation, plates were observed for the growth of endophytic bacteria. Morphologically different colonies were selected while pure culture were prepared, and stored in the refrigerator.

Screening for plant growth promoting substances

Phosphate solubilization

The phosphate solubilising activity of endophytic bacteria was studied by the method described by Pandey et al. (2005). All the endophytic isolates were spot inoculated on Pikovaskaya's agar plates and incubated at 28°C for 3–5 d. After incubation all the plates were observed for clear halo zone formation around the bacterial growth.

Silicate solubilization

The silicate solubilising activity of endophytic bacteria was studied using the method described by Bunt & Rovira (2006). All the endophytic isolates were spot inoculated on Bunt & Rovira medium plates and incubated at 28°C for 5–7 d. After incubation all the plates were observed for clear halo zone formation around the bacterial growth.

Salt tolerance on the growth of endophytic bacteria

To study the effect of sodium chloride on the growth of endophytic bacteria, nutrient agar medium was prepared by supplementing it with different concentrations of NaCl and the endophytic bacterial isolates were inoculated into it. All the plates were incubated at 28°C for 5 d and observed every 24 h (Arunachalam & Gayathri 2010).

Screening for enzymatic activity

All the endophytic bacterial isolates were screened for cellulase and chitinase enzyme activity through the cellulose agar and chitin agar plate methods, respectively. All the isolates were spot inoculated on respective enzyme screening media and incubated at 28°C for 5–7 d. After incubation, cellulose production was screened using a reagent 1% congo red and 1M NaCl. (Sahu et al. 2005: Maria et al. 2005).

Starch hydrolysis

All the endophytic bacterial isolates were screened for starch hydrolysis on starch agar. All the isolates were spot inoculated on starch agar media and incubated at 28°C for 5–7 d. After incubation, results were noted by using a reagent 1% Iodine Cho et al. (2007).

Antimicrobial activity

For the production of antimicrobial compounds, No. 3 medium was prepared and about 10% of inoculum was transferred into it. All the test tubes were incubated in a rotary shaker at 95 rpm for 120 h at 28°C. After incubation, 2 mL of culture broth was taken and separated by centrifugation at 10,000 rpm for 10 min. After centrifugation, the culture supernatant was collected and used for antimicrobial activity testing. The antimicrobial activity of the culture supernatant was tested with the agar using the well diffusion method with nutrient agar medium. Test cultures used in this study were human pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Proteus* s*pecies*, *Klebsiella species*, *Pseudomonas species*, *Salmonella typhi*, *Candida,* and plant pathogens such as *Fusarium*, *Curvalaria*, *Penicillium* and *A.niger*. All the pathogenic cultures were obtained from the research laboratory at the Department of Microbiology, Faculty of Agriculture, Annamalai University, Chidambaram. The lawns of the test cultures were prepared on nutrient agar plates using sterile cotton swabs. A 5 mm well was made and 100 µl of culture supernatant was added to it. All the plates were observed for zone of inhibition after incubation at 37°C for 24 h (Gayathri et al. 2010).

Growth on NFB medium of endophytic bacteria

To study the growth on NFB medium of endophytic bacteria, a nitrogen free basal medium was prepared by supplementing it with malic acid, and the endophytic bacterial isolates were inoculated. All the plates were incubated at 28°C for 3 d and observed for the growth of endophytes every 24 h.

Carbohydrate fermentation test

To study the fermentation of different carbohydrates by endophytic bacteria, three different carbohydrates were used for this test: glucose, sucrose and lactose. The basal medium was supplemented with the respective carbon

source at 0.5% concentration. All the endophytic bacterial isolates were inoculated and incubated at 28°C for 24 h and observed for the growth and colour change of the broth culture after incubation.

Results and Discussion

Plant samples and endophytes isolated

All the bacterial isolates were markedly different from terrestrial bacterial isolates. There are numerous reports available on endophytic fungi in mangroves (Chen et al. 2003; Lin et al. 2001). In India, a number of reports recorded the diversity of endophytic bacteria and fungi in medicinal plants (Ananda & Sridhar 2002), but from the available literature there are no reports on the endophytic bacteria from sugarcane and banana, particularly in Tamil Nadu. In general, endophytic bacteria occur in lower population densities than rhizospheric bacteria or bacterial pathogens (Hallmann et al. 1997; Rosenblueth & Martinez-Romero, 2006).

The endophyte isolates obtained in the present study are given in Table 25.1 below. The procedure for the isolation of endophytes yielded about nine root isolates, eight stem isolates, and seven leaf isolates from mangrove plant samples. About four and two root isolates, four and three stem isolates and two leaf isolates were obtained from sugarcane and banana plant samples, respectively.

Table 25.1. Number of samples and endophytes isolated

Sl. No	Plant/ Samples	Root(R) Isolates	Stem(S) Isolates	Leaf(L) Isolates	Total Isolates
1.	Mangrove(M) 1	2	2	2	6
2.	Mangrove(M) 2	2	2	1	5
3.	Mangrove(M) 3	4	2	2	8
4.	Mangrove(M) 4	1	2	2	5
Total Mangrove isolates		**9**	**8**	**7**	**24**
5.	Sugarcane(S) 1	4	4	2	**10**
6.	Banana(B) 1	2	3	2	**7**
	Total isolates	**15**	**15**	**11**	**41**

Salt tolerance by the endophytic bacteria

The percentage of endophytic bacterial isolates to different salt concentrations are given in Table 25.2 below. More than 90% isolates from all plant samples showed good growth up to 7.5% salt concentration. About 62.50% and 37.50% of isolates from mangrove plants showed fair growth at 10% and 12.5% salt concentration, while 40% isolates from sugarcane showed fair growth above 7.5% salt concentration. Isolates obtained from banana plant samples showed about 57.14% isolates at 10% salt concentration, while 28.57% isolates at 12.5% salt concentration showed fair growth.

Table 25.2. Salt tolerance on the growth of endophytic bacteria

Sl. No	Concentrations (In %)	Mangrove Isolates	Sugarcane Isolates	Banana Isolates
1.	2.5	100%	100%	100%
2.	5.0	100%	100%	100%
3.	7.5	100%	90%	100%
4.	10.0	62.50%	40%	57.14%
5.	**12.5**	**37.50%**	**40%**	**28.57%**

Carbohydrate fermentation by endophytic bacteria

The pattern of glucose utilization was in the order of 71.42%, 40.00% and 33.33% by the isolates obtained from banana, sugarcane and mangrove plant samples. The order of sucrose utilization by isolates was 60.00% (sugarcane isolates), 42.85% (banana isolates), and 25.00% (mangrove isolates). The isolates from mangrove (25.00%) and banana (28.57%) utilized lactose, while no isolate from sugarcane utilized lactose (see Table 25.3 below).

Table 25.3. Carbohydrate fermentation by endophytic bacteria

Sl. No	Sugars used	Mangrove Isolates	Sugarcane Isolates	Banana Isolates
1.	Glucose	33.33%	40.00%	71.42%
2.	Sucrose	25.00%	60.00%	42.85%
3.	Lactose	25.00%	0%	28.57%

PGPR activities by endophytic bacteria

Table 25.4 below reveals the PGPR activities such as Phosphate Solublisation, Silicate Solublisation, Cellulase Production, Chitinase Production, Starch Hydrolysis, Growth on NFB Medium and Antimicrobial activity by the bacterial isolates. Endophytic bacteria residing within plant tissues has been reported as promoting plant growth directly or indirectly via the production of phyto hormones, biocontrol of host plant diseases or improvement of plant nutritional status (Pandey et al. 2005). Endophytic bacteria possesses the capacity to solubilize phosphates and it was suggested by the authors that the endophytic bacteria from soyabean may also participate in phosphate assimilation (Rosenblueth & Martinez-Romero 2006). In the present study, most of the isolates from all the samples showed good PGPR activity.

Table 25.4. PGPR activities by endophytic bacteria

Sl. No	PGPR Activities	Mangrove Isolates	Sugarcane Isolates	Banana Isolates
1.	Phosphate Solubilisation	58.33%	50.00%	57.14%
2.	Silicate Solubilisation	54.16%	30.00%	57.14%
3.	Cellulase Production	41.66%	40.00%	42.85%
4.	Chitinase Production	37.50%	40.00%	42.85%
5.	Starch Hydrolysis	62.50%	60.00%	0%
6.	Growth on NFB Medium	29.16%	50.00%	14.28%
7.	Antimicrobial Activity	41.66%	40.00%	42.85%

About 50.00%–58.33% of isolates from all three plant samples showed phosphate solubilisation activities. More than 50.00% of isolates showed silicate solubilisation activity in the mangrove and banana isolates. The cellulase and chitinase activity were demonstrated by 37.50%–42.85% of isolates. Kumerasen & Surya Narayanan (2002) studied the endophytic assemblage in young, mature and senescent leaves of *Rhozospora apiculata* and their possible role in mangrove litter degradation. The results of the present study indicate litter degradation, since it has a wide range of enzymatic activities. Further, these endophytic isolates will be a potential source for extracellular enzymes.

The starch hydrolysis was demonstrated by mangrove (62.50%) and sugarcane (60.00%) isolates, while none of the banana isolates showed starch hydrolysis. About 40.00%–42.85% of isolates from all three plant samples showed antimicrobial activities.

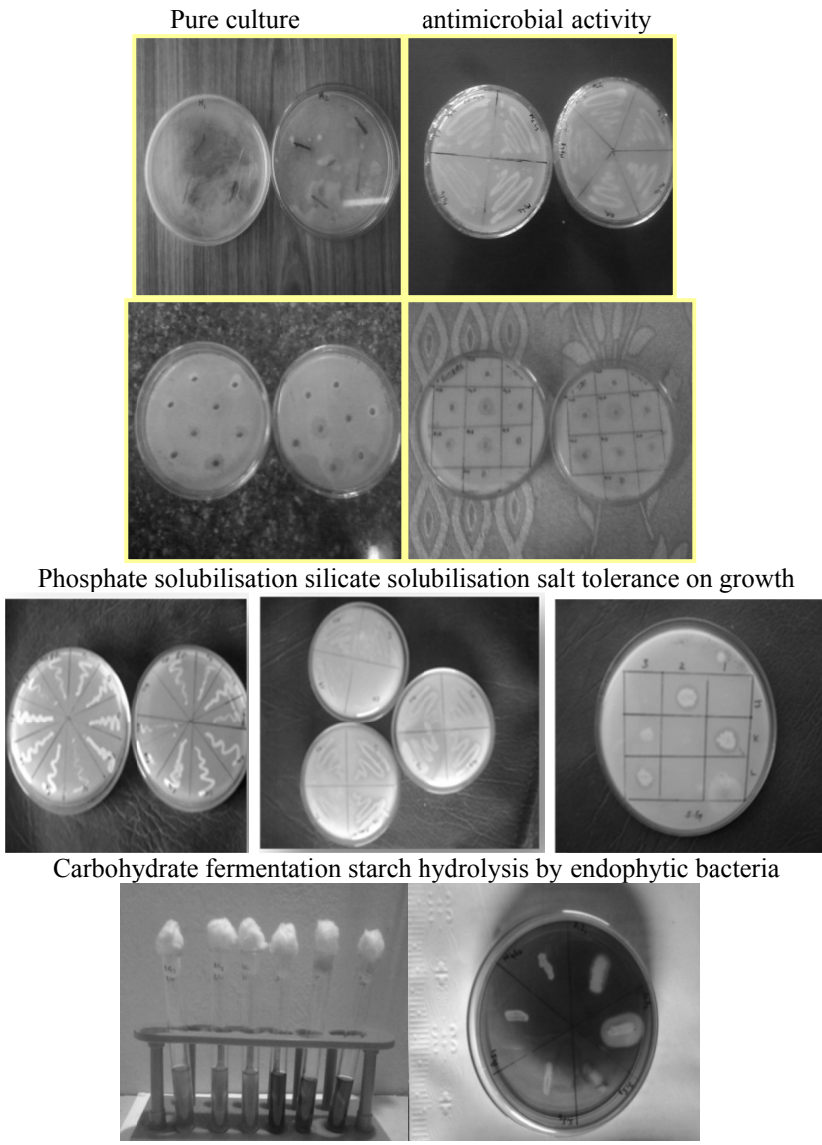

Fig. 25.1. Various PGPR traits of mangrove isolates

Endophytes are the chemical synthesizers within plants. Many of them are capable of synthesizing bioactive compounds that can be used by plants in defence against pathogens, and some of these compounds have been proved as useful for drug discovery. Up to now, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents, antivirals, antidiabetic agents and other bioactive compounds in different functional roles (Guo et al. 2008). In the present study the antimicrobial activity of endophytic bacteria was tested by the agar well diffusion method. Most of the endophytes from all three plants showed good antimicrobial activity.

The present investigation on endophytic bacteria isolated from root, stem and leaf of mangrove, banana and sugarcane provides opportunities to use the endophytic bacteria as a good plant growth promoting bacteria, compared to other normal PGPR organisms. Detailed investigations on the bacterial endophytes of mangrove, sugarcane and banana will be needed to prove the potential PGPR through exploring their numerous high-value metabolites.

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CHAPTER TWENTY-SIX

SURVIVAL OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN COMPOUND CARRIER FORMULATIONS AND THEIR APPLICATION TO MAIZE

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Abstract

The agricultural manipulation of symbiotic and free-living plant growth-promoting rhizobacteria has become a significant component of modern agricultural practice in many countries since plant growth-promoting rhizobacteria (e.g. *Azotobacter, Azospirillum* and *Pseudomonas*) was put forward for the first time in the early twentieth century. Utilizing potential PGPRs as inoculants has received considerable attention. However, the quality control of inoculants must be considerable in the expansion and commercialization of inoculants, and directly determines the effects of PGPR in greenhouses or field experiments. The number of viable cells is a key factor of success of inoculation, and a large amount of the literature has evaluated the maintenance of cell viability and stability, as well as selecting cheap carriers to extend the shelf life of beneficial plant bacterial inoculants for agricultural crops. Peat-based inoculants have been widely used for thirty years; however, little is known about mushroom residue-based inoculants. Meanwhile, more research focused on a single carrier (such as peat, compost etc.) is needed, and the selection of a compound

carrier formulation for plant growth promoting rhizobacteria has seldom been reported. The present chapter evaluates the shelf life and colonization efficiency of plant growth promoting rhizobacteria (PGPR) strains on compound carrier formulations, and their effects on the hay yield and NP content of maize (*Zea mays* L.). A mixture of three plant growth promoting rhizobacteria strains (*Bacillus* sp. Jm170, *Pseudomonas* sp. Jm92 and *Azotobacter* sp. Lx191) was inoculated to compound carrier formulations, composed of five carriers (peat, charcoal, cultivate soil, mushroom residue and potato starchy residue) which were used as alternatives to peat. The results show that compound carrier-based inoculants F1 (peat + charcoal = 1:1.5), F3 (peat+charcoal+cultivate soil = 1:1:0.5) and F4 (peat+mushroom residue+cultivate soil = 0.5:1:1), stored at room temperature, provided a longer shelf life than peat-based inoculants after 6 months of storage, and viable bacteria reaching 10^9 cfu/g inoculants. Field experiments indicated that the effects of the compound carrier-based inoculants on the hay yield and NP content of maize are superior to control (without inoculation), increasing by 8.2%, 6.0% and 5.0% on average. Meanwhile, the application of the compound carrier-based inoculants had an advantage over peat-based inoculants, and F3 was the best formulation of inoculants. Compared to control, all the compound carrier-based inoculants had a significant effect on soil microbial quantity composition, including bacteria, fungus and actinomyces, especially for the amount of bacteria, and inoculants formulated by F3 showed stable colonization ability. In conclusion, F3 can be used to produce PGPR inoculants for sustainable agriculture.

Introduction

Maize (*Zea mays* L.) is considered one of the oldest crops in the world, emerging as a domesticated plant in the southwest and northwest of China around the eighteenth century. In general, excessive fertilizer use guarantees a maize yield. However, continuous application of synthetic fertilizers has continuously increased soil pH, which is why most of the soils in northern China are saline with a pH of more than seven. Meanwhile, extensive studies have demonstrated that maize is suitable for cultivation in soil that is neutral, with a pH value of 7, or that is slightly acidic. Considering a series of environment problems, along with the negative effects on animal and human health, there is need for the application of inoculants because they reduce the soil pH and are environmental friendly.

The agricultural manipulation of symbiotic and free-living plant growth-promoting rhizobacteria has become a significant component of modern agricultural practice in many countries since plant growth-promoting rhizobacteria (PGPR) was put forward for the first time in the early twentieth century. Utilizing potential PGPR as inoculants has received considerable attention. Inoculants, however, are well recognized as an important component of integrated plant nutrient management for sustainable agriculture and hold great promise for improving crop yields. The beneficial effect of plant growth promoting rhizobacteria, particularly those belonging to the genus *Pseudomonas*, *Azospirillium*, *Azotobacter* and *Bacillus*, in enhancing growth and overall plant establishment is well established (Joseph et al. 2007). Indeed, they produce metabolites such as plant growth regulators that directly promote growth and facilitate nutrient uptake by plants. Furthermore, these bacteria are an essential component of the rhizosphere of many plants and are known to colonize the rhizosphere of maize. Especially in the northwest of China wherein the fertility characteristics of soil show it to be abundant in potassium and deficient in nitrogen and phosphorus. As a matter of fact, nitrogen deficiency symptoms can be eased through N-fertilizer supply while phosphorus deficiency is hard to improve because of P fertilizer existing in bound forms of phosphate like $Ca_3(PO_4)_2$, which plants find difficult to assimilate (Zhang et al. 2010). In the meantime, phosphorus deficiency has also led to lower uptakes of nitrogen and potassium (Rao et al. 2002). Hitherto, the use of phosphorus inoculants is more strategically significant for sustainable agricultural development, which could not only increase the availability of phosphate by solubilization but also improve the efficiency of biological nitrogen fixation and increase the availability of Fe, Zn and so on through the production of plant growth promoting substances (Muhammad et al. 2012).

According to Berg & Smalla (2008), a set of parameters including biotic (i.e. plant species), abiotic (i.e. soil types), rhizosphere competence (i.e. plant hormones), and positive and negative interactions (i.e. pathogens) influences the community of rhizosphere microbes. Such parameters can importantly affect inoculants. As for inoculants expansion and commercialization, the quality control of inoculants must be considerable in their expansion and commercialization, directly determining the effects of PGPR in greenhouses of the field experiments. There are two factors determining the quality control of inoculants: one is the selection of the appropriate PGPR strains under different conditions, while the other is an appropriate inoculants carrier with the right formulation for the successful colonization of PGPR. To date, the literature has observed the selection of

PGPR in different habitats, while the selection of compound carrier formulation for plant growth promoting rhizobacteria was seldom reported.

Ben (2002) reported that an appropriate inoculants carrier should have the following characteristics: a high water-holding capacity, chemical and physical uniformity, non-toxic to the microbial strain and environmentally safe. At the same time, these materials should maintain near neutral or readily adjustable pH and be easy to obtain locally and at reasonable cost. At present, various carrier materials including charcoal soil mixture (Abhinav et al. 2011), wastewater sludge (Ben et al. 2002), vermiculite (Ilibazilah et al. 2011), alginate (Pankaj et al. 2008; Russo et al. 2011), sawdust (Abhinav et al. 2011), compost (Zhang et al. 2010; Li et al. 2011), rice husk (Anandham et al. 2007), and polymer (Marinete et al. 2012) have been used for developing PGPR inoculants. Peat-based inoculants have been widely used for thirty years, however little is known about mushroom residue-based inoculants. Meanwhile, more researches have focused on a single carrier (such as peat, composting, etc.), and the selection of compound carrier formulation for plant growth promoting rhizobacteria was seldom reported.

The present study aims to answer the question of whether plant growth promoting rhizobacteria (PGPR) can survive in compound carrier formulations, and which carrier formulation is the best on the basis of its application on maize. We therefore set out to evaluate viable bacteria on different carrier formulations and their application on maize in terms of the height, aboveground biomass, NP content and soil microbial quantity composition.

Materials and Methods

Bacterial strains

Bacillus sp. (Jm170), *Pseudomonas sp.* (Jm92) and *Azotobacter sp.* (Lx191), isolated from the rhizosphere of wheat (*Triticum aestivum*) and alfalfa (*Medicago sativa*) in a semi-arid area of the country, have been reported for their efficiency to promote plant growth and disease control (Feng et al. 2009; Rong et al. 2011) and their characters are shown in Table 26.1 below.

Table 26.1. Strains for test

Strain code	Scientific name	Host plant	Function	Nitrogenase activity (C_2H_4nmol/m·h)	P-solubilization capacity (mg/L)	IAA secreting ability (μg/mL)
Jm170	*Bacillus* sp.	Medicago sativa	P-solubilization	58.97	178.2	4.34
Jm92	*Pseudomonas* sp.	Medicago sativa	P-solubilization	75.34	132.60	47.25
Lx191	*Azotobacter* sp.	Triticum aestivum	P-solubilization	-	200.02	54.36

Note: Nitrogenase activity, P-solubilization capacity and IAA secreting ability were measured by high performance liquid chromatograph (HPLC), molybdenum blue colorimetric method and S2 colorimetry method, respectively.

Physico-chemical characteristics of carrier formulation

Charcoal, peat and cultivate soil were obtained from a flower market in Lanzhou City, Gansu Province. Mushroom residual were supplied by local peasants and their main ingredients were cotton seed hull and hypha. Potato starchy residue was achieved from the company in Dingxi city. Physical parameters were determined, such as pH by a pH meter (3305), moisture and water-holding capacity, and chemical analysis of nitrogen (N) by the Kjeldahl method (K9860, Hlanon), phosphorus (P) by the spectrophotometric method (SP-752, Spectrum), and potassium (K) using a flame photometer (AP130, Anachip) were studied.

Table 26.2. Physical and chemical properties of the carrier

Materials	Water content (%)	pH	Total nitrogen (g/kg)	Available phosphorus (g/kg)	Total potassium (g/kg)	Available potassium (g/kg)
Peat	10.65	5.27	6.7	0.18	24.11	9.07
Charcoal	2.49	8.26	1.5	0.28	15.54	2.72
Cultivate soil	50.39	7.65	12.0	0.36	28.94	8.97
Mushroom residue	6.73	7.75	16.5	1.90	31.06	8.34

Preparation of inocula

Tested strains were grown separately in 250-mL flasks containing 100 ml of LB (10 g peptone, 5 g Yeast extract, 5 g NaCl, pH 7.0-7.2.) medium on a rotary shaker at constant rotation (150 rpm) at 28°C. The mixed bacteria suspension (MBS) consisted of three uniform bacteria suspensions at 1:1:1 volume ratio. 150 g of seven carrier formulations on a mass ratio, namely peat (control), F1 (peat+charcoal = 1:1.5), F2 (peat+potato starchy residue = 1:1.5), F3 (peat+charcoal+cultivate soil = 1:1:0.5), F4 (peat+mushroom residue+cultivate soil = 0.5:1:1), and F5 (peat+potato starchy residue+cultivate soil = 0.5:1:1), were sterilized in a 1000 mL conical flask in triplicate in an autoclave at 121°C for 30 mins, and inoculated with 50 ml MSB culture of initial density 2×10^8 cfu/ml, and then packaged in heat-sealed polyethylene bags. Inoculants were stored at 28°C.

Viable cell count of inoculants in carrier-based materials

A 10 g sample from each inoculant was suspended in a 150 ml conical flask containing 90 ml sterile 0.85% saline solution and then diluted 10^{-7}, 10^{-8} and 10^{-9}. A 50µl suspension was incubated on the plate at 28°C and the colony-forming units (cfu/g) were counted after 3 d. These viability tests were carried out with fresh preparations as well as preparations stored at 4°C at an interval of 1 month up to a period of 6 months of storage.

Field experiment

This experiment was conducted in Xiangquan (35°35′N and 104°36′E with an altitude 2304 m), Dingxi City, China from April to September, 2012. The average temperature and annual precipitation in the region are 6.9°C and 400 mm, respectively. Some of the soil properties were: pH 8.0, TOC 0.89%, total N 0.68g/Kg, available N, P, and K, 48.6, 8.35, and 182.5 mg/Kg, respectively. All plots of the experimental field were treated with 75 Kg/ha phosphate fertilizer following soil tests before sowing.

The layout of the trial was a randomized complete block design (RCBD) with 3 replications and 6 treatments. The area of each plot was 10m × 3.0m. Maize (variety Cheng 3359) was used as a test species. The inter-row distance and within-row distance of maize were 30 cm and 20 cm, respectively. The 150 g maize seeds were inoculated with 100 g tested carrier-based inoculants. Then, the inoculated seeds were dried in a shed (to avoid direct sunshine) and sowing was immediately performed by

hand. In order to prevent cross infection between treatments, the un-inoculated control plots were sown beforehand. At maturity, a 2 × 2 m^2 area of unsampled four central rows of each plot was hand-harvested. The plants were air dried and biomass dry weight, grain yield, nitrogen and phosphorus contents in grain samples were determined. Meanwhile, a 0–20 cm soil sample was collected from the rhizosphere of maize to measure soil microbial quantity composition, including bacteria, fungus and actinomyces.

Statistical analysis

A one-way ANOVA and a Duncan multiple range test were used to evaluate the significant difference in the concentration of different study sites. A probability at the level of 0.05 or less was considered significant (Bailey 1981). The standard error of the mean, variance and ANOVA statistics were calculated using SPSS 16.0 statistical software.

Results and Discussion

Enumeration of viable bacteria in various formulations under storage

The feasibility of using PGPR inoculants in greenhouses or the field is determined largely by carrier formulation, shelf life and delivery techniques (Albareda et al. 2008; Khavazi et al. 2007). The development of cost effective, friendly and readily available commercial formulations for beneficial microbes was extremely urgent. Viable bacterial counts, in the single and compound based carriers, kept at 28ºC, were determined 30, 60, 90, 160, 120, 150 and 180 d after inoculation (see Fig. 26.1 below). The results showed that the cell viability of MSB in all carrier-based inoculants decreased with the extension of storage time. The number of viable bacteria in six carrier formulations accorded with the quality criterion of agricultural microbial fertilizer of China (NY227-94). In addition to F2, the viable bacteria in F1, F3 and F4 were significantly higher than peat (as control). F3 proved the most superior of the carriers tested as they maintain the higher number of cells, and the number of bacteria can reach about 10^9 cfu/g of carrier after inoculating for 180 d.

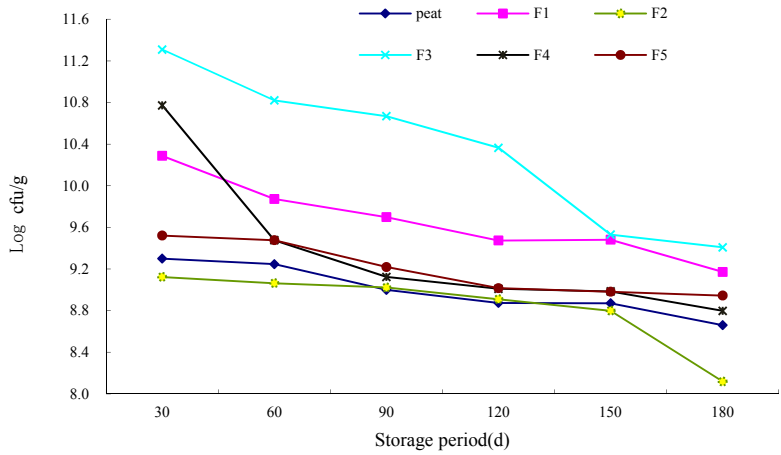

Fig 26.1. Survival of viable bacteria of various based carriers under storage

Solid-based carriers provide the micro-environment and protect the transported micro-organism from hostile conditions and maintain strains over an acceptable period, which is generally difficult to obtain (Aeron et al. 2011). The viable bacteria of inoculants were taken as a parameter to judge the promising application of inoculants. Many factors may have effects on the visible cells of inoculants, such as temperature (Ilibazilah et al. 2011), sterilisation method (Khavazi et al. 2007), and pH (Niu et al. 2003). However, formulation plays a significant role in determining the efficacy of inoculants and carriers, with a close to neutral pH being one of the desirable properties. In the present chapter, compound carrier-based inoculants have a distinct advantage for sustaining the cell population, the reasons being that they addressed a suitable environment for microbial growth (neutral pH and soluble nutrients), avoided regulating the pH value of the carrier to simplify the procedure of inoculant production, and took full advantage of agricultural wastes (e.g. mushroom residue and potato starch residue).

Effects of inoculants in different formulations on height, hay yield, crude protein and P content of maize

While PGPR from cereals and their ability to promote plant growth have been extensively studied, much less information has been published on their effects on nutritive value for animal feed. As maize is used as forage

for animals in the region, the change in nutritive value due to the inoculums should be taken into account. In the present study, we tried to analyze the changes in feed value due to the use of bacterial inoculums. The observations on parameters indicated the application of inoculants increased hay yield, CP and P content by 8.2%, 6.0% and 5.0% on average compared to control (without inoculation), whereas in terms of maize height no significant differences were found ($P > 0.05$). F3-based inoculants recorded the maximum hay yield (23900 Kg/hm^2), CP content (10.53%), and P content (0.652%), respectively, and were followed by F1 and F4. The application of different inoculants has a significant difference on hay yield, CP content and P content ($P < 0.05$), and means that the nutritional quality was improved by using the inoculants. We suggest that this probably arises from an increase in available nutrients from soil and the greater proportion of highly-digestible cell contents from the use of the bacterial inoculants. Abhinav (2011) reported that sawdust-soil was found to be the most efficient carrier material for the PGPR strain *Pseudomonas fluorescens PS1*, followed by other carriers, and their inoculants can increase overall rapeseed plant growth as well as reduce stem blight in mustard with improved yield. Esitken et al. (2010) showed that the use of PGPB significantly increased fruit yield, plant growth and leaf P and Zn content, while Hamidi et al. (2008) showed that fresh weight, number of leaves above the ear, yield of silage forage, and vegetative growth of maize all increased with the application of PGPR.

Indeed, the application of carrier-based inoculants is susceptible to climate conditions (temperature, pH, protozoa, indigenous flora), and continuous field experiments in different regions should be carried out to extend F3-based inoculants on a large scale.

Table 26.3. Effects of inoculants in different formulations on height, hay yield and NP content of maize

Growth parameters	CK	peat	F1	F2	F3	F4	F5
Plant height (cm)	289a	291a	306a	292a	302a	305a	285a
Hay yield (kg/hm^2)	19800c	21800b	23100ab	20200c	23900a	22800ab	21900b
Crude protein (%)	8.93b	9.32 b	9.69ab	9.41b	10.53a	9.47 b	8.87b
Phosphorus (%)	0.576c	0.580c	0.575c	0.623b	0.652a	0.639a	0.620b

Note: Different letters mean significant difference at level of 0.05.

Effects of inoculants in different formulations on soil microbial composition

The reason that inoculants can promote plant growth is that beneficial bacteria (PGPR) are prone to colonize in the rhizosphere and root surface or the intercellular spaces of the host plant. The number of soil microbials, treated by carrier-based inoculants, was determined, and the results indicate that inoculants have a significant effect on the soil microbial composition, including bacteria, fungus, actinomycetes and that the total soil microbials varied depending on carrier formulation. The number of bacteria in all treatments (with the exception of F5) was higher than that of the control, while fungus and actinomycetes were superior to the control. The maximum number of bacteria, fungus and actinomycetes were obtained by F3, the number reaching 24.25×10^5 cfu/g, 1.50×10^3 cfu/g, 1.24×10^5 cfu/g, followed by F4. In general, the change of total soil microbials was in accord with bacteria, because the application of inoculants significantly increased the number of bacteria in the roots. A wide range of soil microbials was used to enhance the availability of different nutrients by utilizing different mechanisms including the production of different enzymes, and biochemicals are produced by soil microbes among which polysaccharides are the ones with the highest impact on binding soil particles, which promote plant growth.

Table 26.4. Effects of inoculants in different formulations on soil microbial composition

Treatments	Bacteria (cfu/g)	Fungus (cfu/g)	Actinomycetes (cfu/g)	Total soil microbials (cfu/g)
CK	3.29×10^5	0.05×10^3	0.18×10^5	3.47×10^5
Peat	4.51×10^5	0.71×10^3	0.45×10^5	4.96×10^5
F1	7.66×10^5	0.47×10^3	0.54×10^5	8.20×10^5
F2	3.17×10^5	0.30×10^3	0.65×10^5	3.82×10^5
F3	24.25×10^5	1.50×10^3	1.24×10^5	25.49×10^5
F4	7.69×10^5	0.35×10^3	1.18×10^5	8.87×10^5
F5	1.85×10^5	0.30×10^3	0.60×10^5	2.45×10^5

In our work, it was difficult to reflect the colonization ability of PGPR without marking PGPR. However, the use of specific mark methods, including the fluorescence mark and antibiotic resistance mark, can effectively solve this problem. Therefore, how PGPR successfully colonize in soil needs further study.

Conclusion

There were differences in the efficacy of the bacteria for each formulation. F1 (peat+charcoal = 1:1.5), F3 (peat+charcoal+cultivate soil = 1:1:0.5) and F4 (peat+mushroom residue+cultivate soil = 0.5:1:1), stored at room temperature, provided a longer shelf-life than peat-based inoculants after 6 months of storage and viable bacteria reaching 10^9 cfu/g inoculants. Field experiments indicated the effects of the compound carrier-based inoculants on the hay yield and NP content of maize as superior to control (without inoculation), increasing by 8.2%, 6.0% and 5.0% on average. Meanwhile, the application of the compound carrier-based inoculants had an advantage over peat-based inoculants, and F3 was the best formulation. Compared to control, all the compound carrier-based inoculants have a significant effect on soil microbial quantity composition, including bacteria, fungus and actinomyces, especially for the amount of bacteria, and inoculants formulated by F3 showed a stable colonization ability. In conclusion, F3 can be used to produce PGPR inoculants for sustainable agriculture.

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POSTER PRESENTATIONS

CHAPTER TWENTY-SEVEN

INDUCTION OF SYSTEMIC RESISTANCE AGAINST *PHYTOPHTHORA CAPSICI* AND *PECTOBACTERIUM CAROTOVORUM* SCC1 BY TREATMENT OF ITURIN DERIVATIVES IN CHILI PEPPER

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Abstract

Five derivatives of cyclic lipopeptides (CLP)s, known as Iturin-A analogs, were identified from a promising plant growth-promoting rhizobacteria (PGPR), *Bacillus vallismortis* strain EXTN-1 (EXTN-1), based on growth promotion and induced systemic resistance (ISR) on various crops. Indirect protection of the plants through ISR by treatment with Iturin-A analogs possessed different degrees of suppression of phytophthora blight caused by *Phytophthora capsici* and soft rot caused by *Pectobacterium carotovorum* SCC1 in chili-pepper (*Capsicum annum* L.). Iturin-A analogs derived from culture filtrate of EXTN-1 were purified using column chromatography and reverse-phased HPLC, and were identified as five different Itruin-A analogs based on NMR spectral analysis. Among all the Iturin-A analogs, the treatment with Itruin-A2 at lower concentration (0.1 ppm) by foliar spray induced systemic resistance against *P. capsici* when compared to water-treated control upon challenge with encystspores of *P. capsici*. However, when Iturin-A analogs were tested for antibiosis assay,

only Iturin-A6 and Iturin-A8 possessed antibiosis activity at higher concentrations. This suggests that the ISR activity was not on the basis of antibiosis, but through some other mechanism. The result of RT-PCR for defence-related gene expressions revealed that the expression levels were varied at various concentrations for all the defence genes in three-week-old chili pepper after pathogen challenge. These results suggest that the Iturin analogs from EXTN-1 are involved in the activation of plant defence.

Introduction

The biological control of plants by microorganisms is one of the promising and alternative ways to use pesticides, which are often expensive and have adverse effects on humans (Leroux 2003). It is well known that plant growth-promoting rhizobacteria (PGPR) help plants to induce resistance against pathogens by secreting biologically active compounds. Due to their immobility, plants can develop defence mechanisms against plant pathogens by interacting with beneficial rhizobacteria in the soil environment (Cui et al. 2005). Phytophthora blight of chili-pepper caused by *Phytophthora capsici* is a soil-borne pathogen that causes seedling blight, foliar blight, root rot and pod rot in nearly all cultivars of *Capsicum annum* (Goldberg 1995). Members of the *Bacillus* genus are considered to be microbial factories for the production of a vast array of biologically active molecules, potentially inhibitory for the growth of phytopathogens (Lisboa et al. 2006). Several strains of *B. subtilis* and *B. amyloliquefaciens* have been reported to produce lipopeptides that possess strong antifungal activity with low toxicity, high biodegradability and as being environmental friendly (Kim et al. 2010).

In recent years, research on the biological control of phytophthora blight and soft rot diseases in chili pepper has been expanded in response to growing concerns about the side effects of fungicides as environmental pollutants. The need to reduce pesticide application to food crops and the concern for environmental pollution call for alternative methods for disease control by the use of biocontrol agents (BCAs). Iturin is one of the cyclic lipopeptides (CLPs) antibiotics against various plant pathogens, and some of the CLPs are non-antibiotics. Iturins are usually produced by *Bacillus subtilis* and other closely related Bacilli, e.g. *B. amyloliquefaciens* (Souto et al. 2004). The Iturin group comprises iturin A-E. Itruin A, the best-known member, was isolated from *B. subtilis* (Stein 2005). The potential for iturin derivatives in the protection of plants through ISR is being studied in the plant system for the first time. Iturins are a family of

lipopeptides extracted from the culture media of various strains of *Bacillus* spp, and exhibit strong antifungal activities against a wide variety of pathogenic yeasts and fungi (Maget-Dana & Peypoux 1994). The present study aims to investigate the potential of Iturin-A analogs derived from EXTN-1 to control phytophthora blight caused by *P. capsici* in chili-pepper through ISR.

Materials and Methods

Rhizobacteria were isolated from the rhizosphere soil of chili peppers in Korea. From a total of 2,300 strains of rhizobacteria, the strain EXTN-1 was selected based on its potential to inhibit the growth of various pathogens *in vitro* (Park et al. 2007). From solid culture media TSA (10 L), EXTN-1 were harvested and extracted with methanol (MeOH), and the extract was concentrated under the rotary vacuum evaporator. In total, seven compounds were identified as cyclic lipopetides (CLPs) based on NMR and Mass spectral data. The purified iturin-A analogs were screened for antibiosis assay against major fungal plant pathogens. The inhibition zone was recorded in diameter after seven days of incubation at 28 °C. *P. capsici* inoculum was prepared as described by Ploetz et al. (2002). To prepare *P. carotovorurm* SCC1 inoculum, 10 ml of SDW was poured on a TSA culture plate, scraped with a sterile plastic loop and adjusted to a final concentration of 1×10^8 cfu/ml (OD_{600}=0.8) before application.

Chili pepper (*Capsicum annum* L.) cv. Hanbyul seedlings at first-branch stage were used in this study. For the induced protection of chili pepper from *P. capsici* and *P. carotovorum* SCC1, the Iturin-A analogs were applied by leaf infiltration at different concentrations (0.1, 1.0, 10 ppm) under greenhouse conditions. One week later, the treated plants were challenged with encystspore suspensions of *P. capsici* (1×10^5 zoospores/mL). Phytophthora blight incidence (%) was recorded 7 d after challenge inoculation. For ISR activity against soft rot disease the square plate assay was performed. The chili pepper leaves were treated with the SCC1 suspensions and the disease incidence (%) was recorded 48 h after incubation at 28 °C. Defence gene expression against *P. capsici* by iturin-A analogs was investigated in 3 wk old chili pepper seedlings at various concentrations using a RT-PCR analysis. The data were subjected to analysis of variance using SAS JMP software, SAS Institute, USA (SAS 2007). Significant differences in treatment means on each sample data were determined using LSD at $P = 0.05$. For each experiment, data were analyzed separately. The results of one representative experiment are shown.

Results and Discussion

The HPLC analysis revealed that there were five major peaks of compounds in EXTN-1 (see Fig. 27.1a below). Samples from each peak were condensed and assayed for antifungal activity against major plant fungal pathogens. The active fractions obtained by a sephadex C-18 column and reverse-phased HPLC were subjected to NMR and Mass spectral analysis for structural elucidation (see Fig. 27.1b below). The present study investigated the potential for Iturin-A analogs to control *P. capsici* and SCC1 in chili pepper. The results show that the Iturin-A analogs are important components of the of *B. vallismortis* EXTN-1 in the biocontrol of phytophthora blight and soft rot of chili pepper. This is the first report on iturins, derived from EXTN-1 in disease suppression through ISR in the plants. Previously, *Bacillus* strains have been reported to produce biosurfactant lipopeptides that suppress the growth of phytopathogenic fungi (Cho et al. 2003).

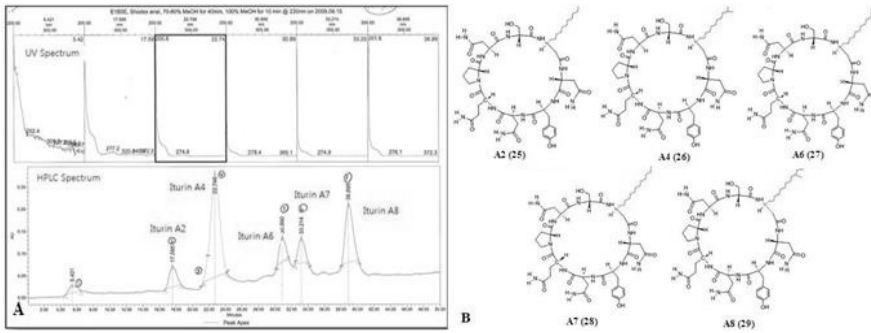

Fig. 27.1 (a) HPLC chromatogram of compounds (Iturin-A analogs) obtained from culture extract of EXTN-1. (b) Isolated and identified CLPs Itruin A analogs derived from EXTN-1 extract based on NMR and Mass spectrum analysis from the fraction 5. The molecular formulae for Iturin A2 ($C_{48}H_{75}N_{12}O_{14}$+H), Iturin A4 ($C_{49}H_{77}N_{12}O_{14}$+H), Iturin A6 ($C_{50}H_{78}N_{12}O_{14}$+H), Iturin A7 ($C_{50}H_{78}N_{12}O_{14}$+H), and Iturin A8 ($C_{51}H_{80}N_{12}O_{14}$+H) were obtained from these structures.

The Iturin-A analogs were screened for antibiosis assay under *in vitro* conditions against major plant pathogenic fungi that cause severe diseases in the plants. The antibiosis activity of five different Iturin-A analogs of EXTN-1 at 500 ppm concentration was tested against selected major plant pathogenic fungi on PDA plates (see Fig. 27.2 below).

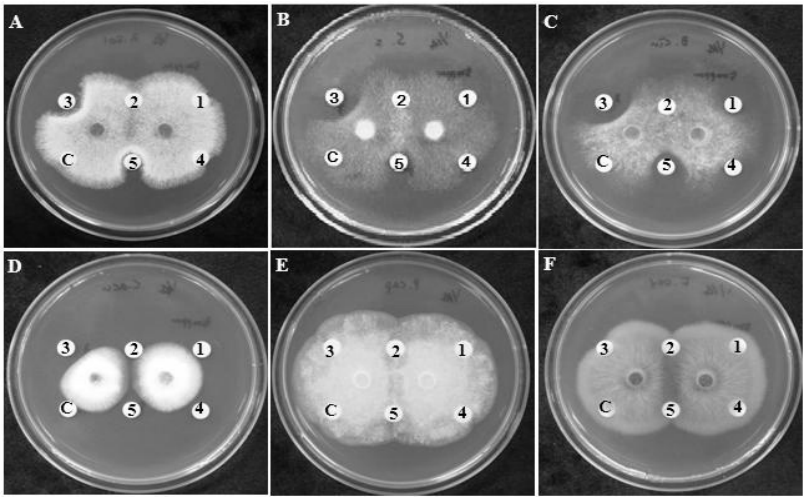

Fig. 27.2. *In vitro* antibiosis assay of Iturin analogs derived from *Bacillus vallismortis* EXTN-1 against major fungal plant pathogens on PDA plates
(a) *Rhizoctonia solani* (b) *Sclerotinia sclerotiorum* (c) *Botrytis cinerea* (d) *Colletotricum acutatum* (e) *Phytophthora capcisi* (f) *Fusarium oxysporum*

From these results, we suggest that CLPs derived from EXTN-1 would seem to be good biocontrol agents against *P. capsici* and SCC1. Various CLPs from bacteria such as *Pseudomonas* spp (Bruijin et al. 2007) or *Bacillus* spp (Zhang et al. 2012) have been described. Treatments with Iturin-A analogs significantly ($P < 0.05$) reduced the phytophthora blight and soft rot infections caused by *P. capsici* and *P. carotovorum* SCC1, respectively, under greenhouse conditions when compared to water-treated control and chemical inducer BTH (0.1 mM). Various concentrations (0.1, 1.0, and 10 ppm) of the five Iturin-A analogs were tested for the suppression of *P. capsici* in chili pepper (see Table 27.1 below). All five Itruin-A analogs were found to be effective in suppression of *P. capsici* at various concentrations upon challenge inoculation with zoospores of *P. capsici*. In the case of soft rot disease suppression by iturin-A analogs, there was higher reduction (7.5%) of soft rot by iturin-A8 at 0.1 ppm, and similar results were observed in iturin-A4 at 1.0 ppm concentration and BTH (0.1 mM) when compared to water-treated control (see Table 27.2 below). From our previous study, *P. capsici* of black pepper was drastically reduced by EXTN-1 when compared to water-treated control in Vietnam.

Table 27.1. Disease suppression of phytophthora blight caused by *Phytophthora capsici* in chili pepper by spraying iturin-*A analogs*

Treatment	Lesion area/leaf (%)				
	Iturin A2	**Iturin A4**	**Iturin A6**	**Iturin A7**	**Iturin A8**
Control	88.0				
BTH 0.1mM	44.0				
0.1 ppm	18.0	38.0	56.0	46.0	36.0
1.0 ppm	28.0	4.0	16.0	20.0	58.0
10 ppm	0	16.0	16.0	82.0	20.0
LSD (*P*=0.05)	43.9	47.4	51.4	49.1	56.5

Table 27.2. Disease suppression of soft rot caused by *Pectobacterium carotovorum* SCC1 in chili pepper by spraying iturin-A analogs

Treatment	Lesion area/leaf (%)				
	Iturin A2	**Iturin A4**	**Iturin A6**	**Iturin A7**	**Iturin A8**
Control	37.0				
BTH 0.1mM	8.50				
0.1 ppm	14.0	11.5	11.0	10.0	7.5
1.0 ppm	39.0	8.5	49.0	12.0	10.0
10 ppm	33.0	26.5	18.0	14.5	43.5
LSD (*P*=0.05)	15.8	14.6	14.1	9.2	13.2

In order to ascertain the enhancement of ISR activity of Iturin-A analogs, the defence gene expression in chili pepper against *P. capsici* was studied using RT-PCR analysis. Overall, compared to water-treated control, all the Iturin-A analogs could express the defence genes at all concentrations to suppress *P. capsici* disease development through induced systemic resistance, and all the defence genes were expressed either strongly or moderately upon pathogen challenge.

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CHAPTER TWENTY-EIGHT

INVESTIGATIONS ON BIOACTIVE SECONDARY METABOLITES OF *TRICHODERMA* SPP AND THEIR EFFICACY AGAINST AFLATOXIN CONTAMINATION IN GROUNDNUT

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Abstract

Aflatoxin contamination in groundnut is considered to be a major qualitative problem affecting human and animal health. In our study, *Trichoderma* isolates collected from six groundnut growing districts of southern India were tested against *Aspergillus flavus*. *In vitro* assays revealed that 48 isolates were significantly antagonistic to the *Aspergillus flavus* toxigenic strain (AF 11-4). These isolates belonged to 10 different species of *Trichoderma*. Based on our preliminary screening for cellular enzyme production, seven potent *Trichoderma* isolates were selected for secondary metabolite production. Secondary metabolites were extracted and tested against *A. flavus* under *in vitro* conditions. The effect of secondary metabolites in reducing seed infection by *A. flavus* was studied *in vitro*. Based on our results, *T. harzianum* (T 20) and *T. longibrachiatum* (T 2) were selected to fractionate secondary metabolites produced through HPLC. Two prominent peaks with retention times of 2.21 and 8.52 min

were collected for the isolate T 20 and three prominent peaks with retention times of 1.38, 2.17 and 6.89 min were collected from the isolate T 2. These fractions were further analyzed using FTIR and Mass Spectrometry followed by NMR for the grouping of compounds and structural identification, respectively.

Introduction

Groundnut (*Arachis hypogaea*) is an important legume cultivated and utilized in more than 100 countries worldwide and is an important source of protein. Aflatoxin contamination, resulting from *Aspergillus flavus* infection, is a major limiting factor affecting the quality and trade of groundnut (Wu et al. 2008). The biological control of *A. flavus* infection and subsequent aflatoxin contamination is one of the most effective and feasible ways of managing this menace. The *Trichoderma* species are effective in the control of soil- or seed-borne fungal diseases in several crop plants (Kubicek et al. 2001). Most of the effective *Trichoderma* strains produce both cell wall lytic enzymes and secondary metabolites against the disease-causing fungi (Vinale et al. 2008; Vinale et al. 2006; Rahman et al. 2011). Earlier studies indicate that antifungal metabolites are extracted from *Trichoderma* isolates using different solvents and were tested against *A. flavus* under *in vitro* conditions (Jantarach & Thanaboripat 2010). In our previous study, we established the mechanism of biocontrol of *A. flavus* in groundnut in different species of *Trichoderma* (Srilakshmi et al. 2011). However, in the present study we aimed at extracting secondary metabolites from seven potent *Trichoderma* isolates and tested their efficacy in inhibiting *Aspergillus flavus* growth under *in vitro* conditions.

Materials and Methods

The antagonistic 48 *Trichoderma* spp were isolated and identified as *T. harzianum*, *T. fertile*, *T. fasiculatum*, *T. koningii*, *T. viride*, *T. atroviride*, *T. hamatum*, *T. aureoviride*, *T. pseudo koningii* and *T. longibrachiatum* using standard protocols. Seven elite strains showing high antagonism were used for biocontrol studies. They are T. harzianum (T 20), *T. pseudokoningii* (T 29), *T. hamatum* (T 47), *T. viride* (T 24), *T. koningii* (T 83), *T. longibrachiatum* (T102) and *T. viride* (T 179). All were used for the extraction of antifungal metabolites using standard protocols.

Determination of optimal concentration of ethyl acetate extract on the growth of *A. flavus* (Af 11-4) by the agar well method

Ethyl acetate extract from all seven isolates were dissolved in DMSO to final concentrations of 25, 50 and 100 mg/ml. Agar wells were made with cork borer and all four concentrations tested on potato dextrose agar against *Aspergillus flavus* one concentration/one well. An *A. flavus* spore suspension (10^6 spores/ml) was added and the plates were incubated for 5 d. As a control, DMSO was added to one of the plates instead of *A. flavus* spore suspension (10^6 spores/ml). After incubation the colony diameter was measured in both treated and control PDA plates.

In vitro antagonistic study using solvent extracts from seven *Trichoderma* isolates on the growth of *A. flavus* (Af 11-4) using the agar well method

The extracts from three different solvents—Ethyl acetate, Methanol and Hexane—were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml. Agar wells were made on PDA plates using cork borer and an *in vitro* assay was done using *A. flavus* spore suspension (10^6 spores/ml) as a pathogen. A total of 36 plates were taken for each solvent extract at four plates per treatment. The plates were incubated for five days, following which the colony diameter was measured.

Seed infection study and method of inoculation

For the *in vitro* seed colonization study (IVSC) a toxigenic isolate of *A. flavus* (Af 11-4) was selected due to the high aflatoxin production capacity in culture. Kernels of both J 11 (Resistant) and JL 24 (Susceptible) genotypes were used and screened for *A. flavus* colonisation and aflatoxin production with the dry seed resistance test using standard procedures. Individual seeds were scored for surface colonization by *A. flavus* and colonization severity (Thakur et al. 2000).

Determination of aflatoxin content using ELISA

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was used to quantify the total aflatoxins (Waliyar et al. 2005).

Fractionation of secondary metabolites using HPLC

Trichoderma species were grown in liquid potato dextrose broth and incubated in the dark for three weeks at 28°C. After incubation the culture was filtered and secondary metabolites were extracted using ethyl acetate as the solvent.

Separation of components using HPLC

From the mycelial extract 0.5 ml was injected into the C 18 column (25×1cm) with an LC-UV detector (bio analytical system) and monitored at 250 nm. The flow rate was adjusted to 1 ml/min. The fractionated samples were collected in vials. The experiment was performed on an Agilent 1100 series HPLC Instrument using the 35 min Isocratic method. Fractions were collected for the samples T102 and T20. For sample T102 the fractions were collected at 2.5–3.3, 4.8 and 6.8 RT, and for sample T20 the fractions were collected at 3.0–4.2 and 6.3–8.6 RT.

Results and Discussion

Determination of optimal concentration of ethyl acetate extract on the growth of *A. flavus* (Af 11-4) by the agar well method

Ethyl acetate extracts of seven different *Trichoderma* species showed variation in activity against *A. flavus* (AF 11-4) at the different concentrations used. There was increased inhibitory activity with an increase in concentration. Maximum inhibition was seen at 100 mg/ml concentration for *Trichoderma* isolates. Among all the species isolates, T 20 and T 102 showed maximum growth reduction (1.8 cm and 2.0 cm) of *Aspergillus flavus* growth when compared to control at a concentration of (100 mg/ml). *T. koningii* (T 83) also showed maximum reduction of *Aspergillus flavus* growth (2.6).

In vitro antagonistic study using solvent extracts from seven *Trichoderma* isolates on the growth of *A. flavus* (Af 11-4) using the agar well method

Of the three solvent extracts, ethyl acetate extracts from culture filtrate of isolate T 102 showed maximum reduction (2.4) in colony diameter when compared to the control, showing 4.9 cm reduction followed by T 20 (2.5). Similarly, extracts from the isolates T 24, T 58, T 47 and T 83 also

showed considerable reduction in the colony diameter compared to the control.

Methanol solvent extracts of both the isolates T 20 and T 102 showed maximum reduction in colony diameter (2.9) of *A. flavus* when compared to control, which shows a colony diameter of 4.3 cm. Isolate T 58 also showed maximum reduction (3.3) of growth compared to control, which significantly differs from isolates T 20 and T 102. Similarly, extracts from the isolates T 12, T 83 and T 47 also showed considerable reduction (3.5, 3.5 and 3.6) in the colony diameter compared to control. These three were on a par with each other. Among all the isolates, T 24 showed the least reduction in colony diameter (3.8) compared to control.

Isolate T 20 showed a significant reduction in the growth of *A. flavus* (2.4) compared to control, which significantly differs from other isolates. Isolate T 102 also showed a significant growth reduction (2.6) of *A. flavus* compared to the control. Isolate T 24 also showed a considerable reduction (3.9) of growth compared to the control, which significantly differs from isolates T 47, T 83, T 58 and T 12. Isolates T 83 and T 47 are on a par with each other. Among all the isolates, T 12 showed the least reduction in colony diameter (4.5) compared to the control.

Seed colonization severity in *A. flavus* inoculated JL 24 and J 11 genotypes treated with the metabolites of seven different *Trichoderma* species

Seeds were scored individually for surface colonization by *A. flavus* and severity of infection was recorded using the scale as mentioned in materials and methods. Groundnut seeds treated with the isolates T 20 and T 102 showed no mycelial growth and sporulation on both the genotypes JL 24 and J 11 when compared to the control (4.5 and 1.25). Among other isolates, isolate T 12 showed reduced *A. flavus* infection followed by isolate T 83 in the case of the JL 24 genotype. Isolates T 24 and T 58 showed reduced seed infection severity in the case of the J 11 genotype, whereas in the case of the JL 24 genotype these isolates showed high seed infection severity (see Table 28.1 below).

Table 28.1. Seed colonisation severity of *A. flavus* treated with seven different treatments of *Trichoderma* species using the groundnut genotypes JL 24 and JL 11

Trichoderma isolate	Seed colonization severity	
	JL 24 (Sensitive)	J 11 (Resistant)
T 12	0.8	0
T 24	2.5	0.25
T 58	3.8	0.25
T 47	2.3	0.5
T 83	1.0	0.5
T 20	0.0	0
T 102	0.0	0
Uninoculated Control	0.0	0
Inoculated Control	4.5	1.25
CD	1.199	0.579
SE (D)	0.581	0.281
SE (M)	0.411	0.198

Aflatoxin content in susceptible (JL 24) and resistant (J 11) groundnut genotypes treated with different metabolites of *Trichoderma* species

Among the seven *Trichoderma* treatments isolates, T 20 and T 102 showed reduced aflatoxin content in both the genotypes JL 24 (14.58 and 15.28) and J 11 (6.36 and 5.83) compared to the control. Among the other five isolates, T 12 and T 24 showed reduced aflatoxin content (18.24 and 19.24) in the genotype JL 24 and (8.06 and 15.32) in the case of J 11 when compared to the control. Among all the isolates T 47 showed the highest aflatoxin content in both the genotypes (24.05 and 22.80) compared to the control (Table 28.2).

Of all the isolates tested, two were effective in controlling the growth of *A. flavus*. When three different solvents were used for extracting metabolites from culture filtrates of seven *Trichoderma* species, ethyl acetate extracts of *T. harzianum* showed a promising ability against *A. flavus*. However, methanol extracts of *T. longibrachiatum* also gave considerable growth reduction against *A. flavus*. Methanol extracts of *T. asperellum* and *T. longibrachiatum* gave the best antimicrobial activity (Vizcaino et al. 2005). Comparing the efficacy of metabolites extracted from seven different *Trichoderma* species using three different solvents, ethyl extract metabolites from two different *Trichoderma* species (T 20

and T 2) showed potent growth reduction of *A. flavus* compared to other species. In this chapter, we collected five fractions from both the isolates and tested their antifungal activity. Furthermore, fractions were submitted to FTIR analysis for chemical grouping of the compounds and the collected potent fractions of HPLC against *Aspergillus flavus* were presented. The selected fractions were sent for compound identification by mass spectrometry and NMR analysis.

Table 28.2. Evaluation of *Trichoderma* isolates in reducing aflatoxin contamination in groundnut (genotypes JL 24 and JL 11)

***Trichoderma* isolate**	**Aflatoxin content (ppb)**	
	JL 24 (Sensitive)	**J11 (Resistant)**
T 12	18.24	8.06
T 24	19.24	15.32
T 58	24.12	8.34
T 47	24.05	22.80
T 83	20.78	24.51
T 20	14.58	6.36
T 102	15.28	5.83
Uninoculated Control	11.29	1.16
Inoculated Control	27.27	25.30
CD	10.303	6.842
SE (D)	4.994	3.296
SE (M)	3.532	2.330

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CHAPTER TWENTY-NINE

EFFICACY OF *PSEUDOMONAS FLUORESCENS* STRAINS IN ENHANCING GROWTH AND YIELD OF PEANUT

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Abstract

Growth promotion and yield enhancement by PGPR in various crops is a well-established phenomenon. *Pseudomonas fluorescens* are gram negative PGPR that have wide applicability due to various modes of action. The present study focuses on the evaluation of various *P. fluorescens* strains on growth promotion and yield in peanut. Eleven *P. fluorescens* strains were isolated from peanut rhizosphere and evaluated for their growth promoting effects on cultivar (Narayani). A greenhouse study was conducted in a RCBD fashion with 12 treatments (including control) and four replications. Prior to seeding, the peanut seeds were treated with PGPR strains at a concentration of 1×10^9 cfu/ml. Control seeds were treated with water as seed treatment. The pots were harvested after 95 d. Biometric characteristics such as plant height, root growth and yields were recorded. Our results demonstrate the efficacy of the *P. fluorescens* strains in enhancing root growth, plant height and yields of peanut over control under controlled conditions. The data revealed that one strain, IFT 30, showed higher plant height and root growth. Overall, our results indicate that seed bacterization with Pf strains enhanced growth in peanut. The strain IFT-30 was found to be superior among the tested Pf

strains with highest root length, root and shoot dry weights, and with a proportionate root/shoot ratio. Further, IFT-30 has a significantly higher number of filled pods/plant and recorded the highest pod yields/plant over others. Our future studies are directed at assessing the extent of antagonism of this superior Pf strain in combatting stem rot and collar rot, aflatoxin contamination of peanut, and inducing the drought tolerance of peanut.

Introduction

Peanut is an important oilseed crop earning a great deal of revenue in India. Globally, India accounts for 31% of the total peanut area in the world (24.6 mha) and 22% of the total production (35.7 mt). The productivity of peanut is only 897 Kg/ha compared to Brazil, China and the USA. The main reason for this is that 60% of the crop is grown under rainfed conditions and in sub-marginal soils. The crop is frequently prone to abiotic stresses which include moisture and high temperature stress. Hence, the productivity is low due to poor root, shoot growth and low nutrient uptake. The exploration of biological methods to sustain crop growth and yield in peanut is a research priority.

Presently, peanut yields in India are hampered by several biotic stresses, of which plant pathogens are a major part. Biological control of peanut diseases using Plant Growth Promoting Rhizobacteria (PGPR) is gaining importance. These PGPR strains offer an environmentally sustainable approach to increasing crop production and soil health. Among the various PGPRs, *Pseudomonas fluorescens* are widely used in controlling plant diseases and enhancing yields. The plant growth-promotion by fluorescent Pseudomonads is done by both direct and indirect mechanisms. A direct mechanism which enhances the plant growth includes the production of phytohormones, solubilization of phosphates (Antoun & Kloepper 2001) and increase in the uptake of iron by the production of siderophores (Chaiharn et al. 2009; Gupta et al. 2002) and volatile metabolites. Indirect mechanisms enhance plant growth through the synthesis of HCN (Dowling & Gara 1994), antibiosis, competition for space and nutrients, parasitism or lysis of pathogen hyphae, inhibition of pathogen growth by enzymes or toxins, and through induced systemic resistance (ISR) (Nandakumar et al. 2001). In our present study we have screened to identify native rhizospheric fluorescent Pseudomonads for their growth-promoting activities in peanut. The long-term goal is to identify potential Pseudomonads contributing to yield

enhancement in peanut besides being effective against major soil borne diseases.

Materials and Methods

Rhizospheric soil samples were collected from peanut and *P. fluorescens* were isolated using standard protocols. Confirmation of PGPR strains was carried out using standard procedures. These PGPR were further screened for their efficacy in containing major soil borne pathogens in peanut under *in vitro* conditions. Superior strains with high antagonism were selected for growth promotion studies under greenhouse conditions. The growth promoting characterizations of these superior strains were also carried out using standard procedures for traits such as production of Indole Acetic Acid (IAA), siderophores, cellulose and phosphate solubilization capacity.

A greenhouse study was conducted in an RCBD fashion with 12 treatments and 4 replications to evaluate these superior PGPRs for enhancement of growth and yields. A popular peanut cultivar "Narayani" was used in the present study. Prior to seeding, seeds were treated with PGPR strains at a concentration of 1×10^9 cfu/ml. Eleven PGPR strains were screened in this experiment. Control seeds were treated with SDW. The seeds were later sown and seedlings were allowed to grow under GH conditions in plastic pots using standard procedures. The seedlings were allowed to grow for up to 95 d and then harvested. Biometric characteristics such as plant height and root traits such as root length, root dry weight, and shoot dry weight were recorded at harvest. Further, plant height, number of filled pods/plant and pod yield/plant were recorded. The percentage kernel oil and protein content in seeds were recorded using standard protocols.

Results and Discussion

The highest root lengths were recorded with Pf strain IFT-30 (33.8 cm) and were significantly superior over others. This was followed by strains IFT-24, IFT-34, IFT-17, IFT-14, IFT-12 and IFT-32 with no significant differences in root lengths among them. In general, the majority of Pf strains have caused superior root lengths over the control (27 cm) except IFT-1, IFT-2 and IFT-11 (see Table 29.1 below).

Root dry weights were highest with IFT-30 (7.1 g/plant). This was followed by strains IFT-12, IFT-14 IFT-24 with no significant differences among them. In general, all the strains have shown significantly superior

root dry weights over the control except IFT-11. Control seedlings have recorded a root dry weight of 2.1 g/plant (see Table 29.1 below).

The highest shoot dry weights were recorded in ITF-30 treated plants (29 g/plant) and were significantly superior over others, followed by IFT-17 (24.1 g/plant) and IFT-11 (21.8 cm). No significant differences in shoot dry weights compared to control (13.1 g/plant) were recorded with strains IFT-1 and IFT-14. The root/shoot ratio is lowest and proportionate in treatments with seed bacterization by IFT-12, IFT-14 and IFT-30 (see Table 29.1 below).

Plant heights at harvest were highest in IFT-17 (29.1 cm), IFT-32 (28.5cm) and IFT-34 (28.3 cm) with no significant differences among them. In general, all the strains have recorded significantly higher plant heights over the control except IFT-2 (19.8 cm). Control seedlings have recorded a plant height of 21.0 cm. The number of filled pods/plant was highest with strains IFT-30 (15.3 cm) and IFT-14 (15.0 cm). In general, all the PGPR strains resulted in significantly more filled pods/plant over the control. The control pots recorded a mean number of filled pods of 7.5/plant (see Table 29.1 below).

Table 29.1. Efficacy of various *Pseudomonas fluorescens* isolates as seed treatment in promoting root and shoot growth in peanut

PGPR Identity	Root Length (cm)	Root Dry Weight (g/plant)	Shoot Dry Weight (g/plant)	Root:Shoot Ratio
IFT-1	21.6^{e}	2.3^{e}	13.1^{h}	1:6
IFT-2	23.6^{d}	2.6^{de}	20.9^{d}	1:8
IFT-11	24.6^{d}	2.0^{f}	21.8^{c}	1:11
IFT-12	28.8^{c}	4.1^{b}	17.2^{f}	1:4
IFT-14	30.0^{bc}	3.8^{bc}	15.1^{gh}	1:4
IFT-16	28.5^{bc}	2.5^{de}	19.6^{de}	1:8
IFT-17	29.6^{bc}	2.7^{d}	24.1^{b}	1:9

IFT-24	30.8^{b}	4.0^{b}	21.6^{cd}	1:5
IFT-30	33.8^{a}	7.1^{a}	29.0^{a}	1:4
IFT-32	25.8^{cd}	1.9^{g}	15.9^{g}	1:8
IFT-34	30.3^{bc}	2.3^{e}	21.8^{c}	1:9
Control	27.0^{c}	2.1^{f}	13.1^{h}	1:6

Seeds of peanut (CV: Narayani) were bacterized with Pf strains at 1×10^{9} cfu/ml.
Values are means of four replications.
Means followed by a common letter in the columns are not significantly different according to LSD (at $p < 0.05$)

The pod yield/plant was highest in plant seeds bacterized with IFT-30 (50 g) and was significantly superior over others. This is followed by IFT-17 (42.1 g) and IFT-14 (37.3 g). The strains, IFT-11, IFT-12 and IFT-14 also recorded higher pod yields/plant compared to the control. Control plants registered a pod yield of 22.3 g/plant (see Table 29.2 below).

There were no significant differences in the oil and protein percentages of kernels, including the control plants. The oil percentage ranged from 47.1 to 48.4, whereas the protein percentage ranged from 26.0 to 26.6. Overall, our results indicate that seed bacterization with Pf strains enhances growth in peanut. The strain IFT-30 was found to be superior among the tested Pf strains with highest root length, root and shoot dry weights, and with a proportionate root/shoot ratio. Further, IFT-30 has a significantly higher number of filled pods/plant and recorded the highest pod yields/plant over others. Our future studies are directed at assessing the extent of antagonism of this superior Pf strain in combatting stem rot and collar rot pathogens and also in reducing aflatoxin contamination of peanut.

Table 29.2. Efficacy of various *Pseudomonas fluorescens* isolates as seed treatments in promoting seedling height and yield in peanut under greenhouse conditions

PR Identity	Plant height(cm)	No. of Filled Pods/Plant	Pod yield/Plant (g/plant)
IFT-1	26.0^{c}	8.3^{e}	20.1^{f}
IFT-2	19.8^{f}	9.6^{cd}	14.9^{g}
IFT-11	24.8^{de}	10.6^{c}	30.2^{d}
IFT-12	26.8^{c}	14.0^{b}	35.9^{cd}
IFT-14	28.4^{ab}	15.0^{a}	37.3^{c}
IFT-16	24.6^{de}	9.3^{d}	24.6^{e}
IFT-17	29.1^{a}	14.0^{b}	42.1^{b}
IFT-24	25.0^{d}	9.0^{de}	19.0^{f}
IFT-30	27.3^{b}	15.3^{a}	50.0^{a}
IFT-32	28.5^{ab}	6.6^{g}	5.8^{h}
IFT-34	28.3^{ab}	9.6^{cd}	23.5^{e}
Control	21.0^{f}	7.50^{f}	22.3^{ef}

Seeds of peanut (CV: Narayani) were bacterized with Pf strains at 1×10^{9} cfu/ml. Values are means of four replications. Means followed by a common letter in the columns are not significantly different according to LSD (at $p < 0.05$).

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CHAPTER THIRTY

UTILIZATION OF *PSEUDOMONAS FLUORESCENS* AND *BACILLUS SUBTILIS* FOR THE ROOT KNOT NEMATODE MANAGEMENT OF CHILLI AND THEIR EFFECT ON CHILLI GROWTH

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Abstract

Pseudomonas fluorescens and *Bacillus subtilis* have long been studied for conferring beneficial effects on a wide variety of plants against several pathogens. In the current study, *P. fluorescens* and *B. subtilis* were investigated against the root knot nematode in chilli, and their effects on growth parameters (plant height, fresh and dry weight of root, fresh and dry weight of shoot) were computed. The experiments were conducted in a net house and a growth chamber. The results demonstrate that a greater increase was observed in the case of *P. fluorescens*, an increase in plant height (15.2%), fresh weight of shoot (44%), and fresh weight of root (65.75%) in the growth chamber were recorded, while in the net house plant height was observed to increase by 27.6%, fresh weight of shoot by 62.66%, and fresh weight of root by 49.15%. The nematode population was decreased by 35.32% by *P. fluorescens* in the growth chamber while *B.subtilis* decreased the nematode population up to 30.84%. A siderophore assay was performed to confirm the siderophore production from these bacteria using a Chrome Azurol Sulphonate (CAS) agar plate assay which revealed that both were positive in the test.

Introduction

Chilli (*Capsicum annum* L.) is among the most important vegetables around the world, and after potato and tomato it is the one most consumed. It is not only an important component in food, but is also used for quintessence production.

Chilli is known to be invaded by several pathogens causing substantial losses in the crop yield. These include bacteria, viruses, fungi and nematodes. Among plant parasitic nematodes, root knot nematodes (RKN) are the best-known phytonematodes throughout the world. They are polyphagous and implicated in causing yield losses to many vegetables. Because of the very prominent and specific symptoms of root galls or knots formed at infection sites, these nematodes were amongst the first plant parasitic nematodes to be recognized.

Chilli plants affected by root knot nematodes exhibit a slow development and a stunted appearance. If the infestation appears early it gets severe, leaves becoming yellow-green to yellow, tending to droop, and the plant wilting. The roots become galled, which is the most characteristic symptom of infection. In the case of severe infection there may be a complete loss of plant vigour resulting in heavy yield losses. The approximate distribution of *Meloidogyne*s pp. in the agricultural soil of Pakistan is *M. incognita* 85%, *M. javanica* 10%, and other *Meloidogyne*s pp. about 5% (Anwar et al. 2007).

Rhizobacteria are potential biocontrol agents first reported by Kloepper & Schroth (1978). Since then, these rhizobacteria are one of the most attended-to issues throughout the world, and there is much advancement in the ecology, rhizosphere dynamics, root colonization, disease suppression, crop growth enhancement, etc. These rhizobacteria are known to increase crop yield by several mechanisms. The biological control is far better than chemical control and is environmentally safe, offering a durable, safe and cost-effective alternative to seed- and soil-applied chemicals.

In the current study, two rhizobacterial isolates, *Pseudomonas fluorescens* and *Bacillus subtilis*, were exploited against the root knot nematode (*Meloidogynejavanica*) in a net house and a growth chamber, and their effect on chilli growth was also observed.

Materials and Methods

Sampling and extraction of root knot nematodes

Sampling from chilli plants was done by uprooting the whole plant from the soil using a spade. One hundred grams of soil and roots from each sample were processed for the isolation of nematodes. Nematodes were extracted by using Whitehead & Hemming tray methods (Whitehead 1986) and the sieving method. After extraction, nematodes were picked by using a bamboo needle followed by the killing and fixing of nematodes. Nematodes were identified on the basis of perennial pattern.

Multiplication of nematodes

To multiply the culture of root-knot nematodes, the most susceptible variety of aubergine (Black Beauty) was used. A three-week-old nursery was transplanted in earthen pots containing 2.5 kg, 1:2 sandy loam soil sterilized with formalin, and one plant per pot was planted. One week after transplanting, these plants were inoculated with approximately 2,000 freshly hatched, second-stage juveniles of *Meloidogynejavanica.* Tap water was used to irrigate young seedlings throughout the period of study. A temperature range of 20°C–30°C was recorded.

Rhizobacterial isolates

The rhizobacterial isolates (*Pseudomonas fluorescens* and *Bacillus subtilis*), previously studied by Haq et al. (2011) in tomato against root knot nematode, were used in this study. The cultures were maintained on King B (KB) media at 30 ±2°C.

Siderophore production

Qualitatively, siderophores were detected by assay using the universal CAS assay (Schwyn & Neilands 1987). The assay is based on the competitive exchange of Fe^{3+} between a strong chelator (siderophore) and weak chemical iron chelator HexaDecylTrimethyl Ammonium Bromide (HDTMA), resulting in colour change of CAS reagent from blue to orange or golden yellow, irrespective of type of siderophores.

In vitro antagonistic activity of rhizobacteria against *M. javanica*

The inoculum of *P. fluorescens* and *B. subtilis* was prepared by transferring a loopful of bacteria from a 5 d old culture to 100 mL KB liquid medium and incubated at room temperature on a shaker (150 rpm) for 48 h. The bacterial cells were centrifuged at (4500×g) for 15 min. Supernatant was discarded and pellet was suspended in sterile $MgSO_4$(0.1M). For the preparation of cell-free culture filtrate, the bacterium was grown at 30ºC in KB Liquid medium for 48 h in the dark and centrifuged twice at 2,800 rpm for 20 min. Pellets were discarded and culture filtrate was collected in a sterilized beaker prior to use. The filtrate was spread over KB medium for the presence or absence of bacterial cells. No bacterial growth was observed on any of the plates.

M. javanica was maintained on egg plants in a glass house. Egg masses were handpicked from infested roots and kept in autoclaved distilled water. Juveniles were collected three days after incubation. Nematicidal activity of bacterial culture filtrate was determined by transferring 2 mL of filtrate in a 3 cm cavity glass slide along with 25–30 surface sterile juveniles per mL. The number of dead juveniles was counted after 24 and 48 h. The mean percentage of dead larva was estimated. The juveniles were considered to be dead when they did not move on probing with a fine needle (Cayrol & Frankowski 1979).

Development of induced systemic resistance

Two root systems, each with bacteria (*P. fluorescens* and *B. subtilis*) and nematodes, were inoculated separately. The seedlings were uprooted from sterilized soil after three weeks, and seedlings were washed with tap water and their roots split into two halves with a dissecting scalpel. Each of the root systems was transplanted into separate plastic pots. One root system was treated with cell suspension of a bacterial strain prepared in distilled water. The ringer solution was used as a control treatment. After one week the other half root system was treated with the freshly hatched juveniles of *Meloidogyn*e spp. and the nematode was observed after 21 d of inoculation.

Efficacy of *P. fluorescens* and *B. subtilis* against *M. javanica* under growth chamber and net house conditions

The bacteria were grown on liquid KB medium for 48 h, and culture was shaken at room temperature. Chilli seeds were surface sterilized in 1% $Ca(OCl)_2$ for 3 min, then rinsed several times with distilled water, and were then treated with an aqueous cell suspension of the bacteria using 1% gum Arabic as a sticking agent. The process yielded 4×10^9cfu/seed.

Three treated seeds were sown in each pot, and after germination one seedling was retained per pot. For root dip treatment, roots were dipped in bacterial suspension and one seedling was planted in each pot. Seed and root treated with sterile $MgSO_4$ (0.1 M) without the bacterium served as control. For application of the bacteria as a soil drench, a 25 ml aqueous cell suspension prepared in sterile $MgSO_4$ (0.1M) was drenched onto the soil and three seeds were sown into the pot. One seedling per pot was retained after emergence in each trial. Soil drenched with 25-ml sterile $MgSO_4$ (0.1M) served as control.

One week after seedling emergence, each pot was inoculated with 1,000 freshly hatched juveniles of *M. Javanica* that were added to a hole made between the seedlings in the soil of each pot. Treatments and controls were replicated four times. Eight weeks after nematode addition, the root system was washed and the number of galls induced by *M. javanica* on the entire root system was recorded. The root-knot nematodes in bacteria in treated and untreated pots were extracted using a modified Baermann funnel technique, and the treatments used were: T1 = *P. fluorescens*; T2 = *P. fluorescens*+ *M. javanica*; T3 = *B. subtilis*; T4 = *B. subtilis* + *M. javanica*; T5 = *M. javanica*; and T6 = No bacteria + No nematode

Results and Discussion

Antagonistic activity of PGPR

In vitro evaluation of *P. fluorescens* and *B. Subtilis* revealed them to be antagonistic against *M. javanica*. Both isolates produced compounds that resulted in the death of *M. javanica*. Maximum mortality was observed by *P. fluorescens* which gave 58% juvenile mortality after 48 h as compared to control, while *B. subtilis* exhibited 42% mortality.

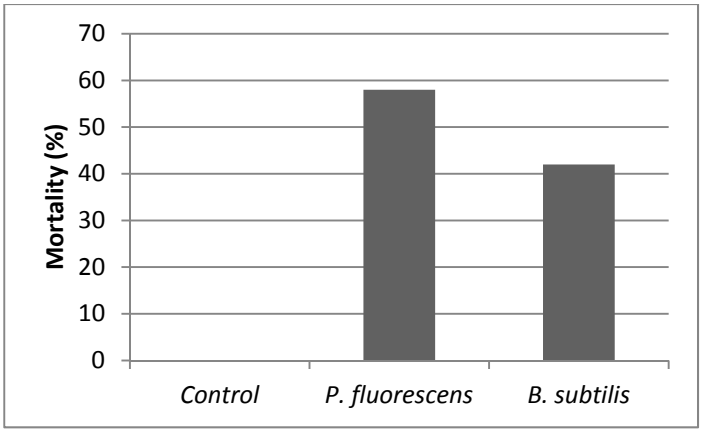

Fig. 30.1. *In vitro* antagonistic activity of PGPR against *Meloidogyne javanica*

Siderophore production

Qualitatively, siderophore was detected in the culture supernatant by a Universal Chemical Test (Schwyn & Neilands 1987). It turned red-orange when the supernatant and CAS reagent were added equally, which confirmed the siderophore production.

Development of induced systemic resistance

The separate application of *P. fluorescens* and *B. subtilis* to one half of the split root section reduced nematode attack to the other (non-bacterized nematode treated) half of the root system in both isolates (data not shown).

Efficacy of *P. fluorescens* and *B. subtilis* against *M. javanica* under growth chamber

The treatment comparisons of each treatment show that *P. fluorescens* was more dominant than the other treatments. The highest control of nematode along with the greater increase in growth parameters were shown by *P. fluorescens*, followed by *B. subtilis*. No juveniles were observed in root, soil and growth parameters, i.e. plant height, fresh weight of shoot and fresh weight of root, that were recorded to be 11.95 cm, 0.88 g, 0.26 g respectively in *P. fluorescens*. The treatment of rhizobacteria with nematodes was observed to be nearly equal to treatment without any

bacteria and nematode application, while root weight was highest in treatment with only nematode application due to galls formation. The data is shown in Table 30.1 below.

Table 30.1. Effect of induced systemic resistance by rhizobacteria in growth chamber against *Meloidogyne javanica* in chilli

Treatments	Plant height (cm)	Fresh wt. of shoot (g)	Fresh wt. of root (g)	No. of juveniles /250 gm of soil	No. of juveniles /g of fresh root	No. of egg masses/ root	Galling index
P. fluorescens	11.95a	0.88a	0.26d	0.0c	0.0d	0.0d	0.0c
P. fluorescens + nematode	10.21c	0.76c	0.42b	301b	50c	11c	3.1b
B. subtilis	11.11b	0.81b	0.20e	0.0c	0.0d	0.0d	0.0c
B. subtilis + nematode	10.01c	0.71d	0.42c	325b	59b	15b	3.3a
Nematode alone	9.50d	0.56e	0.50a	462a	93a	20a	3.6a
No bacteria +No nematode	10.40c	0.72c	0.16e	0.0c	0.0d	0.0d	0.0c

Efficacy of *P. fluorescens* and *B. subtilis* against *M. javanica* in net house conditions

The effect of *P. fluorescens* and *B. subtilis* on the growth parameters of chilli and the development of root knot nematode disease was measured in a pot experiment in a net house. The results reveal that chilli performed better in the net house as compared to the growth chamber, and the pronounced treatment was observed to be *P. fluorescens* followed by *B. subtilis*. Application of *P. fluorescens* exhibited the highest increase in plant growth parameters and best control was also seen by this treatment (see Table 30.2 below). The number of nematode juveniles was observed to be highest in the treatment with only nematode application (451 juveniles), while all other treatments were found to be better. The highest control of nematode juveniles was recorded in *P. fluorescens*, and no juvenile was observed. Similarly, *B. subtilis* was the second best treatment.

Table 30.2. The effect of induced systemic resistance by rhizobacteria in net house on plant growth and *Meloidogynejavanica* on chilli

Treatments	Plant height (cm)	Fresh wt. of shoot (g)	Fresh wt of root (g)	No. of juveniles /250 gm of soil	No. of juveniles /g of fresh root	No. of egg masses/root	Galling index
P. fluorescens	12.15a	0.94a	0.29d	0.0	0.0d	0.0c	0.0c
P. fluorescens + nematode	10.32c	0.78c	0.46b	301	295c	15b	3.2b
B. subtilis	11.29b	0.83b	0.23e	0.0a	0.0d	0.0c	0.0c
B. subtilis + nematode	10.11c	0.74d	0.42c	319	339b	19b	3.5a
Nematode Alone	9.6d	0.59e	0.59a	1370a	451a	28a	3.8a
No bacteria +No nematode	10.52c	0.77c	0.20e	0.0d	0.0d	0.0c	0.0c

In our study, increase in the growth of chilli was observed with the application of rhizobacteria to a considerable extent, especially with *P. fluorescens*. Increase in the growth parameters of the chilli plant was as a result of the increased synthesis of auxin, which is a growth promoter. There is always a positive correlation of growth parameters and IAA production by the rhizobacteria. The production of auxin by rhizobacteria has been reported by many species, and *Pseudomonas spp*. has always been found to produce more auxin, thus increasing plant height and root and shoot weights. This may imply that auxin produced by PGPR isolates caused improvement in root weight, shoot weight, plant height and more biomass production. However, other mechanisms through which PGPR plays role in the growth of plants should also be considered. Glick (1995) viewed that the mechanism most commonly invoked to explain the various effects of PGPR on plants is the production of phytohormones, and IAA may play the most important role in growth promotion. Treatment with *P. fluorescens* showed the greatest increase in plant height, perhaps because it is an IAA producer. Phosphate solubilization and IAA production by *Pseudomona*s *spp.* have also been reported (Débora et al. 2007).

In a study by Tariq et al. (2009), the highest increase in growth of chilli and nematode control was observed by *Pseudomonas* spp. *Pseudomonas* spp. has been reported to suppress root knot infection on chilli, watermelon, guar, pumpkin (Parveen et al. 1998), okra (Ara et al. 1997) and tomato (Siddiqui & Ehteshamul 2001). There are reports that plant

growth promoting endophytic rhizobacteria is involved with host plants in mutual interaction (Pandey et al. 2005).

P. fluorescens suppressed root-knot nematode greater than *B. subtilis* both in the growth chamber and the net house. Moreover, nematicidal activity of *Pseudomonas* was enhanced due to the secondary metabolites such as DAPG, pyoluteorin and hydrogen cyanide secreted by *Pseudomonas spp.*, which act as biocontrol factors against nematodes (Gallagher & Manoil 2001). The biosynthesis of hydrogen cyanide is affected by environmental factors including iron, phosphate and oxygen concentrations (Knowles & Bunch 1986), and may be the cause of difference in the antagonistic activities of different strains. In our study, *B. subtilis* was also observed to be the potential biocontrol agent of root knot nematode. *B. subtilis* is known to produce siderophore and our study confirms the early findings, as colour change was observed during CAS assay. Based on the results presented here it can be concluded that *P. fluorescens* and *Bacillus subtilis* have conferred resistance against root-knot nematodes in chili.

The present study focuses on the utilization of PGPRs in chilli against the root knot nematodes and their possible effects on its growth. It was revealed that the *P. fluorescens* and *Bacillus subtilis* are the potential biocontrol agents suppressing the activity of *Meloidogyne javanica* to a considerable extent. It can also be used as an important management measure which must be integrated along with other control measures to overcome the devastating pathogen.

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CHAPTER THIRTY-ONE

DISEASE SUPPRESSION OF FUNGAL ROOT PATHOGENS OF CHICKPEA USING ANTAGONISTIC RHIZOBACTERIA AND NEEM CAKE

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Abstract

Rhizobacteria are well known for their beneficial effects that may influence plants directly or indirectly through several mechanisms. This study was conducted to evaluate *Pseudomonas fluorescens* and *Bacillus subtilis* with and without neem cake, and to explore their biocontrol activity against the root-infecting pathogens of chickpea (*Fusarium solani*, *F. oxysporum* and *Macrophomina phaseolina*) that are causing huge losses worldwide. A dual culture plate technique was used to examine the biocontrol activity of the rhizobacterial isolates, revealing that the isolates are antagonistic. The overall performance of *P. fluorescens* was better than *B. subtilis*, showing a mean zone of inhibition of 1.52 cm followed by *B. subtilis* with 1.28 cm. These isolates, individually and in combination, were applied to the soil with and without neem cake under laboratory and greenhouse conditions for disease suppression and growth promotion using a soil application method. Treatments were applied at the time of sowing and data was collected after 40 d. Studies demonstrated the isolates to be antagonistic in both pot tests and greenhouse conditions. The treatment of isolates in combination (*P. fluorescens* + *B. subtilis*) was observed to be best among other treatments. Soil amended with the neem cake exhibited better performance of the rhizobacteria with 90% disease

suppression than soil without neem cake with 75% suppression by the combined treatment of *P. fluorescens* + *B. subtilis*. Growth promotion was also dominant in the neem cake amended soils.

Introduction

Chickpea (*Cicer arietinum* L.) is a major source of human and animal food and the world's third most important pulse crop after beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) (Nikam et al. 2007). It is mostly grown under rain-fed conditions in arid and semi-arid areas around the world (Millan et al. 2006). Pakistan is the third major chickpea producer in the world after India and Turkey (Dusunceli et al. 2007). It has hosted a number of pathogens, among which *Fusarium oxysporum*, *F. solani* and *Macrophomina phaseolina* are important. Annual chickpea yield losses from Fusarium wilt vary from 10% to 15% (Jalali & Chand 1992; Casas & Diaz 1985). *M. phaseolina* is endemic to temperate and tropical regions of the world and can infect over 500 different hosts (Wyllie 1998). Similarly, *F. solani* is one of the most serious diseases which causes severe yield loss, i.e. 60%–70% under favourable conditions (Tewari & Mukhopadhyay 2003). Though many control measures are devised for these pathogens, they do not work. Resistant varieties are one of the most effective methods but the phenomenon of race evolution of the pathogen is a great problem for the pathologists, resulting in resistance breakdown. Biological control is emerging as an alternative strategy for disease management which is also ecology-conscious and environmentally friendly.

The rhizosphere provides the initial barrier against pathogen attack of the roots (Weller 1988). Plant growth-promoting rhizobacteria (PGPR) present in the rhizosphere have the ability to improve plant growth by colonising the root system and pre-empting the establishment of harmful rhizosphere microorganisms (Glick 1995; Kloepper 2003). Rhizosphere microorganisms provide biocontrol through mechanisms such as the production of antibiotics (Hebbar et al. 1992; Bender et al. 1999), iron sequestering compounds, siderophores (Dwivedi & Johri 2003; Siddiqui 2006), extracellular hydrolytic enzymes (Fridlender et al. 1993), other secondary metabolites such as hydrogen cyanide (HCN) (Gardener et al. 2000; Pal et al. 2000; Validov et al. 2005; Singh et al. 2006) and induced systemic resistance (Liu et al. 1995).

The objectives of this study are to evaluate the rhizobacteria with and without neem cake amendment against the root-infecting pathogens of chickpea using the soil treatment method, and to observe the disease

suppression and growth improvement in chickpea as a result of application of rhizobacterial isolates, both individually and in combination.

Materials and Methods

Isolation of fungi

Potato Dextrose Agar (PDA) medium was used for the isolation of the fungal pathogens of chickpea. Infected root samples of chickpea were collected from the field and cut into small pieces following treatment with 1% sodium hypochlorite for surface disinfection. These pieces were placed on to a medium and incubated at 28 ±2°C.

Pathogenicity test of fungi

Surface sterilized chickpea seeds were grown in pots filled with sterilized soil, and a fungal inoculum of each pathogen was prepared by macerating 100 g wet mycelium in 1 l of water. The 10 mL of each fungus inoculum were added to each plant. The root dip method was also employed and seedlings were uprooted carefully and dipped in fungal suspension. Plants were kept under controlled conditions and observed for symptom development.

Rhizobacterial isolates

The rhizobacterial isolates (*P. fluorescens* and *B. subtilis*) used in previous studies by Inam et al. (2011) for root knot nematode management were used in this study.

Preparation of Neem cake

Neem cake (NC), a byproduct from the extraction of oil from neem seed, was dried, crushed and converted into fine powder using a grinder fitted with a 2 mm pore size sieve. The moisture content of dry cake powder was 6%. The powder was stored in metal containers at 4°C for experimental use. The 2% w:w of neem cake in soil was applied.

In vitro evaluation of antagonistic rhizobacteria

The antagonism of rhizobacterial isolates was confirmed by the dual-culture plate technique. PDA plates were separately inoculated with *F.*

oxysporum, *F. solani* and *M. phaseolina* and each challenged with *P. fluorescens* and *B. subtilis* at a concentration of 10^8 cfu/ml. The plates were incubated at 29 ±2°C and the zone of inhibition was measured after 3 d.

Laboratory evaluation of rhizobacteria

Two experiments were conducted in which antagonistic rhizobacteria were applied with and without neem cake soil amendment. The surface sterilized seeds of chickpea were sown in sterilized soil mixture (3 soil: 1 sand: 1 compost). Neem cake was applied as a treatment at 2% w:w in soil. Antagonistic rhizobacteria were applied as individually and combined treatments by the soil application method, i.e. T1 = control; T2 = *P. fluorescens*; T3 = *B. subtilis*; T4 = *P. fluorescens* + *B. subtilis*. Ten ml of suspension of each treatment were applied at a concentration of 10^7 cfu/ml and the seeds were sown. Fungal inoculum was also applied to the soil. The experiment was replicated thrice and data was collected after 40 d. Data of disease incidence (DI), biocontrol efficiency (BE), fresh weight of root (FWR), and dry weight of root (DWR) were recorded.

Greenhouse evaluation of rhizobacteria

The same procedure was used as in the laboratory experiments, wherein antagonistic rhizobacteria were applied with and without neem cake soil amendment. Surface sterilized soil was used for growing the seeds and the soil application method was exploited. This experiment was also replicated thrice and data was collected after 40 d. Data of disease incidence (DI), biocontrol efficiency (BE), fresh weight of root (FWR), and dry weight of root (DWR) were recorded.
The following formulae were used for calculating BCE and DI (Guo et al. 2004):

$$\text{BCE (\%)} = \frac{\text{Disease incidence on control} - \text{Disease incidence on treatment group}}{\text{Disease incidence on control}}$$

Disease incidence (DI) was calculated with the following formula (Guo et al. 2004):

$$\text{DI (\%)} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}}$$

Results and Discussion

In vitro evaluation of rhizobacteria

P. fluorescens and *B. subtilis* were evaluated for antagonism against each fungal pathogen, i.e. *M. phaseolina*, *F. oxysporum* and *F. solani*. The average zone of inhibition of *P. fluorescens* was calculated as 1.52 cm and that of *B. subtilis* as 1.28 cm. Particularly, *P. fluorescens* showed the highest zone of inhibition in *M. phaseolina* (2.00 cm) followed by *F. oxysporum* (1.41 cm). *B. subtilis* performed best against F. solani with the zone of inhibition at 1.68 cm (see Table 31.1 below).

Table 31.1. *In vitro* effect of rhizobacterial isolates on pathogens

Isolates	Inhibition zone (cm)			
	F. oxysporum	*F. solani*	*M. phaseolina*	Mean
Control	0b	0d	0c	0
P. fluorescens	1.41 b	1.15 c	2.00 a	1.52
B. subtilis	1.02 b	1.68	1.14	1.28

Pathogenicity test of fungi

All pathogens isolated from disease samples were re-isolated in a pathogenicity test that confirmed them to be the real cause of the disease. That the pathogens incited disease in the plants was evident from the symptoms that appeared on the roots after 20 d of inoculation.

Laboratory evaluation of rhizobacteria

All treatments were found to be effective with varying results. The treatment of isolates in combination was found to be the most effective against all the pathogens, while varying results were observed in individual treatments. The combination (*P. fluorescens* + *B. subtilis*) expressed the best control against the pathogens with a maximum 90% efficacy with neem cake, while 86% disease control was the highest when observed without neem cake (see Table 31.2 below). The data show that neem cake amended soil has performed relatively better than non-amended soil. The highest disease incidence was recorded in the control and the best biocontrol efficiency was observed in the combined treatment of *P. fluorescens* + *B. subtilis* in the case of all pathogens. The highest BCE was observed against M. phaseolina, i.e. 90.40%, and similarly the highest

Table 31.2. The effect of rhizobacterial isolates on the root pathogens and growth of chickpea plants with and without neem cake under laboratory conditions

Treatments		With neem cake				Without neem cake			
		D.I (%)	BCE (%)	FWR (g)	DWR (g)	D.I. (%)	BCE (%)	FWR (g)	DWR (g)
F. oxysporum	Control	73.33	0	1.03	0.16	76.00	0	0.98	0.14
	P. fluorescens	27.67	62.16	1.19	0.19	30.33	59.92	1.15	0.17
	B. subtilis	30.00	58.89	1.17	0.18	30.67	59.46	1.13	0.17
	P. fluorescens + *B. subtilis*	13.00	82.22	1.23	0.20	17.67	75.76	1.20	0.18
F. solani	Control	77.67	0	1.04	0.16	79.00	0	1.01	0.15
	P. fluorescens	26.67	65.59	1.18	0.19	28.33	64.13	1.16	0.17
	B. subtilis	23.00	70.49	1.17	0.19	24.33	69.20	1.14	0.17
	P. fluorescens + *B. subtilis*	14.33	81.64	1.26	0.20	19.33	74.16	1.24	0.18
M. phaseolina	Control	80.00	0.00	1.01	0.16	77.33	0	0.99	0.14

P. fluorescens	24.33	69.66	1.18	0.19	25.33	67.21	1.15	0.17
B. subtilis	29.00	63.86	1.07	0.17	26.00	66.47	1.10	0.16
P. fluorescens + *B. subtilis*	7.67	90.40	1.21	0.19	18.67	75.24	1.19	0.17

increase in the fresh and dry weight of roots was observed in this treatment. In soil without neem cake amendment, a similar trend was observed and the best treatment was observed to be the *P. fluorescens* + *B. subtilis*.

Greenhouse evaluation of rhizobacteria

After the laboratory evaluation the isolates were treated under greenhouse conditions and similar results were observed. All the pathogens were controlled in all treatments as compared to control. The treatment *P. fluorescens* + *B. subtilis* was found to be significant when compared to control. Disease incidence was lowest in this treatment in all pathogens while the BCE of this treatment against *F. oxysporum*, *F. solani* and *M. phaseolina* was recorded as 77%, 81% and 89%, respectively, in neem cake amended soil. In soil without neem cake amendment the highest BCE against each pathogen was found to be 68%, 70% and 70%, respectively. Similarly, FWR and DWR were also highest in *P. fluorescens* + *B. subtilis* treatment (see Table 31.3 below).

Improvements in plant growth and disease resistance to a broad array of plant pests can be accomplished using PGPR (Kloepper et al. 2004). The concept of introducing PGPR into the rhizosphere is based on the hypothesis that their establishment in the relatively clean environment of the planting media would afford them an opportunity to develop stable populations in the seedling rhizosphere that, when transferred to fields, will confer resistance against diseases. This is attributed to rhizobacteria, due to which it competes with other microorganisms by establishing itself into the rhizosphere, and with other microbial communities existing there. The PGPR, including *Pseudomonas* and *Bacillus* spp., have been reported to stimulate the development of healthy root systems (Germinda & Walley 1996) and rapid root colonization by beneficial bacteria (Bolton et al. 1990). This might be the reason that a larger population of antagonistic rhizobacteria in the rhizosphere has overcome the disease pathogen due to competition for space and food. In our study the application of PGPR has shown remarkable deviation from the control and thus it could be implied that these PGPR were effective root colonizers that enabled them to establish themselves more rapidly than other microbial communities.

Both the isolates were found to be effective against all the fungal pathogens with varying abilities to antagonize them. Different researchers have also reported that *P. fluorescens* was an effective biocontrol agent for different fungal pathogens (Meyer et al. 1992; Buysens et al. 1996; Reimmann et al. 1988; Singh et al. 2006). *P. fluorescens* could act as

strong elicitors of plant defence reactions (M'Piga et al. 1997). Recent studies imply that prior application of fluorescent pseudomonads strengthens host cell wall structures, resulting in the restriction of pathogen invasion in plant tissue (Benhamou et al. 2000; Chen et al. 2000; Conrath et al. 2002; Dwivedi & Johri 2003).

In another study by Kumar et al. (2007), *P. fluorescens* inhibited the mycelial growth of the *M. phaseolina* under *in vitro* conditions and reduced the disease severity. It also significantly increased the biomass of the chickpea plants, as well as the shoot length, root length and protein content of the chickpea seeds. The observations revealed that *P. fluorescens* is quite effective in reducing the charcoal rot disease both in the field and the greenhouse, and in significantly increasing seed yields. In our study, similar observations were recorded where *P. fluorescens* was found to be an effective biocontrol agent which suppressed not only the charcoal rot pathogen *M. phaseolina* but also *F. solani* and *F. oxysporum*. Moreover, it also increased the fresh and dry root weight. Srivastava et al. (2001) reported that treatment of *P. fluorescens* decreased the disease severity of *M. phaseolina* (33%) by increasing chitinases and glucanases. Isolates in our study also showed antifungal activity which may be associated with the production of antifungal metabolites. The absorption of these metabolites by chickpea roots may be another reason for the reduced disease levels and increased plant growth.

Species of *Bacillus* are common inhabitants among the resident microflora of inner tissues of various species of plants, including cotton, grape, peas, spruce and sweetcorn, where they play an important role in plant protection and growth promotion (Berg et al. 2005). In our study, *Bacillus subtilis* was found to be an efficient biocontrol agent that, if applied in a consortium, could provide a promising biocontrol of pathogens. In our study, *B. subtilis* controlled the pathogens in dual culture plate assay and also under laboratory and greenhouse conditions. This biocontrol ability of *B. subtilis* is not well understood but it is assumed that it may be due to antibiotic compounds (Joshi & McSpadden 2006).

In our study, the treatment of isolates in combination was found to be more effective than individual isolates. More disease suppression and more growth promotion were observed throughout the experiment in combined treatments. In general, treatments performed better in laboratory experiments than in greenhouse conditions, which may be due to the controlled conditions that favoured PGPR. The sterilization of soil enabled the PGPRs to fight specific organisms, which is not possible in the field.

Table 31.3. The effect of rhizobacterial isolates on the root pathogens and growth of chickpea plants with and without neem cake under greenhouse conditions

Treatments		With neem cake				Without neem cake			
		D.I (%)	BCE (%)	FWR (g)	DWR (g)	D.I (%)	BCE (%)	FWR (g)	DWR (g)
F. oxysporum	Control	75.33	0	0.99	0.15	79.00	0	0.98	0.15
	P. fluorescens	29.67	60.58	1.14	0.17	33.33	57.62	1.15	0.17
	B. subtilis	32.00	57.61	1.13	0.17	33.33	57.61	1.14	0.17
	P. fluorescens + *B. subtilis*	17.33	77.00	1.18	0.18	24.67	68.49	1.21	0.18
F. solani	Control	78.00	0	1.07	0.16	78.00	0	0.99	0.15
	P. fluorescens	28.00	64.18	1.15	0.17	29.33	62.27	1.15	0.18
	B. subtilis	25.00	67.96	1.14	0.17	29.33	62.57	1.12	0.17
	P. fluorescens + *B. subtilis*	14.33	81.72	1.22	0.19	23.33	70.33	1.24	0.19
M. phaseolina	Control	81.33	0	1.01	0.16	75.67	0	0.99	0.15
	P. fluorescens	24.67	69.72	1.18	0.18	30.00	60.10	1.13	0.17
	B. subtilis	30.00	63.22	1.07	0.17	30.67	59.43	1.07	0.16
	P. fluorescens + *B. subtilis*	8.67	89.33	1.21	0.19	22.33	70.14	1.20	0.18

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CHAPTER THIRTY-TWO

EFFICACY OF *PSEUDOMONAS FLUORESCENS* P60 IN ORGANIC LIQUID FOR SUPPRESSION OF *SCLEROTIUM ROLFSII* IN CUCUMBER

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Abstract

This study aims to determine the potential of *Pseudomonas fluorescens* in organic liquid broth for the suppression of cucumber stem rot caused by *Sclerotium rolfsii.* Studies were conducted on the formulation of *P. Fluorescens* in liquid organic broth, its effect on sclerotial germination and delivery of the formulated bacteria for suppression of stem rot disease in cucumber seedlings. Assays were conducted both *in vitro* and *in vivo* using a completely randomized factorial design. The factors comprised the type of organic liquid formula composed of a control treatment, and broths of snail, chicken, catfish, chicken meat, beef bones and rat. The second factor is the period (0, 1, 10 and 100 h) of soaking sclerotia in these broths for their effect on sclerotial germination. Assays were repeated four times. The results showed that all animal broths tested can be used as liquid formulation for *P. fluorescens* P60. The snail broth with a population of 98.3×10^9 cfu/g soil was proved to be the best formulation at a soaking time of 100 h. Generally, fish is the best broth application for the use formula in the field, the suppression of sclerotial germination and suppression of stem rot disease. Our results also showed that *P. fluorescens* P60 formula may increase cucumber plant height, plant wet weight, dry weight of plant, root wet weight, dry weight of roots and root length, respectively, at 146.83%, 86.62%, 112.5%, 87.88%, 140% and 159.68%, compared to control.

Introduction

Cucumber (*Cucumis sativus* L.) is one of the Cucurbit vegetables (Cucurbitaceae) widely used as a vegetable, depending on the species (Linayanti et al. 2000). The Central Bureau of Statistics (2012) notes that from 2007 to 2011, the production of cucumbers in Indonesia declined from 581.206 t to 521.535 t. This is due to land conversion and the attacks of plant pest organisms.

One of the plant pathogens attacking cucumber plants is Sclerotium rolfsii, causing stem rot disease. These pathogens can form sclerotia as resting or dormant structures, and so are difficult to control (Hadar et al. 1981; Punja 1985). The pathogen *Sclerotium rolfsii* Sacc. is distributed in tropical and sub-tropical regions of the world where high temperatures prevail. The fungus has a wide host range of 500 species in about 100 families (Aycock 1966; Punja 1985; Azhar et al. 2003).

One of alternative disease controls that is safe and environmentally friendly is using the biological agent *Pseudomonas fluorescens* (Landa et al. 2002a/b; Obradovic 2005; McSpadden Gardener 2007). Strain P60 of *P. fluorescens* has been tested for several plant pathogens (Soesanto 2003; Soesanto & Termorshuizen 2004; Soesanto et al. 2010). Applications of biological agent *P. fluorescens* P60 in the field are still experiencing problems, especially on the formulation of biological agents with its carrier material. Formulation is still relatively expensive, difficult to obtain and difficult to apply in the field. In addition, the medium used is generally in the medium-scale laboratories which are limited and expensive, such as King's B (Landa et al. 2002b; Lee et al. 2005; Goud & Muralikrishnan 2009), tryptic soy agar (Milus & Rothrock 1997), nutrient broth (Obradovic 2005), or mannitol-glutamate yeast (Choi et al. 2006). Therefore, cheap and easily applied carrier media for the formulation of *P. fluorescens* P60 are needed. The formulation of organic liquid derived from animal broth has the potential to be developed as a carrier substance *P. fluorescens* P60 to formulation because it contains high nutrient broth, and animal broth contains lots of protein.

This study aims to determine the potential of organic liquid formula *P. fluorescens* P60, the effect of immersion time on germination sclerotia, and how best to apply organic liquid formula in the control of stem rot of cucumber.

Materials and Methods

Preparation, propagation, and infestations of *S. rolfsii*

S. rolfsii is grown aseptically on PDA medium and incubated at room temperature for 5 d. Furthermore, the fungi are propagated on rice straw pieces (± 1 cm long) that have been sterilized and put in plastic bags, and then incubated at room temperature for 1–2 weeks to form sclerotia. Infestations of sclerotia took place a day before planting, with 10 sclerotia placed in Trico porous fabric bags, sized 2 × 2 cm, tied at the end to facilitate observation, and then buried in the ground next to cucumber seedlings.

Preparation and propagation of antagonist

Bacterial antagonists *Pseudomoans fluorescens* P60 are propagated on King's B medium in Petri dishes aseptically, and then incubated for 2 × 24 h at room temperature. Furthermore, colonies formed are moved to King's B broth medium in Erlenmeyer flasks and shaken with a shaker (Selecta) at 150 rpm for 3 d at room temperature.

Preparation of growth media and cucumber seedlings

Planting medium is prepared by mixing soil, sand and manure in a 1:1:1 ratio, and placed in a 0.5 kg polybag. Seeds of cucumber (Red Arrow) are previously soaked in sterile water for ± 20 min and then planted in the planting medium in a polybag.

Preparation of animal broth

Animal broth used for the multiplication of antagonistic bacteria is created using catfish, chicken meat, local chicken, beef bones, snails and a field mouse. As much as 1 Kg of the material is then boiled in 4 l of water, after which 0.02 g of shrimp paste is added and stirred until homogeneous. Furthermore, the broth is filtered with cotton and immediately put into sterilized plastic jerrycans using 80% alcohol, and rinsed with sterile water. The jerrycan broth is then sealed and allowed to cool. Once cool, 200 ml / l bacterial suspension of antagonist P. fluorescens P60 is added to each broth in aseptic, and then resealed and incubated at room temperature for 5 d, and whipped frequently.

Experimental design

In vitro test

The design used was a completely randomized design (CRD) factorial. The first factor is the type of organic liquid formula tested, comprising six types of broth and control, the sterile water control (K), snail broth (F1), local chicken broth (F2), catfish broth (F3), chicken meat broth (F4), cow bone broth (F5), and mouse broth (F6). The second factor is the sclerotia soaking time in broth containing P. fluorescens P60, which is 0 h (until evenly moist) (W0), 1 (W2), 10 (W3), and 100 (W4) hours. There were 28 combinations of treatments in total, and each treatment was repeated 4 times.

In planta test

The experimental design used was the factorial randomized block design (RBD). The first factor was the type of organic liquid formula, comprising six types of broth *in vitro* and control, up to as much as 2 treatments. The second factor was the way the application included flushing (A1) and soaking the seeds (A2). The combination of these two factors totalled 16, and each treatment was repeated 3 times.

Observation of variables

In vitro test

The calculation of sclerotia germination (%) after immersion for 0 h (until evenly moist) was 1, 10, and 100 h in some broth, using the formula:

$$P = \frac{a}{b} x100\%$$

P = germination (%), a = the number of geminating sclerotia, b = total number of sclerotia.

In planta test

Variables of the *in planta* observations include the incubation period (days after inoculation, dai), the incidence of disease, the final population of

sclerotia *S. rolfsii*, the final density of *P. fluorescens* P60, and the differences in plant height, root length, plant wet weight, dry weight of plant, root wet weight, and dry weight of roots. The calculation of disease incidence was made using the formula:

$$KP = \frac{n}{N} x100\ \%$$

Description: KP = incidence of disease (%) n = number of diseased plants, N = number of plants observed.

Data analysis

Data were analyzed by an F test to produce the effect of treatment. If they differed they were followed by a DMRT (Duncan's Multiple Range Test) with a 5% level.

Results and Discussion

In vitro test

The results of the statistical analysis show that sclerotia germination was significantly different between treatments and the controls (see Table 32.1 below), but not significantly different among formulas. However, in general the best formula to suppress the germination of sclerotia is F1 (snail broth) at 55.79% with a germination of 43.1%. Germination at F5, F3, F2, F4, F6 and K were presented, respectively, by 48.1%, 48.8%, 50%, 50%, 53.1% and 97.5%. The high emphasis of sclerotia germination in snail broth formula was predicted in the formula to be antagonistic to the growth of both nutrients (proteins) available so that the development and growth are good. Protein is an essential nutrient required for the growth of bacteria, particularly the bacterial antagonist *P. fluorescens* (Palumbo et al. 1971; Singh & Arora 2001; Farjalla et al. 2006), and ribosomal proteins (Ochi 1995) and green fluorescent protein fluorescence (Normander et al. 1999) can be found in species belonging to the genus *Pseudomonas*.

Table 32.1. Effect of treatment on germination of sclerotia *S. rolfsii*

Treatment	Germination of sclerotia (%)	Treatment	Germination of sclerotia (%)
F cal F	49.98 **	W1K	97.5 a
K	97.5 a	W1F1	65.0 d
F1	43.1 c	W1F2	67.5 cd
F2	50.0 bc	W1F3	70.0 cd
F3	48.8 bc	W1F4	70.0 cd
F4	50.0 bc	W1F5	70.0 cd
F5	48.1 bc	W1F6	75.0 bcd
F6	53.1 b	W2K	97.5 a
F cal W	232.10 **	W2F1	17.5 fghi
W0	88.6 a	W2F2	30.0 ef
W1	73.6 b	W2F3	27.5 efg
W2	37.5 c	W2F4	30.0 ef
W3	23.6 d	W2F5	25.0 efgh
F cal WXF	6.75 **	W2F6	35.0 e
W0K	97.5 a	W3K	97.5 a
W0F1	87.5 ab	W3F1	2.5 i
W0F2	90.0 ab	W3F2	12.5 ghi
W0F3	82.5 abc	W3F3	15.0 fghi
W0F4	87.5 ab	W3F4	12.5 ghi
W0F5	87.5 ab	W3F5	10.0 hi
W0F6	87.5 ab	W3F6	15.0 fghi

Description: Numbers followed by the same letter in the same column are not significantly different at DMRT 5% error level. K: control (sterile water), F1: Formula of snail broth, F2: Formula of chicken broth, F3: Formula of catfish broth, F4: Formula of pieces of chicken broth, F5: Formula of beef bone broth, F6: Formula of rat broth, W0: Soaking 0 h (just until evenly moist), W1: Immersion 1 hour, W2: Soaking 10 h, and W3: Immersion 100 h.

Viegas (2009) and Sulistiono (2010) state that broth contains a lot of protein, and even the snail protein content can reach 16–50%, and can even be as high as 66.76% (Sogbesan et al. 2006). Babalola & Akinsoyinu (2009) stated that the crude protein contents of the breeds of snail, *Archachatina Marginata* (AM), *Achatina Achatina* (AA), *Achatina Fulica* (AF) and *Limicolaria* species (LM) ranged from 19.53% for AM to 5.86% for LM. The Department of Animal Husbandry and Fishery, Central Jakarta (1992), pointed out that 100 g of chicken meat contains 18 g protein, 25 g fat and 302 kcal calories. The protein content of 100 g catfish is 11.2 g (Directorate General of Aquaculture 2010). Astawan (2004) stated that the composition of all meats is relatively similar, with beef protein content in the range of 15%–20% of the weight of the material, and meat cholesterol levels at about 500 mg/100 g. The value of rat meat protein was balanced with beef or chicken, with a lower fat content present.

Based on the statistical analysis, long-soaking sclerotia showed very different results. The best time to suppress the germination of sclerotia was after 100 h soaking, followed by 10 h and 1 h (see Fig. 32.1 below). This is because the longer the sclerotia is soaked the larger the limiting factor becomes, which is attached to the sclerotia antibiotics. Antibiotics can then lead to lysis of the sclerotia. Further biochemical reactions of the antibiotics directly after the soaked sclerotia would not be advantageous to the growth and germination of sclerotia (Keel et al. 1992).

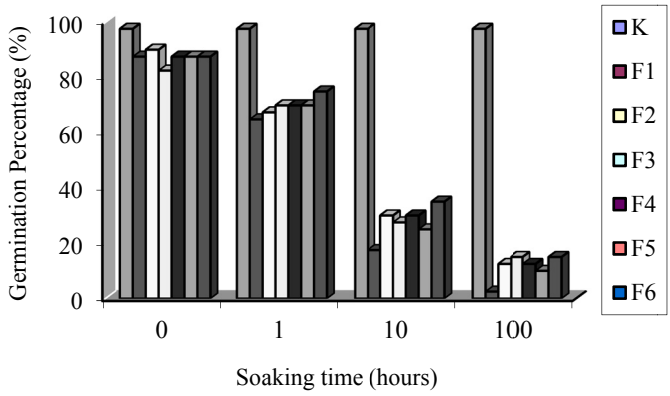

Fig. 32.1. Germination of sclerotia (%) on various soaking times

In planta test

Effect of treatment on patho-system component

Incubation period and symptoms

The results of the analysis showed no significant difference on the incubation period of the disease (see Table 32.2 below). However, in general the treatment of *P. fluorescens* P60 can be seen in organic liquid formula affecting the pathogen *S. rolfsii*, causing stem rot disease. The longest incubation period is in the treatment F2 (chicken broth formula) of more than 29.88 dai or asymptomatic plants, followed by treatment F1 (snail broth formula) at 28.25 dai. This proves that the formula is able to delay the incubation period when compared with the sterile water control (K1) at 12.083 dai and fungicide control (K2) for 17.362 dai. Treatment F2 and F1 were allegedly able to influence the development of the pathogen.

Table 32.2. Effect of treatment on patho-system components

Treatment	Incubation period (dai)[tn]	Disease incidence (%)	Late population of the pathogen (sclerotia)	Late population of the antagonist (cfu/g)
F hit F	1.45	3.66 **	5.35**	111.25 **
K1	12.08	50.00 a	24.11 a	0.0 d
K2	17.36	38.89 a	15.06 b	0.0 d
F1	28.25	16.67 b	9.72 b	98.3 a
F2	29.89	16.67 b	9.83 b	95.7 ab
F3	26.08	16.67 b	11.39 b	91.7 abc
F4	23.83	16.67 b	14.95 b	82.7 c
F5	28.08	16.67 b	15.00 b	84.7 bc
F6	24.50	16.67 b	11.33 b	90.0 abc
F hit A	1.27	2.92	1.19	94.45 **
A1	21.74	27.78	14.71	54.1 b
A2	25.78	19.44	13.14	81.6 a
F hit FXA	0.21	0.32	0.02	4.76 **
K1A1	7.50	55.56	25.22	0.0 d

K1A2	16.67	44.44	23.00	0.0 d
K2A1	18.17	33.33	15.22	0.0 d
K2A2	16.56	44.44	14.89	0.0 d
F1A1	27.50	22.22	10.67	82.3 b
F1A2	29.00	11.11	8.78	14.3 a
F2A1	30.44	22.22	10.78	76.0 bc
F2A2	29.33	11.11	8.89	15.3 a
F3A1	23.50	22.22	12.22	74.3 bc
F3A2	28.67	11.11	10.56	109.0 a
F4A1	19.33	22.22	15.78	60.7 c
F4A2	28.33	11.11	14.11	104.7 a
F5A1	27.50	22.22	15.78	69.0 bc
F5A2	28.67	11.11	14.22	100.3 a
F6A1	20.00	22.22	12.00	70.7 bc
F6A2	29.00	11.11	10.67	109.3 a

Description: Numbers followed by the same letter in the same column are not significantly different at DMRT 5% error level. K1: control with sterile water, K2: control with fungicides, A1: Application soak seeds, A2: Application flush formula. F1: Formula of snail broth, F2: Formula of local chicken broth, F3: Formula of catfish broth, F4: Formula of chicken broth, F5: Formula of beef bone broth, F6: Formula of rat broth.

The growth of *P. fluorescens* P60 in the formula did influence the germination of sclerotia. The bacteria attach to the sclerotia and cause lysis because antibiotics are produced, so as to delay the incubation period. According to Prabowo et al. (2006), the delay in incubation occurred because of the incubation period of competition between pathogens with antagonistic organisms. One such competition is the antibiosis mechanism, thus causing pathogens to take longer to infect plants (Keel et al. 1992; Dowling & O'Gara 1994).

The applications show that the incubation periods are not significantly different (see Table 32.2 below), but in general tend to have the ability to flush the application better than wading applications. On the application of flush, the incubation period can be delayed until 25.78 hsi, whereas only 21.74 hsi soak applications. It is thought that the flush application has a greater population of *P. fluorescens* P60 than the application of soak. Soaking seeds causing bacteria are more concentrated around the seeds,

especially when the seeds begin to germinate, so *P. fluorescens* P60, in flushing applications, suppresses the development of pathogenic *S. rolfsii* faster. This is in line with Vidhyasekaran et al.'s (1997) research results.

The results of the combination formula to soak or flush applications are also not significantly different to the incubation period (see Table 32.2 below). The longest incubation period is in the treatment F2A1 (application of soak chicken broth formula) at 30.443 dai and F2A2 (applications of pour chicken broth formula), i.e. 29.333 dai or asymptomatic plants. This is presumably because the bacteria *P. fluorescens* P60 in the formula is able to influence the growth of pathogens. It is also consistent with the results of research *in vitro*, where the germination of sclerotia after immersion in some broths, particularly snail and chicken, has high levels of inhibition of germination of sclerotia.

Disease incidence

The results of the analysis of incidence of the disease suggest that the use of broth in reducing the incidence of the disease compared with the controls (K1 and K2) comprised significantly different formulas, but that there was no significant difference between formulas. The smallest disease incidence in F1 formula (snail broth), F2 (chicken broth) and F5 (beef bone broth) is equal to 16.67% (see Table 32.3 below). This suggests that the bacterium *P. fluorescens* P60 in the formulas F1, F2 and F5 can reduce the incidence of stem rot disease reaching 66.67%, compared with the use of fungicides that can only push to 22.22%.

The high-nutrient broth allowing the bacteria to grow and thrive is an antagonist, so when applied it can compete with and suppress *S. rolfsii*. In addition, the effectiveness of *P. fluorescens* P60 in suppressing *S. rolfsii* is presumably because these bacteria produce secondary metabolites. Secondary metabolites, such as siderophore, pterin, pyrrole and fenazin, play an important role, with siderophore used as fungistasis and bakteriostatis (Dowling & O'Gara 1994; Soesanto et al. 2011).

The soaking and flushing application forms based on the results of the analysis did not differ significantly in reducing the incidence of disease (see Table 32.2 below). However, applications have a greater tendency to suppress the incidence of the disease in wading applications. This is evident from the low incidence of the disease in flush applications at 19.44%, which is lower than the 27.78% of soak applications. These results also correlate with the incubation period, which tends to be longer in the flush applications. It is thought to have a higher population of flush applications *P. fluorescens* P60 than the cucumber seed soaking application. The high population of *P. fluorescens* P60 on the application

flush means that bacteria have a greater ability to spread, mainly due to the flow of water spray. This is supported by Soesanto & Termorshuizen (2004), who state that *P. fluorescencs* P60 is able to sustain itself on rhizosper, increase its population, produce compounds inhibiting pathogens, and make plant roots easier to colonize.

Late population of *P. fluorescens* P60 and sclerotia

The results of statistical analysis show significantly different results between applications for the soak. The population density is highest at the end of the antagonist UPK 115.3 × 109 /g soil treatment contained in F2A2 (applications of local chicken broth formula). The high population of antagonists on the application flush is allegedly because of some trait or ability of the antagonist that supports life. According to Duffy & Weller (1995), *P. fluorescens* is able to utilize root exudates, colonize roots, maintain the population, remain strongly attached to the roots, and is durable on the ground. Cook et al. (1998) state that one of the properties of the bacteria *P. fluorescens* P60 is its ability to colonize around the rhizosphere faster and maintain the population.

Meanwhile, the results of the analysis of the population of sclerotia in the single treatment showed that the K1 control (sterile water) without the use of *P. fluorescens* P60 demonstrate highly significant different results with a combination of other treatments, with either flush or soak applications and K1 (control fungicide). Populations appear most at the end of the sclerotia K1 treatment (control) for 24 points/polybag. This is due to the absence of antagonistic microbes that act as competitors, so the pathogen can infect the cucumber stem bases. In addition, pathogens can utilize nutrients in the soil without any competition from microbial antagonists, particularly in the use of ferric ions which many pathogens use as an essential nutrient required for kevirulennya. According to Lucas (1998), sclerotia require iron as an important nutrient for its virulence.

The lowest sclerotia population densities present in the treatment F3A2 (*P. fluorescens* formula watering broth made from the snail carrier) were 9 points/polybag. This is presumably because the bacteria *P. fluorescens* P60 can act as a competitor pathogen. Competition happens in obtaining nutrients. According Widiyanto (2003), carbon and nitrogen compounds are absolutely necessary to synthesize the components that will improve the lives and growth of vegetative cells, and the formation of a particular structure. The low population of sclerotia on treatment is because bacterial antagonists have obtained carbon and nitrogen nutrients and also require the fungus *S. rolfsii*. In addition, *P. fluorescens* produces siderophore iron ion binding, and antibiotics or activity that inhibit the growth of sclerotia

(Dowling & O'Gara 1994), thus causing *S. rolfsii* to neither grow or thrive.

Effect of treatment on growth components

Differences in crop height

The results of statistical analysis showed that for plant height there is a real difference of each treatment with the control. The highest plant height in the F1 treatment is 20.29 cm, showing the mean treatment with *P. fluorescens* P60 to be higher than without *P. fluorescens* P60 (K1 and K2), because *P. fluorescens* P60 can stimulate plant growth and is able to suppress the growth of pathogens.

According to Dowling & O'Gara (1994), *P. fluorescens* can stimulate plant growth by producing salicylic acid and indole acetic acid (IAA) as a plant growth hormone. Furthermore, according to the nature of the bacteria *P. fluorescens* P60 is a biological agent as well as a PGPR. This is in accordance with the opinion of Soesanto et al. (2010; 2011) that the bacterium *P. fluorescens* P60 can provide beneficial effects for plant growth and development.

Wet and dry weights of crop

The results of the data analysis of wet and dry weight of plants showed significant differences between combination treatment with the control and inter-control, but not for dry weight. Pramudiana (1998) reported that the addition of the *P. fluorescens* rhizosphere origin of corn, tomatoes, caisin, scallion, potatoes, celery, carrots and cabbage plants tends to increase the wet weight.

Wet and dry weights were higher in the combination treatment F2A2 (applications for chicken broth formula) and are attributed to sclerotia-infected plants as well as *P. fluorescens* P60 to produce growth hormones. This is supported by the opinion of Landa et al. (2002a) that *P. fluorescens* acts as a PGPR which can produce growth hormones, including auxin, giberallin and cytokines.

Wet and dry weights of root

The analysis showed that for the wet weight of real roots there are differences of each treatment with the control (see Table 32.3 below).

Table 32.3. Effect of treatment on growth components

Treatment	Difference of crop height (cm)	Wet weight of crop (g)	Wet weight of root (g)	Dry weight of crop (g)	Dry weight of root (g)	Root length (cm)
F hit F	2.72 *	2.37 *	2.55 *	3.85 **	3.87 **	3.31 *
K1	8.22 c	1.419 c	0.033 b	0.083 b	0.005 b	2.533 b
K2	8.94 bc	1.492 b	0.040 ab	0.077 b	0.006 b	2.872 b
F1	20.29 a	2.476 a	0.061 a	0.147 a	0.011 a	6.450 a
F2	18.29 a	2.648 a	0.063 a	0.170 a	0.012 a	6.572 a
F3	16.98 ab	2.523 a	0.060 a	0.147 a	0.010 a	6.006 a
F4	18.21 a	2.297 a	0.058 a	0.141 a	0.009 a	5.750 a
F5	18.82 a	2.409 a	0.062 a	0.147 a	0.011 a	6.372 a
F6	17.89 a	2.349 a	0.057 a	0.144 a	0.009 a	5.750 a
F hit A	0.96	1.40	0.46	7.01 *	3.37	2.07
A1	14.975	2.073	0.052	0.116 b	0.008	4.833
A2	16.931	2.330	0.056	0.148 a	0.010	5.743
F hit FXA	0.17	0.78	0.14	1.25	0.18	0.02
K1A1	7.467	1.507	0.033	0.069	0.005	2.267
K1A2	8.977	1.330	0.034	0.098	0.006	2.800
K2A1	10.620	1.946	0.044	0.104	0.006	2.545
K2A2	7.257	1.038	0.037	0.050	0.006	3.200
F1A1	18.867	2.249	0.060	0.128	0.010	5.989
F1A2	21.713	2.702	0.063	0.166	0.011	6.911
F2A1	16.933	2.301	0.060	0.130	0.011	6.111
F2A2	19.647	2.996	0.065	0.211	0.013	7.033
F3A1	14.753	2.167	0.059	0.126	0.009	5.633
F3A2	19.200	2.878	0.061	0.169	0.011	6.378
F4A1	16.733	2.099	0.056	0.123	0.008	5.256
F4A2	19.677	2.495	0.061	0.160	0.010	6.244
F5A1	17.647	2.193	0.059	0.127	0.010	5.778

F5A2	19.967	2.625	0.063	0.167	0.012	6.967
F6A1	16.780	2.121	0.050	0.124	0.008	5.089
F6A2	19.010	2.577	0.063	0.164	0.011	6.411

Description: Numbers followed by the same letter in the same column are not significantly different at DMRT 5% error level. K1: control with sterile water, K2: control with fungicides, A1: Application of soak seeds, A2: Application of flush formula. F1: Formula of snail broth, F2: Formula of local chicken broth, F3: Formula of catfish broth, F4: Formula of chicken broth, F5: Formula of beef bone broth, F6: Formula of rat broth.

The highest wet root weight in treated F2A2 (applications of local chicken broth formula) is 0.064 g. The high wet weight of plant roots on F2A2 treatment in the allegedly given formula can suppress plant pathogens so that a physiological process is not interrupted, and the translocation of nutrients from the soil into the plant is undisturbed. The mechanism of *P. fluorescens* P60, besides the suppression of pathogens, can also be attributed to the indirect effect that can stimulate the growth of plant roots and act as a PGPR, which can produce growth hormones including auxin, giberallin and cytokinin (Landa et al. 2002).

The results of the analysis on root dry weight also showed significantly different results between combination treatments with control. The greatest root dry weight in the treatment is 0.012 g F2A2, while the lowest root dry weight in the treatment of K1 is 0.005 g. The higher root dry weight of plants in the F2A2 treatment allegedly given to suppress the pathogen does not provide the opportunity for pathogens to penetrate crops. This is further explained by Leong (1986) in that in conditions of limited Fe^{3+}, *P. fluorescens* is able to tie in with a strong siderophore compound. Iron is an essential element of growth, as it serves as a cofactor for enzyme oxidation and reduction. Many pathogens require iron as an essential nutrient for kevirulenannya, thus causing the production of siderophore when Fe3 + is not available to the pathogen. As a result, pathogens can be suppressed and the plants can grow optimally (Santoso et al. 2007).

Root length

The results of the analysis of root length generally indicate significant differences between the control and treatment combination. The sizes of the longest roots on the combination treatment of F2A2 and F1A2 are 7.033 and 6.911 cm, respectively. When compared with the control, the shortest root is 2.267 cm. The high value of root length in the treatment of

P. fluorescens shows that these bacteria can produce growth hormones such as auxin, giberallin and cytokines. Gibberellin plant hormones are needed as a stimulus to the growth of the roots, so the presence of these bacteria may increase the length of the roots of cucumber plants.

Conclusion

All organic liquid formula made from animal broth formulation can be used for the antagonistic bacterium *P. fluorescens*. The best formula is the snail broth with a late population of 98.3 × 109 cfu/g soil.

The period of sclerotia immersion in the formula *P. fluorescens* affects germination, and the longer the soaking, the better suppression of germination. The best suppression of germination is soaking 100 h on the carrier of snail broth at 97.95%.

Generally, flush is the best application for the use of the formula in the field. This is seen from the incubation period, the incidence of the disease, the final density of sclerotia, the final density of *P. fluorescens* P60, the difference in plant height, root length, wet weight and dry weight of the plant, and the wet weight and root dry weight, respectively, for 25.78 dai, 19.44%, 13 sclerotia, 81.6 × 109 cfu/g soil, 16.93 cm, 5.74 cm, 2.33 g, 0.15 g, 0.055 g and 0.009 g.

Applications of *P. fluorescens* P60 formula can suppress sclerotia germination, the incubation period, the incidence of disease, and the final population of sclerotia, respectively, for 55.79%, 147.35%, 66.67% and 59.68%.

Applications of *P. fluorescens* P60 formula may increase the difference in crop height, wet weight, dry weight, root wet weight, root dry weight, and root length at 146.83%, 86.62%, 112.5%, 87%, 88%, 140% and 159.68%, respectively.

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CHAPTER THIRTY-THREE

SCREENING, IDENTIFICATION AND RELATED CHARACTERISTICS OF THREE STRAINS OF PHOSPHATE SOLUBILIZING BACTERIA FROM ALFALFA RHIZOSPHERE

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Abstract

Presently, there is little documented information on the occurrence of plant growth promoting rhizobacteria (PGPR) for producing bacteria inoculant (biofertilizer) in alfalfa (*Medicago sativa*), except rhizobium, even though alfalfa is one of the most widely cultured and important perennial leguminous forage with high yield and quality in the world. Our work focuses on isolated plant growth promoting rhizobacteria strains from the rhizosphere of alfalfa. The PGPR abilities of phosphate solubilizing based on phosphate solubilizing zone and phospho-molybdate blue colorimetric methods were determined, and we also analyzed PGPR capabilities of secreting indole acetic acid (IAA) through the Salkowski Colorimetry method. The results indicated that there were three strains—MS-27, MS-40 and MS-47—showing high abilities to solubilize different phosphorus sources of $Ca_3(PO4)_2$, $AlPO_4$, $FePO_4 \cdot 4H_2O$ and egg yolk phosphatidylcholine (EYPC), and to secrete IAA. The three strains showed a strong ability to solubilize $Ca_3(PO4)_2$, followed by $AlPO_4$, then

$FePO_4 \cdot 4H_2O$ and egg yolk phosphatidylcholine (EYPC). The quantity of phosphate solubilizing of Ca-P, Al-P, Fe-P and EYPC was 557.48-614.18 mg/L, 35.11-42.21 mg/L, 0-17.67 mg/L and 2.19-6.73 mg/L, respectively. All three strains secreted IAA without tryptophan, and the IAA secreting content of MS-40, MS-27 and MS-47 was 18.59 mg/L, 14.36 mg/L and 13.37 mg/L, respectively. The three stains were identified by the 16S rDNA sequence, and MS-27 falls in the genus of *Enterobacter* sp., MS-40 and MS-47 in *Pantoea* sp. The three strains each have the applied potential to be a PGPR inoculant for alfalfa in the study region.

Introduction

Phosphorus (P) is one of the major essential macro-nutrients for biological growth and development. Most agricultural soils contain large reserves of phosphorus, mostly accumulated as a consequence of regular applications of P fertilizers. However, a large portion of the soluble inorganic phosphate in fertilizers is rapidly inmobilized in the soil and becomes unavailable to plants (Rodríguez & Fraga 1999). For over a century, agricultural microbiologists and microbial ecologists have studied the ability of some bacteria to dissolve poorly soluble calcium mineral phosphates (Ca-P) (Goldstein et al. 1999). Phosphate-solubilizing bacteria (PSB) mobilize insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots (Sashidhar & Podile 2010). Recently, phosphate solubilizing bacteria have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield (Young 1994; Young et al. 1998; Goldstein et al. 1999; Fasim et al. 2002). Plant growth-promoting rhizobacteria (PGPR) are soil and rhizosphere bacteria that can benefit plant growth through different mechanisms (Glick 1995), and the P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant P nutrition.

Phosphate solubilizing bacteria (PSB) have been used as a biofertilizer since the 1950s (Kudashev 1956; Krasilinikov 1957). These microorganisms secrete different types of organic acids, such as carboxylic acid (Deubel & Merbach 2005), thus lowering the pH in the rhizosphere (He & Zhu 1988) and consequently dissociating the bound forms of phosphate like $Ca_3(PO_4)_2$ in calcareous soils. P-solubilizing bacteria (PSB) can solubilise and mineralize P from inorganic and organic pools of total soil P, and may be used as inoculants to increase P-availability to plants (Kucey et al. 1989; Richardson 1994, 2001; Illmer et al. 1995; Whitelaw et al. 1999).

Bacteria currently recognized as PSB belong to the genera *Pseudomonas*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, *Acinetobacter*, *Erwinia* and *Pantoea* (Rodríguez & Fraga 1999; Torres et al. 2008; Peix et al. 2009). However, the P-solubilizing mechanism has been studied mostly in species of the genera *Pseudomonas* and *Erwinia* (Liu et al. 1992; Vyas & Gulati 2009).

Alfalfa is the most cultivated forage legume in the world, and worldwide production was around 436 million tons in 2006. The US is the largest alfalfa producer in the world, but considerable production is found in Canada, Argentina (primarily grazed), Southern Europe, Australia, South Africa and the Middle East. China has become one of the leading alfalfa cultivating regions in Asia. Alfalfa has a wide range of adaptations, and can be grown from the very cold northeastern edge area of the Tibetan plateau to high mountain valleys, from rich temperate agricultural regions to the Karst terrain area in Guizhou, China. As a result, a bulk of insoluble phosphorus has been left in the soil by agrochemicals.

The objective of this chapter is, through determination and estimation, to supply the basic material of potential PSB for inoculants of alfalfa which can enhance the phosphorus availability in the region and yield of alfalfa locally.

Materials and Methods

Bacterial strains

The three tested strains were screened for the strains of rhizobacteria on alfalfa growing in the Maiping region, Guzhou province, China (E 106° 31' 59.9982", N 26° 28' 59.9982"), which were provided by the Engineering Laboratory of Guzhou Provincial Institute of Grassland Science, and were encoded as MS-27, MS-40 and MS-47.

Culture medium

The inorganic phosphorus source medium with calcium phosphate and the organic phosphorus source medium with EYPC were selected in the initial screening of P-solubilizing bacteria. The P-solubilizing ability was examined by the liquid medium mentioned above, the King medium without tryptophan was utilized for measuring the abilities of secreting IAA, the NFM medium was selected for testing whether strains produced acid or alkali, and the LB medium was chosen for conserving strains.

The inorganic phosphorus source medium comprised glucose 10.0 g, Sodium chloride [NaCl] 0.2 g, Ammonium sulfate [$(NH_4)_2SO_4$] 0.5 g, Magnesium sulfate [$MgSO_4 \cdot 7H_2O$] 0.1 g, Potassium chloride [KCl] 0.2 g, Manganese sulfate [$MnSO_4$] 0.03 g, Ferric sulfate [$FeSO_4 \cdot 7H_2O$] 0.03 g, yeast extract 0.5 g, agar 20.0 g, distilled water 1000 mL, pH 6.8~7.2, and insoluble phosphorus source 3.0 g. The phosphorus source included Calcium phosphate [$Ca_3(PO_4)_2$], Aluminum phosphate [$AlPO_4$], and Ferric sulfate [$FePO_4 \cdot 4H_2O$].

The organic phosphorus source medium comprised glucose 10.0 g, Ammonium sulfate [$(NH_4)_2SO_4$] 0.5 g, Sodium chloride [NaCl] 0.3 g, Potassium chloride [KCl] 0.3 g, Ferric sulfate [$FeSO_4 \cdot 7H_2O$] 0.03 g, Manganese sulfate [$MnSO_4 \cdot 4H_2O$] 0.03 g, Calcium carbonate [$CaCO_3$] 5.0 g, yeast extract 0.5 g, agar 20.0 g, and distilled water 1000 mL, pH 7.0~7.5. The phosphorus source included egg yolk phosphatidylcholine (EYPC) 0.2 g.

Measurement of P-solubilizing ability

After sterilization, 50 mL medium with phosphorus were poured into a 150 mL Erlenmeyer flask and inoculated in 0.5 mL bacterial suspension, whose concentration ranked 10^7~10^8 cuf/mL. The medium without inoculant of bacterial suspension was the control. Every treatment was repeated 3 times. The cultural condition was 28ºC in a 150 r/min constant temperature shaker, within 7 d. The cultured medium was centrifuged for 10 min under a rotative velocity of 10,000 r/min, and the supernatant was decanted and the effective phosphorus content determined by Mo-Sb Colorimetry. In this case, the solubilized phosphorus content equal to the effective phosphorus content subtracted the content of control, taking P_2O_5 mg/L as the unit. At the same time, the pH of bacterial suspension was tested.

After activation, the strains were inoculated in Petri dishes of the inorganic phosphorus source medium with calcium phosphate and the organic phosphorus source medium with EYPC, with every 4 points in one dish, and then cultured in 28ºC, observing and measuring the P-solubilizing zone diameter (HD), and colony diameter (CD) at 5d, 10d and 15d. Finally, the better strain was screened by calculating the ratio of the two.

Determination of secreting IAA and the acidity or alkalinity of strains

The Salkowski colorimetry was utilized to measure the content of secreting IAA. After sterilization, 30 mL liquid medium were inoculated in 0.5 mL of bacterial suspension. The medium without inoculant of bacterial suspension was the control. Every treatment was repeated 3 times. The cultural condition was 28ºC with a 125 r/min constant temperature shaker, within 7 days. The centrifugal condition was similar to the measurement of the P-solubilizing ability. The composition of S2 colorimetric solution was Ferric chloride 4.5g, 10.8 moL/L sulfuric acid 1000 mL. The same proportion of supernatant and the S2 colorimetric solution were mixed. After the mixture had been at rest in darkness for 30 min the absorbency was measured under 530 nm wavelength, and the content of IAA was measured via the standard curve (mg/L).

Identification of bacteria strains by 16S rDNA sequence analysis

The reagent box of DNA genetic group extract was purchased from Baosheng Biotech Co. Dalian. The positive direction of designed bacteria general primer was 5'-GAGAGTTTGATCCTGGCTCAG-3', opposite direction 5'-CTACGGCTACCTTGTTACGA-3', supplied by the Shanghai Jierui Biotech Co. The composition of 25 μL PCR reacting system was 10 × buffer (Mg^{2+} plus) 2.5 μL, dNTP 2.0 μL, primer (10 μM) each 1μL, template 1.0 μL(50 ng/μl), Taq DNA Polymeras 0.25 μL, ddH_20 17.25 μL. The condition of reaction was predenaturing in 94ºC for 5 min denaturing in 94ºC for 30 s, annealing in 45ºC for 30 s, elongation in 72ºC for 120 s, cycling 30 times in 25ºC for 2 min. PCR product was detected via 1.0% agarose gel horizontal electrophoresis and reclaimed by reagent box of Get Extraction Kit (50) D-2500-01 provided by OMEGA BIO-TEK. After detecting the purity, the reclaimed product was delivered to Shanghai Jierui Biotech Co. to determine the sequence. The results were sent to GenBank to compare with the Blast database, after multiplex sequence comparison with Clustal 1.8 software, eventually constructing an evolutionary tree of strains with Mega 2.0 software.

Statistical analysis

A Duncan multiple range test was used to evaluate the significant difference in the concentration of different study sites. A probability level

of 0.01 ($P < 0.01$) or less was considered significant. The standard error of the mean, variance and Duncan statistics were calculated using SPSS 16.0 statistical software.

Results

Screening of the strains

After screening by the P-solubilizing zone from 51 strains of alfalfa rhizosphere soil, 3 strains demonstrated the good abilities of P-solubilizing on media of calcium phosphate and egg yolk phosphatidylcholine (EYPC) through obvious and large zones which appeared on the culture media.

The value of HD/CD varied from different culture media of different strains, and also varied from different culture days of the same strain on the same culture media (see Figs. 33.1 and 33.2 below).

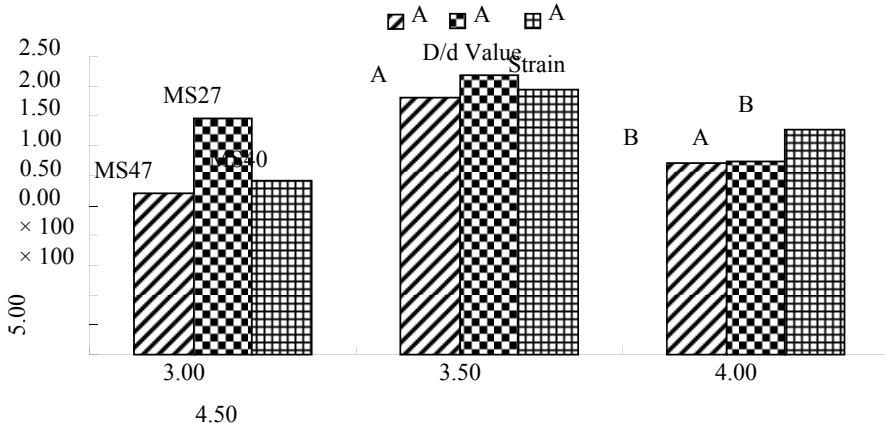

Fig. 33.1. The value of HD/CD on medium with calcium phosphate by 3 strains

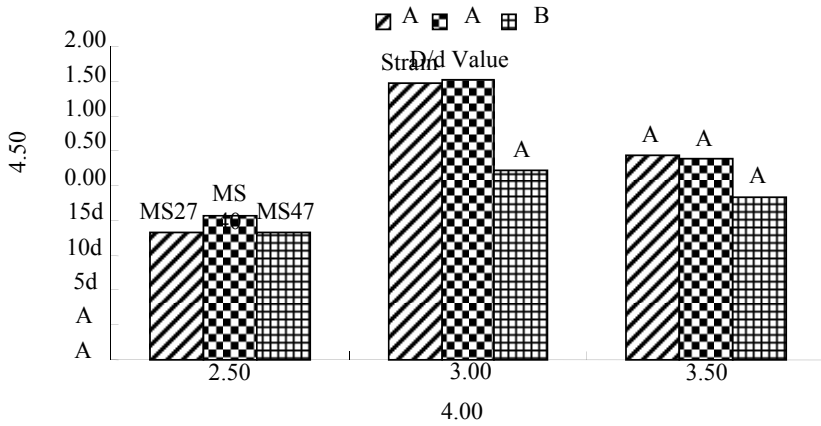

Fig. 33.2. The value of HD/CD on medium with EYPC by 3 strains

On the calcium phosphate medium, the value of HD/CD of strains MS-27, MS-40 and MS-47 all exceeded 2.00. After 15 culture days, MS-40 had the maximum value of HD/CD, followed by MS-47 and MS-27. The value of HD/CD of strains MS-27 and MS-40 possessed the same rule of alteration—an increase from 5 d to 10 d, a reduction from 10 d to 15 d—but MS-47 had a different rule—an increase from 15 d (see Fig. 33.1 below). Among these 3 strains there was a dramatic extent of change of value of HD/CD—the ratio of increasing was 46.13% during the period of 5d to 10d, and the rate of decreasing was 26.51% from 10d to 15d. But for the other 2 strains the rate of change was relatively steady.

On the EYPC medium the value of HD/CD displayed the identical trend of alteration on the calcium phosphate. MS-40 possessed the highest value of HD/CD, and all were higher than 2.50 after 5 d, 10 d and 15 d culture, and also higher than the other 2 strains in the same culture condition.

The type of soil of strain source and the characteristics of the colony are recorded in Table 33.1 below.

Table 33.1. Colonial characteristics and sources of P-solubilizing bacteria

Code of strain	Sources	Colonial characteristics	Growth rate
MS-27	RP、HP	The size of colony between 0.39–0.52 cm, irregular form, milky white colour, semi-humid and flat, semi-transparent, untrim edge	Fast, colony can be seen by naked eyes in 12 h
MS-40	NRS	The size of colony between 0.41–0.59 cm, round form, pale yellow colour, semi-humid and flat, non-transparent, radial groove edge	Fast, colony can be seen by naked eyes in 12 h
MS-47	RS、RP、HP	The size of colony between 0.52–0.72 cm, round form, yellow, semi-humid and flat, non-transparent, smooth edge, slight musty odour in late phase	Fast, colony can be seen by naked eyes in 12 h

Note: NRS: soil away from roots; RS: soil adhering to roots; RP: soil of rhizoplan or surface of roots; HP: soil of histoplan or interior of roots.

The P-solubilizing abilities of PSB on different insoluble phosphates

The abilities of P-solubilizing on 4 insoluble phosphates

The result of the P-solubilizing content of 3 strains on 4 different media of varied phosphorus sources are shown in Table 33.2 below. The P-solubilizing content varied from different strains on different or similar insoluble phosphates, and also varied in different insoluble phosphates of the same strain.

Table 33.2. The P-solubilizing ability of strains on 4 different insoluble phosphates

Code of strain	**The content of P-solubilizing (mg/L)**			
	Al-P	Fe-P	Ca-P	EYPC
MS-27	35.11 ±5.60^{B}	17.67 ±1.05^{C}	577.90 ±7.03^{A}	2.70 ±0.10^{D}
MS-40	92.67 ±2.08^{B}	0.00^{D}	557.48 ±8.15^{A}	2.19 ±0.14^{C}
MS-47	42.21 ±4.00^{B}	6.41 ±0.74^{C}	614.18 ±7.16^{A}	6.73 ±0.16^{C}

For the aspect of the same strain on different phosphates, the strain MS-27 possessed the highest P-solubilizing ability $Ca_3(PO4)_2$ and the content could reach 577.90 mg/L, followed by $AlPO_4$ (35.11 mg/L), then $FePO_4 \cdot 4H_2O$ (17.67 mg/L) and EYPC (2.70 mg/L). The P-solubilizing ability of $Ca_3(PO4)_2$ was evidently higher than the last 2 types of phosphate ($P < 0.01$).

The content of solubilizing 4 different insoluble phosphates of MS-40 gave it the order Ca-P>Al-P>EYPC>Fe-P. There was slight solubilizing on EYPC and none on Fe-P. By contrast, there was a high content of solubilizing on Ca-P (557.48 mg/L), which was more than six times on Al-P (92.67 mg/L) ($P<0.01$).

Similar to the former 2 strains, the content of solubilizing phosphate of MS-47 was high on Ca-P (614.18 mg/L), and again higher than the other 3 phosphates Al-P (42.21 mg/L), Fe-P (6.41 mg/L) and EYPC (6.73 mg/L). The content was slight and alike on the latter 2 insoluble phosphates.

The influence of pH value of culture medium reduced by PSB

After 7 d culture the pH value of each strain on each medium of a different phosphorus source also changed. Except for the strain of MS-40 on EYPC, the others all demonstrated a reduction compared with the neutral status 7 days prior (see Fig 33.3 below).

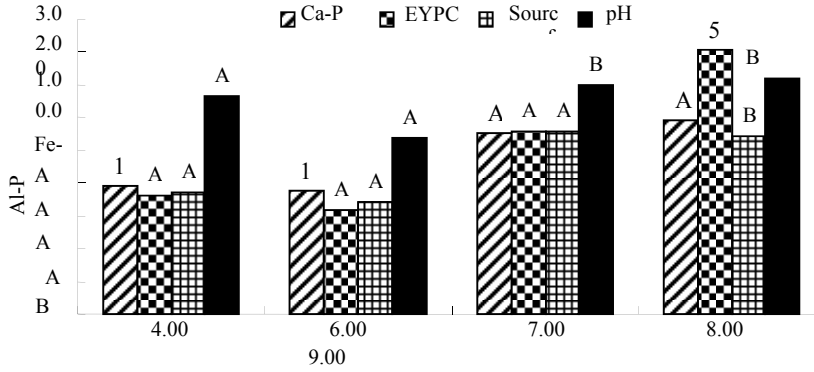

Fig. 33.3. The pH value during the culture period of four types of insoluble phosphorus

Several phenomena happened in 4 types of media of different phosphorus sources. First of all, the pH value of media which utilized Ca-P and EYPC as phosphorus sources decreased less than those media that utilized Al-P and Fe-P as phosphorus sources. The pH value of Al-P phosphorus source medium was between 3.50–4.00, of Fe-P was 3.00, of Ca-P was 3.00, and EYPC was between 5.00–6.00, which decreased relatively little. Secondly, the pH value of the control treatment without PSB was maintained around 7.00. Thirdly, the pH value of the strain of MS-40 on the EYPC medium was around 8.00, which differed from others (see Table 33.3 below).

Table 33.3. The ability to produce IAA and acid or alkaline

Code of strain	IAA		Acid or alkaline		
	Colour	Amount ($mg \cdot L^{-1}$)		Colour	pH
MS-27	+	13.37 ± 0.53^{A}	alkaline	Blue	7.88
MS-40	+++	18.59 ± 0.86^{A}	alkaline	Blue	8.61
MS-47	++	14.36 ± 1.23^{A}	alkaline	Blue	8.04

Note: "+" = low pink; "++" = pink; "+++" = deep pink.

The ability to secrete IAA and produce acid or alkaline

The culture media of 3 strains all changed their colour when mixed with the S2 colorimetric solution—MS-27 turned pale pink, MS-47 turned pink, and MS-40 turned deep pink (see Fig. 33.4 below). It showed that MS-40 might have a more powerful ability for secreting IAA. Under quantitative determination, the content of secreting IAA of MS-40 was highest in 3 strains, reaching 18.59 mg/L, followed by MS-47(14.36 mg/L), and MS-27 (13.37 mg/L).

The result of the ability to produce acid or alkaline of 3 strains can be found in Table 33.3 above and Fig 33.4 below. The culture media of 3 strains all had a pH value above 7.00, the colour turned blue in all, and they all produced alkaline.

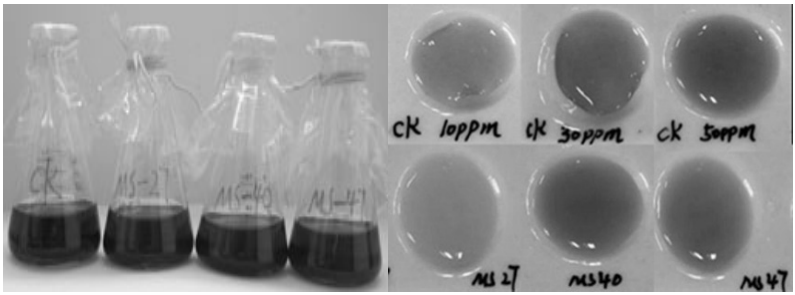

Fig. 33.4 The colour (left) of producing IAA and acid or alkaline (right) of strains

Identification of bacteria strains

The DNA fragment of 1500bp can be seen in which 3 strains of MS-27, MS-40 and MS-473 all displayed this DNA fragment, and the bands were bright and unified. The outcome of the recycled product of PCR, and that the purity of the aimed gene was high and without other bands.

The detected result declared that the gene lengths of MS-27 and MS-40 matched each other, and the number was 1443bp, and that the gene length of MS-47 was 1431bp. Through the homology comparison with the Blast program of GenBank on 16S rDNA of the 3 tested strains, the sequence fragment of MS-27 had 98.82% homology with *Enterobacter cloacae* (EU733519), and was homogeneous with *Enterobacter sp.* (GQ380575) and *Enterobacter ludwigii* (JF783987), whose homology exceeded 98.50%. Upon this comparison, the strain of MS-27 was identified in *Enterobacter* sp., and the registry number in GenBank of this strain was JQ038222.

The sequence of the strains of MS-40 and MS-47 possessed high similarity at 98.41%, and the homology of these 2 strains with *Pantoea* sp. was also high, reaching above 97.99%. These 2 strains were identified in *Pantoea sp.*, and their registry numbers in GenBank were JQ038223 and JQ038224. Furthermore, the phylogenetic tree is displayed in Fig. 33.5 below.

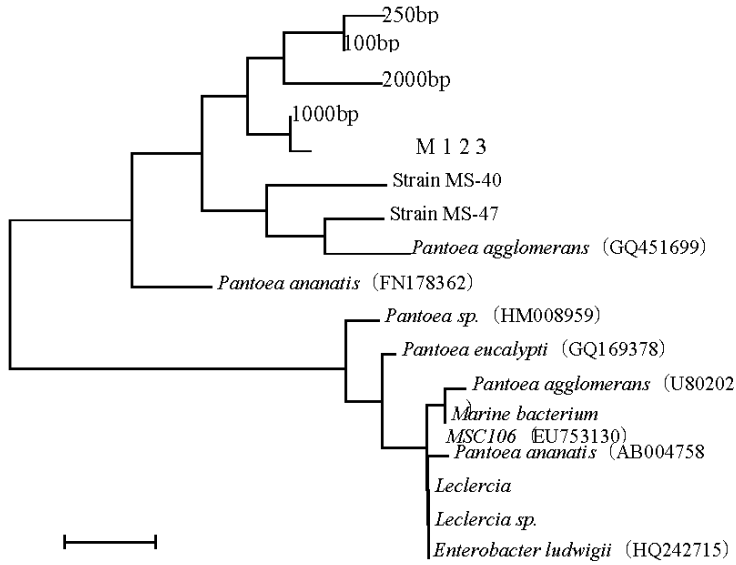

Fig. 33.5. The phylogenetic tree based on the 16S rDNA sequences homology of strains

Discussion

Screening PSB by plate screening methods is a classic approach and has already been utilized by many scholars for many years, such as Bardiya & Gaur (1974), Gupta et al. (1994) and Rodríguez (1999), who stated that the visual detection and even semi-quantitative estimation of the phosphate solubilization ability of PSB have been possible using plate screening methods which show clearing zones around the microbial colonies in media containing insoluble mineral phosphates (mostly tricalcium phosphate or hydroxyapatite) as the single P source. But other researchers like Oliveira (2009) applied a new PSM screening method using a P-Mehlich 1 (Mehlich 1978) phosphorus extractor, and the solubilizing activities and ability were evaluated via this P-Mehlich 1 method.

The variation of pH value was influenced by PSB, and Rodríguez (1999) points out that the phosphohydrolases are clustered in acid or alkaline. The acid phosphohydrolases (several phosphatases), unlike alkaline phosphatases, showed optimal catalytic activity at acidic to neutral pH values. In this chapter we detected that the 3 strains all

possessed the function of alkaline phosphatases, because the soil sample plot in the Guizhou region is full of rainwater, and the pH value of soil there is below 7.00, and only producing alkaline can promote solubilization. Oliveira (2009) found that the *Burkholderia* sp. isolate had the largest reduction in pH in the growth solution to 4.46 at Sete Lagoas, Minas Gerais, Brazil. No correlation was found between pH and Al-P solubilization. This is different to the increase that happened in this study, though the environmental conditions between the two sites were similar regarding humidity but different on soil type and insoluble phosphate content. Chen (2006) found that the solubilization of TCP in the liquid medium of different PSB strains was accompanied by a significant drop in pH (to 4.9 and 6.0) from an initial pH of 6.8–7.0 after 72 h in subtropical soil of Taiwan.

On the aspect of secreting IAA, Afzal & Asghari (2008) found that better root development may be due to the synergistic relationship of the inoculated bacteria for improving root length and weight by producing growth regulators like IAA that, along with P fertilizer, favoured better root development, which enhanced water and nutrient uptake and ultimately helped better shoot development. Ahemad & Khan (2010) found that the *E. asburiae* strain PS2 produced a substantial amount of IAA both in the absence and presence of fungicides. In the medium untreated with fungicides, the *E. asburiae* strain PS2 produced a maximum (32 μg/mL) amount of IAA. However, the IAA released by the *E. asburiae* strain PS2 decreased progressively with an increase in the concentration of each fungicide.

Hameeda et al. (2008) used a strain of Gyaneshwar (1999) called *Enterobacter asburiae* PSI3 in which phosphate-solubilizing activity was used as a reference strain, displaying the long-term utilization of *Enterobacter* sp. genus as PSB. Castagno's study (2011) also identified the strains that showed a high P-solubilizing activity as *Pantoea eucalypti*, a novel species in terms of plant growth promoting rhizobacteria, and many more *Pantoea* sp. genus were studied from their original N-fixing application.

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CHAPTER THIRTY-FOUR

BIO-CONTROL POTENTIAL OF *TRICHODERMA* SPECIES AGAINST *ALTERNARIA BRASSICAE*

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Abstract

The *Alternaria* blight disease, caused by *Alternaria brassicae*, has been observed in different areas of India in recent years. Current biocontrol studies have confirmed the effectiveness of the *Trichoderma* species against many fungal phytopathogens. In this study, biocontrol effects of Trichoderma isolates were evaluated against the *Alternaria brassicae* pathogen. Two Trichoderma species *T. harzianum* and *T. virens* were extensively studied on the inhibition of the *Alternaria brassicae* pathogen of brassica disease. All isolates inhibited the mycelial growth of *Alternaria brassicae* that causes blight disease. Antifungal metabolite was extracted and tested against the mycelial growth of *A. brassicae* spores. The disease severity of blight treated with antifungal preparation extracted from Trichoderma was 20.2% and 29.5% when measured in laboratory conditions, while the severity in the control treatment was found to be 78.5% and 78.4%, respectively. Microscopic studies revealed hyphal coiling (hyperparasitism) of *T. harzianum* and *T. virens* around *A. brassicae* hyphae. Two concentrations of Culture Filterate of these Trichoderma species were tested against the Mycelial Growth of *Alternaria brassicae*. Two dual culture methods were applied to find the inhibition of mycelial growth. The highest inhibition of mycelial growth was 65.80% by *T. virens.* The use of antifungal metabolite was effective in laboratory conditions. The results showed that the mycelial growth of *A.*

brassicae was completely inhibited. Strains of *T. virens* and *T. harzianum* induced metabolic changes in plants that increase resistance to a wide range of plant-pathogenic microorganisms. The highest inhibition of mycelial growth was found to be 64.34% (*T. harzianum*) and 75.86% (*T. virens*), respectively. The results showed that the mycelial growth of *A. brassicae* was 9.4% compared to 98.5% for the control. Trichoderma species have a good antagonistic effect on *A. brassicae*.

Introduction

The present investigation deals with comparative antagonistic behaviour of two different species of *Trichoderma—Trichoderma harzianum* and *Trichoderma viride*—against *Alternaria brassicae*, a common and destructive pathogen of Brassica Biological. The management of plant disease is presently a popular method of disease control. Being environmentally safe and effective disease control agents, biocontrol agents are used widely by farmers in combatting disease and pests. The Trichoderma species have been reported as having the greatest potential as biocontrol agents (Lewis & Papavizas 1991; Haran et al. 1996; Elad 2000; Hermosa et al. 2000). Biocontrol agents are non-pathogenic plant-associated microbes that suppress disease or enhance plant growth. *Trichoderma harzianum* is a fungus, naturally found in soils or associated to other fungi in aerial portions of plants, which has proved to be an efficient biocontrol agent of fungal plant pathogens.

The biocontrol activity of *Trichoderma* on phytopathogens is accomplished by different mechanisms: (a) competition for space and nutrients in order to exclude the presence of the phytopathogen from a certain portion of soil or tissue; (b) mycoparasitism in order to use the metabolic machinery of the phytopathogen for its benefit; (c) production of antibiotics that interfere with cellular functions of the phytopathogen; (d) production of hydrolytic enzymes that degrade the cell wall of the phytopathogen. Therefore, by using one or more of these biocontrol mechanisms, *Trichoderma* interfere with the development of the phytopathogen, thus preventing or suppressing disease. Several products that include *T. harzianum* in their formulations have been developed. These could, at least in part, replace the use of chemicals for the control of phytopathogens preventing or decreasing the environmental pollution and toxic effects on animals and human beings of chemical pesticides. Nevertheless, the biocontrol activity of *Trichoderma* could be affected by other components used at the field level to improve crop productivity, such as plant nutrients and growth regulators. The effect of these growth

regulators on the biocontrol ability of *T. harzianum* was tested against *Alternaria alternata*, a known phytopathogen of several crops, using pure hormones (Indolacetic acid, IAA; Gibberelic acid, GA_3, and Benzylaminopurine [BAP]). This study was initiated to test the antagonistic effect between *A. brassicae* and Trichoderma. With a view to measuring the role of Trichoderma as a bio-control agent against the *A. brassicae* pathogen, this research is presented in this chapter.

Materials and Methods

Pathogens used

Alternaria brassicae was isolated from the infected leaf of brassica and cultured on Potato Dextrose Agar (PDA) medium. Ten day old culture of pathogen was used for each experiment (CSA Kanpur). For pathogen, the isolation was performed by a tissue transplanting technique modified from Agrios (1978). Leaf spot symptoms were cut into small pieces (0.5 × 0.5 cm), dipped in 2% sodium hypochlorite solution for 30 s and rinsed with sterilized water 3 times. The plant samples were dried on sterilized tissue paper before being transferred to a petri dish containing potato dextrose agar (PDA). The petri dish was enfolded with plastic wrap and incubated at room temperature for 2 d. The growing colony was sub-cultured in PDA slant before being kept at 10°C and used as a stock culture. Therefore, it was decided to study symptomatology, the morphology of the pathogen, epidemiology and the effect of the *Trichoderma* species on pathogen growth. Available brassica genotypes were also screened against the disease.

Trichoderma species used

The strains of fungal antagonists were tested and proved by CSA University. For *Trichoderma spp*, the upper 10 cm of the field planting soil was collected after removing the surface plant material. The soil samples were air dried for 2 d and sieved through a 2 mm mesh screen to remove coarse debris. Ten grams of each sieved sample were added to a 250 ml flask containing 90 ml of sterilized water and mixed in a shaker at 1,000 rpm for 30 min. The soil suspension was then diluted 10^{-2} and 10^{-3}, or at the appropriate concentration. 0.1 ml of the diluted solution was dropped onto the surface of Martin's agar in a petri dish. The soil suspension was spread on the surface of the medium with a sterile glass rod. The dishes were sealed with plastic wrap and incubated at room

temperature (27 ±2°C) until the result was measured. Two species of *Trichoderma*, namely *T. harzianum* and *T. virens*, were isolated from the different soil samples, and cultured on culture medium. Five day old culture of *Trichoderma* was used for each experiment. The colony of the pathogen on potato dextrose agar was grey to black conidiphores that were simple or branched, brown, septate and 38 to 46 mm long and 2.4 to 6.5 mm thick. Conidia were formed in branched chains, light brown to dark brown, smooth and muriform, with 1 to 4 transverse and 2 to 3 longitudinal septa and measuring 11.25–31.5 × 4.5–13.5 mm and beak 3.60–9.00 × 12–18 mm.

Collection of culture filtrate of *Trichoderma spp.*

To harvest the filtrate of two *Trichoderma* species, potato dextrose broth was used in a conical flask and *Trichoderma* was cultured for 20 d at 25°C on a rotary shaker at 140~150 rpm, from which filtrate were harvested. To collect the filtrate, the liquid cultures were filtered through two layers of Whatmann no. 1 filter paper to remove hyphal fragments. The filtrates were used for incorporation into PDA and PDB separately. Thus, the samples were ready for further use.

Mycelial growth

The filtrate of two *Trichoderma* species was mixed with PDA and PDB separately to give 25%, 50% and 75% concentrations (v/v) of each. After autoclaving, PDA mixtures were poured into sterilized petri dishes. Mycelial Growth readings were recorded after 10 d of incubation. The experiment was replicated 5 times and mycelial growth was measured (1.5 μ) and kept at 25 °C in a moisture chamber for 24 h. For control, mycelial growth CG was counted in fresh PDA and PDB, respectively. The two readings A1 (control) A2 (treated) of mycelial growth were transformed into percentage inhibition of mycelial growth (PIMG) using the formula

$$PIMG = (A1-A2)/A2*100$$

Dual culture method

Two parameters were observed in this test of percentage inhibition of mycelial growth, with the number of the day taken for the antagonist's total overgrowth of the *A. brassicae* colony on PDA. For each of the two *Trichoderma* species, a 5 mm agar disc was taken from 5 d old culture and

placed at the periphery of the 90 mm culture plates. Then, another agar similar sized disc of *A. brassicae* was placed at the periphery but on the opposing end of the same petri dishes. For the control, *A. brassicae* was placed in a similar manner on fresh PDA. All pairings were carried out 5 times and incubated at 25°C. Antagonistic activity was assessed after 5 d of incubation by measuring the radius of the *A. brassicae* colony in the direction of the antagonistic colony (R2) and the radius of the *A. brassicae* colony in the control plate (R1). The two readings were transformed in to percentage inhibition of mycelial growth using the formula. The number of days taken for the antagonist to overgrow the whole colony of *A. brassicae* was recorded. Observation was continued on the dual culture plates after 5 d of incubation, when mycelial extensions of *A. brassicae* were measured and followed by calculation of percentage inhibition of myceIial growth. The colony overgrowth time was recorded for each and every day, even for the *Trichoderma* that took the longest time to overgrow the *A. brassicae* colony.

To test whether there was significant variation in screening methods with respect to the disc culture placement, two methods were carried out using two species of *Trichoderma*. The first method was as described earlier. In the second method, a 5 mm agar plug of the antagonist *Trichoderma* was placed 2 cm away from the periphery of the petri dish, and an agar plug of the same size test fungus *A. brassicae* was similarly placed 2 cm away from the edge of the petriplate, but opposite to *Trichoderma*. The plates were incubated in the same manner as for method 1. For the control, a single agar plug the same size as *A. brassicae* was placed in a similar manner on a fresh PDA plate, but without *Trichoderma*. The radius of *A. brassicae* mycelium R1 and R2 was recorded and percentage inhibition of mycelial growth determined after 5 d of incubation using the same formula.

Results and Discussion

The three concentrations 25%, 50% and 75% of filtrate of two *Trichoderma* species were tested against the mycelial growth of *A. brassicae*. The highest percentage inhibition growth was found to be 71.43 at 75% filtrate of *T. virens*. The lowest percentage inhibition growth was found to be 9.52 at 25% filtrate of *T. virens*. *T. harzianum* showed moderate inhibition and mycelial growth increased with the increase of filtrate concentration in every case. Among two *Trichoderma* species, filtrate of *Trichoderma* was diffusible as well as antifungal, which inhibited the mycelial growth of *A. brassicae* which was very pronounced

compared to the control plate (see Table 34.1 below). The metabolites of *Trichoderma* could influence the outcome of the decay caused by basidiomycetes in freshly felled pine. Magnus & Dickson (1996), Dennis & Webster (1971) and Jinantara (1995) showed that culture filtrate produced by *Trichoderma* contained inhibitory substances against microorganisms. Among the antibiotics produced by *T. harzianum* were pyridine, anthrequinoesfu, butenolides, isonitrin D and F trichorzianines and ranone (Claydon et al. 1987; Ordentlich et al. 1992). *T. virens* was also shown to produce several other antibiotics such as gliotoxin, gliovirin and valinotricin (Taylor 1986). Two dual culture methods were applied where the percentage of inhibition in mycelial growth (73.77) occurred for *T. harzianum* and *T. pseudokoningii*. In method 2 the highest percentage of inhibition in mycelial growth was 75.54 (PDA) for *T. virens*. The number of colony overgrowth per day was recorded as the lowest (8) in metho-1 for *T. harzianum* and the highest (11) in methods-2 for *T. pseudokoningii* and *T. virens*, and took 9 d to overgrow the colony of *A. brassicae* in both methods (see Table 34.1 below). Jun & Kim (2004) reported that the antifungal activity of *T. virens* and *T. harzianum* to *Pythium spp*. was stronger than that of *T. koningii*. Dharmaputra et al. (1994) tested two isolates of *T. harzianum* and one isolate of *T. viride* against three isolates of *Ganoderma* from oil palms. All three *Trichoderma* isolates inhibited the mycelial growth of the pathogen, but *T. harzanium* showed the best performance among the three isolates. The first screening was to select the most active antagonist against that particular pathogen before a species or a particular isolate of *Trichoderma* could be considered a biocontrol agent. Roinger & Jeffers (1991) and Jinantara (1995) reported that the three isolates of *T. harzianum* possessed different abilities to attack *Sclerotium rolfsii*, and this was in agreement with Henis et al. (1983), who found that different isolates of *T. harzianum* could parasitize sclerotia of *S. rolfsii* at varying percentage inhibition.

Table 34.1. Antimicrobial activity of *Trichoderma* sp.

***Trichoderma* sp used**	**Liquid culture Filtrate**	**Mycelial Growth (mm)**	**Percentage Inhibition Growth**
T. harzianum	30	50	16.45
	50	35	44.70
	75	26	60.00
T. virens	30	45	20.26
Control	50	38	55.67
	75	15	66.00
			65.7(on PDA)

Conclusion

It was confirmed by this experiment that the filtrate produced by two *Trichoderma* species was diffusible and could prevent or inhibit the mycelial growth of *A. brassicae*. Therefore, *Trichoderma* has a large potential effect as a biocontrol agent against the *A. brassicae* pathogen of early blight.

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CHAPTER THIRTY-FIVE

ABOVEGROUND APHID FEEDING ATTRACTS BELOWGROUND PLANT GROWTH-PROMOTING RHIZOBACTERIA AND PRIMES PLANT IMMUNITY IN PEPPER

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Abstract

Plants modulate defence signalling networks in response to different biotic stresses. The present study evaluates the effect of a phloem-sucking aphid on plant defence mechanisms during subsequent pathogen attacks on leaves and that of rhizosphere bacteria on roots. Plants were pretreated with aphids and/or the chemical trigger benzothiadiazol (BTH) 7 days before being challenged with two pathogenic bacteria, *Xanthomonas axonopodis* pv. vesicatoria (*Xav*) as a compatible pathogen and *X. axonopodis* pv. glycines (*Xag*) as an incompatible (nonhost) pathogen. Disease severity was noticeably lower in aphid- and BTH + aphid-treated plants than in the controls. Although treatment with BTH or aphids alone did not affect the hypersensitive response (HR) against Xag 8ra, the combination treatment had a synergistic effect on the HR. The aphid population was reduced by BTH pretreatment and by combination treatment with BTH and bacterial pathogens in a synergistic manner. Analysis of the expression of the defence-related genes *Capsicum annum pathogenesis-related gene 9* (*CaPR9*), c*hitinase 2* (*CaCHI2*), *SAR8.2* and *Iipoxygenase1* (*CaLOX1*) revealed that aphid infestation resulted in the priming of the systemic defence responses against compatible and incompatible pathogens. Conversely, pre-challenge with the compatible

pathogen *Xav* on pepper leaves significantly reduced aphid numbers. Aphid infestation increased the population of the beneficial *Bacillus subtilis* GB03 but reduced that of the pathogenic *Ralstonia solanacearum* SL1931. The expression of defence-related genes in the root and leaf after aphid feeding indicated that the aboveground aphid infestation elicited salicylic acid and jasmonic acid signalling throughout the whole plant. The findings of this study show that aphid feeding elicits plant resistance responses and attracts beneficial bacterial populations to help the plant cope with subsequent pathogen attacks.

Introduction

Plants have survived diverse biotic and abiotic stresses by mounting defence mechanisms (Pieterse et al. 2009) in nature. To overcome biotic stresses caused by the attack of pathogens and herbivorous insects, the development of a more specific, targeted resistance machinery is required (Smith et al. 2009). Among the mechanisms of plant resistance, induced resistance was studied intensively due to its similarity with innate animal immunity, which is different from the so-called constitutive resistance (Pieterse et al. 2009). Plant immunity can be induced in response to feeding by herbivores or infection by pathogens and is mainly regulated by three signalling molecules—salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)—which are interconnected by complex signalling networks and crosstalk phenomena (Pieterse et al, 2009). Generally, JA-mediated responses are directed against herbivores and necrotrophic pathogens, whereas SA-mediated systemic acquired resistance (SAR) responses are active against biotrophic pathogens (Bostock 2005; Heil & Bostock 2002).

In recent studies, resistance was induced aboveground (AG) by whitefly infestation to study the biological effects on both leaf and root belowground (BG) infecting bacterial pathogens. The induction of systemic resistance was confirmed by the significant up-regulation of the SA and JA defence signalling pathway marker genes, *Capsicum annuum pathogenesis-related protein* (*CaPR*)*1*, *CaPR4*, *CaPR10* and *Ca protease inhibitor* (*CaPIN*) in both leaves (AG) and roots (BG) after whitefly feeding. Interestingly, AG whitefly feeding significantly increased the population density of beneficial BG microflora, including gram-positive bacteria, actinomycetes and saprophytic fungi that may induce systemic resistance (Yang et al. 2011). Among BG microbial groups, several gram-positive *Bacillus sp*. strains significantly elicited plant systemic defences against the whitefly population in the tomato field (Murphy et al. 2000).

In the present work we provide new evidence that aphids, which are similar sucking insects to the whitefly, increase plant systemic immunity against the biotrophic bacterial pathogen *Xanthomonas axonopodis*. Furthermore, because both biotrophic pathogens and aphids are known to induce SA signalling, we evaluated whether pre-challenge with compatible and incompatible pathovars of *X. axonopodis* increased plant resistance to aphid feeding in pepper. The activation of signal transduction pathways by aphid infestation was investigated by assessing the transcriptional expression of pepper marker genes for SA and JA after aphid feeding. Conversely, pre-challenge with the compatible pathogen *Xav* on pepper leaves significantly reduced aphid numbers. The bacterial populations of the beneficial plant growth-promoting rhizobacteria *Bacillus subtilis* GB03, the saprophyte *Pseudomonas fluorescens* Pf-5, and the pathogenic *Ralstonia solanacearum* SL1931 were evaluated in the roots after aphid infestation in the leaf (Haas & Defago 2005; Kloepper et al. 2004). Our studies provide a new understanding of tritrophic (insect-plant-beneficial root bacteria) interactions and their role in the induction of defence mechanisms.

Materials and Methods

Plant preparation and disease assay

Pepper (*Capsicum annuum* L. cv. Bukang) was used as the study plant because it interacts with multiple enemies and mutualists representing different guilds, and because the availability of genetic tools allows for the analysis of gene expression patterns under different conditions. The seeds of *C. annuum* were surface-sterilized with 6% sodium hypochlorite, washed four times with sterile distilled water (SDW), and then maintained at 25ºC for 3 d until germination on Murashige and Skoog medium (Duchefa, Haarlem, the Netherlands). The germinated seeds were then planted on soilless media (Punong Horiculture Nursery Media LOW, Punong CO. LTD, Gyeongju, Korea). Plants were grown at 25 ± 2ºC under fluorescent light (12 h/12 h day/night cycle, c. 7000 lx light intensity) in a controlled-environment growth room for seeding growth and transferred to the KRIBB greenhouse facility in Daejeon, South Korea for aphid treatment. Two-week-old pepper plants were drenched with either 10 mL of a solution of 0.5 mM benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (benzothiadiazole = BTH) (Syngenta, Research Triangle Park, NC, USA) or sterile water. At the same time, the aphid *Myzus persicae*, a naturally occurring insect in the greenhouse in

Daejeon, South Korea, in 2010–2011 (referred to as "green peach aphid") was treated as the biological inducer. The aphid was maintained on pepper plants. Cross-phyla-induced plant immunity against bacteria or aphids was investigated by using *Xanthomonas axonopodis* pv. vesicatoria (Yang et al. 2009), a causal pathogen of bacterial leaf spot disease, as a compatible pathogen, and *X. axonopodis* pv. glycines, a causal pathogen in the leaves of soybean (Lee et al. 2004), as an incompatible pathogen. One week after aphid, BTH and BTH + aphid treatments, all plants were inoculated with *X. axonopodis* pv. vesicatoria and *X. axonopodis* pv. glycines on Luria-Bertani (LB, Duchefa, Haarlem, the Netherlands) agar. For experimental use, bacteria were scraped from plates and re-suspended in sterile water.

Quantitative RT-PCR

Molecular evidence for aphid-elicited expression of bacterial resistance-related genes in pepper was obtained using qRT-PCR. The relative mRNA expression of *CaCHI2*, *CaPR9* and *CaLOX1*, which are expressed during incompatible pathogen/chemical-elicited SAR and PGPR-elicited ISR, was measured in the leaves and roots (Yang et al. 2009; Yi et al. 2009). Total RNA was isolated from leaf and root tissues treated with aphids, water, BTH + aphids and BTH one week after each treatment using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions.

Results and Discussion

Induction of plant immunity against bacterial pathogens by aphid infestation

Assessment of disease resistance against the compatible pathogen *X. axonopodis* pv. vesicatoria, which is a bacterial spot pathogen of pepper, showed reduced disease symptoms after two bacterial challenges in aphid- and BTH-treated plants compared to water controls. Water control plants developed severe necrosis 7 d after pathogen challenge on leaves, while plants treated with aphids, BTH or BTH + aphids did not show any visible symptoms. Statistical analysis of disease severity revealed a significant level of plant immunity induced by aphid feeding. *Post hoc* analysis revealed that the greatest resistance against *X. axonopodis* pv. vesicatoria was elicited by aphid alone and BTH + aphid treatment, indicating an additive effect of these treatments. The quantification of bacteria showed similar patterns (data not shown).

Control plant leaves challenged with the pathogen developed significant necrosis within 36 h. By contrast, all three resistance-induction treatments (aphid, BTH and aphid + BTH) caused a significant reduction in the HR index compared to controls. Although no significant differences were observed among the individual treatments, aphid + BTH treatment reduced the HR index to 50% of the control-treated plants.

Induction of salicylic acid- and jasmonic acid-related genes during aphid-elicited plant immunity

To investigate the possible activation of a whitefly infestation mediated plant defence signalling pathway conferring resistance against bacterial pathogens, we employed qRT-PCR. Increased expression of *CaPR9*, *CaCHI2* and *CaLOX1* under incompatible pathogen-induced SAR conditions, and in response to treatment with defence signalling molecules such as SA, JA, ET and abscisic acid, was previously reported (Yang et al. 2009; Yi et al. 2009). The transcriptional expression of both *CaPR9* and *CaLOX1* was significantly up-regulated in *Xav*- and *Xag*- treated aphid-infested pepper leaves. These results suggest that AG feeding by aphids elicited SA and JA/ethylene (ET) dependent defence signalling pathways. BTH only induced the transcription of *CaLOX1* in the leaves challenged by *Xav*. However, the BTH + aphid combination treatment had a synergistic effect on the activation of *CaPR9* in response to *Xav* and *Xag* infiltration, and of *CaCHI2* in response to *Xag* infiltration in the AG parts of the plants. In contrast, under the same conditions the ET response genes *CaCHI2* and JA response *CaLOX1* were significantly repressed compared with BTH or aphid treatment alone. Assessment of the early responses to aphid treatment showed that the transcriptional expression of *CaSAR8.2* and *CaLOX1* genes in aphid-infested pepper leaves increased 2.98- and 3.5-fold compared to control treatment on day 1, while no changes in *CaSAR8.2* and decreased *CaLOX1* expression were detected on day 3. In the root, the *CaLOX1* gene was induced 2.3-fold by aphid treatment on day 1, while no changes were observed on day 3. In contrast, the mRNA level of *CaSAR8.2* in the root did not change on day 1, while a 4-fold increase was detected on day 3 compared to the control treatment.

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CHAPTER THIRTY-SIX

APPLICATION OF BACTERIAL VOLATILES IN PEPPER ROOTS PROMPTS INDUCED RESISTANCE UNDER FIELD CONDITIONS

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Abstract

Bacillus amyloliquefaciens strain IN937a, previously known to thrive inside plant tissues and referred to as an endophyte, was reported to stimulate plant growth and induce systemic resistance (ISR) via its emission of volatile organic compound (VOC). We investigated ISR capacity of the VOCs collected from the strain IN937a against bacterial spot disease caused by *Xanthomonas axonopodis* pv. *vesicatoria* on pepper in the greenhouse. Among 18 bacterial VOCs, 3-pentanol was selected for further experiment. The 3-pentanol was drenched onto the four-week-old pepper before being transplanted to the field. Disease severity was assessed at seven days after pathogen challenge when infiltrated in the pepper leaves at 10, 20, 30 and 40 days post-transplant (dpt). The 3-pentanol treatment significantly increased ISR compared to control treatment at 30 dpt. Taken together, bacterial VOC 3-pentanol can be utilized as a trigger of ISR against a broad spectrum of pathogens under field conditions.

Introduction

In 2003 volatile organic compounds (VOCs) emitted by two *Bacillus* spp. were established as the primary determinants of both plant growth promotion and elicitation of ISR in *Arabidopsis* (Ryu et al. 2003; Lucy et al. 2004; Ryu et al. 2004). Ryu et al. (2003) demonstrated the significant growth promotion of *Arabidopsis* by *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a. Among the VOCs from strain GB03, 2,3-butanediol was the major compound that promoted plant growth and ISR against *Erwinia carotovora* subsp. carotovora. Several mutant lines of *Arabidopsis*, including brassinosteroid- and gibberellic acid-insensitive mutants, as well as auxin-transport-deficient and cytokinin receptor-deficient mutants, were used to elucidate the signalling pathways that promote growth. The tested VOCs did not promote plant growth in *cre1*, a cytokinin receptor-deficient mutant, suggesting that cytokinin signalling is essential for the promotion of plant growth in response to bacterial volatiles (Ryu et al. 2004). Furthermore, a study using The Affymetrix *Arabidopsis* AG GeneChip revealed that volatile emissions from strain GB03 differentially up-regulated more than 600 transcripts, which encoded proteins with various functions, such as cell-wall modification, primary and secondary metabolism, stress responses, and hormone regulation (Zhang et al. 2007). Exposure of *Arabidopsis* to VOCs from strains GB03 and IN937a also resulted in a significant reduction in the severity of disease caused by *Erwinia carotovora* subsp. *carotovora*. The signalling pathway was found to be ethylene dependent but salicylic acid (SA) independent, using *PDF1.2* and *Jin14* indicator genes for ethylene/jasmonate and jasmonate signalling, respectively (Ryu et al. 2004).

In this study we assessed the effect of bacterial volatiles from different bacterial species under greenhouse and field conditions. Through 18 bacterial volatiles we evaluated ISR capacity against bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* in pepper in the greenhouse. From the pre-screening, 3-pentanol was selected and drench-applied into pepper seedlings under field conditions. The ISR capacity of 3-pentanol was measured by symptom development following leaf-infiltration of bacterial suspension and naturally occurring disease symptoms, including bacterial spot and *Cucumber mosaic virus*.

Materials and Methods

Induced resistance against X. *axonopodis* pv. *vesicatoria*

To screen a promising candidate of bacterial volatiles, 18 bacterial volatiles that reported the production from *Bacillus subtilis* strain GB03, *B. amyloliqefaciens* IN937a, and *P. fluorescens* strain 89B61 (Farag et al. 2006). The 5 ml of 18 bacterial volatiles were drench-applied in the crown part of 3-week-old pepper seedling at 10 μM and 100 nM. Bacterial suspensions of 10^6 cfu/ml *X. axonopodis* pv. *vesicatoria* were forced to penetrate pepper leaf backsides using the needleless syringe method at 7 d after volatile application in the greenhouse. The disease severity was measured at 7 d after pathogen challenge. For the ISR test in the field, 10 d after dipping application of BTH and 10 μM 3-pentanol into 6 week old pepper seedlings before transplanting, *X. axonopodis* pv. *vesicatoria* was challenged with the same method as described above. Seven days after the pathogen challenge, the severity of symptoms on the leaf was scored from 0–5, where 0 = no symptoms, 1 = mild chlorosis, 2 = chlorosis only, 3 = chlorosis and mild necrosis, 4 = necrosis, and 5 = severe necrosis of the inoculated area. The experiment was repeated 4 times with 10 replications (one plant per replication).

Statistical analysis

The analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC). Significant effects of treatment were determined by the magnitude of the *F* value ($P = 0.05$). When a significant *F* test was obtained, separation of means was accomplished by Fisher's protected LSD at $P = 0.05$.

Results and Discussion

In this study, we tested 18 bacterial volatiles previously identified from different genera including pseudomond and bacilli. To screen any promising volatile candidate we infiltrated bacterial suspension at relatively high concentrations, such as 10^6 cfu/ml *X. axonopodis* pv. *vesicatoria*. Such high disease pressure allowed us to select a strong candidate to apply ISR capacity of bacterial volatile in the field. Among 18 volatiles, only 3-pentanol treatment showed significant induction of systemic resistance against bacterial spot in the greenhouse.

Furthermore, 3-pentanol did not affect plant growth (data not shown). Normally, the strong induction of systemic acquired resistance by chemical inducer or avirulent pathogen treatments resulted in the reduction of plant growth referred to as "allocation fitness cost" (Heil & Baldwin 2004). Based on the consistent results of 3-pentanol in the greenhouse, 10 μM 3-pentanol was finally selected for further field trials. The pre-treatment of 3-pentanol elicited ISR against a subsequent challenge of *X. axonopodis* pv. *vesicatoria* at 10, 20, 30 and 40 d after 3-pentanol treatment (data not shown). The pathogen population in the plant treated with 3-pentanol was significantly reduced compared to control. At the end of the season, bacterial spot and abnormal virus symptoms broke out across the field. Further identification revealed *X. axonopodis* pv. *vesicatoria* for the bacterial spot and *Cucumber mosaic virus* for virus symptoms. Interestingly, typical CMV symptoms appeared at a significantly lower frequency in the 3-pentanol treated plants. Our results suggest that 3-pentanol will be a strong candidate for a chemical trigger of ISR against bacterial and viral diseases under field conditions.

Regarding the 18 bacterial VOCs with two concentrations (10 μM and 100 nM), as a positive control plants were treated with 0.5 mM BTH. Disease severity (0–5) was measured 7 d after pathogen challenge. "*" indicates statistically significant differences (P=0.05) compared to the control. Data represents the mean and the standard error of the mean.

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CHAPTER THIRTY-SEVEN

ELICITATION OF PLANT SELF-DEFENCE BY VOLATILE ORGANIC COMPOUNDS AGAINST BOTH A BACTERIA PATHOGEN AND AN INSECT PEST IN THE CUCUMBER FIELD

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Abstract

Systemic acquired resistance (SAR) is a plant self-defence mechanism against broad-ranged pathogens and insect pests. Among chemical SAR triggers, plant and bacterial volatiles are promising candidates due to highly effective and cheap chemicals with relatively low concentrations related to agrochemicals. However, in order use them for agriculture, the high evaporation rate after application, plant growth alteration and inconsistent effectiveness are major pitfalls that need to be considered before large-scale application to manage diseases. In this study, we provide new evidence of volatile organic compound (VOC)-mediated SAR against both a bacterial angular leaf spot pathogen *Pseudononas syringae* pv. lachrymans and a sucking insect aphid *Myzus persicae* in the open field without changing plant growth until harvesting the fruit yields. Unexpectedly, the drench of two VOCs—3-pentanol and 2-butanone—into cucumber seedlings caused a significant increase in the number of the ladybird beetle, known to be a natural enemy of aphids. The defence-related gene *CsLOX* was induced in volatile treatments to trigger an oxylipin pathway responding to the emission of green leaf volatile to recruiting natural enemies. Our results demonstrate that VOCs are good

resources to prevent plant diseases and insect damage by elicitation of SAR even in the open field.

Introduction

Induced resistance is an innate plant resistance to a variety of plant enemies, including insect and microbial pathogens (Schneider et al. 1996). Until now, two types of induced resistance have been indicated: systemic acquired resistance (SAR) and induced systemic resistance (ISR). First, systemic acquired resistance (SAR) was discovered by Ross, who found plant systemic induced resistance while studying virus-plant interactions (Ross 1991). In addition to microbe-elicited SAR, later studies revealed that plant and microbe-derived chemical compounds also trigger induced resistance. Scientists also called this type of induced resistance SAR. Such compounds include chitin, ergosterol, glucans, lipopolysaccharides, proteins, peptides, salicylic acid and sphingolipids. Secondly, plant growth-promoting rhizobacteria (PGPR) are a certain group of root-associated bacteria (rhizobacteria) and confer enhanced plant growth and yield under greenhouse and field conditions, eliciting induced resistance referred to as ISR.

In the present study, we attempted to identify effective VOCs with the capacity to elicit SAR for the open field application of insect and bacterial volatiles to control plant pathogens and insects as well as minimize the negative effects of plant growth. Due to the ineffectiveness of a bacterial volatile 2,3-butanediol on induced resistance in cucumber plants, there is a demand to isolate effective VOCs from a greater number of known bacterial volatiles even under open conditions. Here, we provide new evidence on VOC-elicited SAR against both a microbial pathogen and an insect pest at the same time. The drench application of 1 mM 3-pentanol and 0.1 μM 2-butanone on cucumber seedlings consistently triggered plant systemic defence against *Pseudomonas syringae* pv. lachrymans in cucumber. Examination of plant defence responses after VOC treatments revealed the upregulated expression of the *CsLOX1* gene. *CsLOX1*, a cucumber lypoxygenase, is a marker protein of oxylipin pathway for indirect plant defence that releases green leaf volatile to attract natural enemies, protecting the plant from herbivores (Dicke & Baldwin 2010). In conducting experiments, a natural outbreak of aphids occurred. The cucumber applied with VOCs achieved a higher number of ladybird beetles and led to a significant reduction of aphid density than water control. Our results newly indicate that VOCs can be used to manage plant

disease and insect pests through elicitation of induced resistance even in the open field.

Materials and Methods

Plant and bacteria preparation and greenhouse experiment

Cucumber plants (*Cucumis sativus* L. cv. backdadagi) were cultivated in the open field under natural conditions. The seeds were directly seeded into soilless medium (Punong Co. Ltd, Gyeongju, Korea). The germinated seeds were transplanted into large pots (d = 30 cm; height = 30 cm). Chemical treatment eliciting induced resistance in cucumber was carried out as previously described (Lee et al. 2012). Cucumber seedlings were treated by direct drench-application of 50 mL of 1 mM and 10 μM 3-pentanol and 0.1 μM and 10 nM 2-butanone 14 days after seeding (dps). Treatments with 0.5 mM BTH and water were used as positive and negative controls, respectively.

Assessment of angular leaf spot disease and aphid infestation

For pathogen challenge, a culture of the compatible bacterial pathogen *Pseudomonas syringae* pv. lachrymans (OD_{600} = 1 in 10 mM $MgCl_2$) was spray challenged on the cucumber leaves until drop-out at 7 d after drench-application of two chemicals on the cucumber roots 21 d after seeding. The severity of symptoms was scored 0–5 as follows: 0: no symptoms; 1: below 20% diseased area; 2: 21–40 diseased area; 3: 41–60 diseased area; 4: 61–80 % diseased area; and 5 above 81% diseased area of the whole leaf. Bacterial pathogens were cultured overnight at 28°C in King's B medium supplemented with the appropriate antibiotics (100 μg/ml). Chemical treatment on cucumber roots was performed as described previously (Lee et al. 2012). As a positive control, roots were treated with 0.5 mM benzothiadiazole (BTH). Intact cucumber leaves were used for non-stress treatments. Following inoculation with the pathogen, plants were returned to the growth chamber and leaf tissue was harvested 0 and 6 h post-inoculation with *P. syringae* pv. lachrymans. The experiment had a completely randomized design with 10 replications and was independently repeated four times. To investigate whether the two VOCs elicit plant immunity to aphid feeding, we counted aphid numbers that naturally occurred in 2012, Daejeon, S. Korea. 0.5 mM BTH was used as a positive control. The total number of nymph and adult aphids was counted at 34 d after seeding. The experiments were repeated with similar results.

Results and Discussion

3-Pentanol and 2-butanone-elicited SAR against P. syringae and aphid

Drench application of 3-pentanol and 2-butanone resulted in reduction of disease severity at 28 days post seeding (dps) 7 d after the spray-challenge of *P. syringae* pv. lachrymans. Disease severity of cucumber plants treated with 1 mM 3-pentanol, 0.1 μM 2-butanone, and 10 nM 2-butanone caused 24%, 26% and 17% less symptoms than water control, respectively. 10 μM M 3-pentanol application did not show any statistical (P = 0.05) difference. BTH as a positive control displayed a similar level to that of 0.1 μM 2-butanone.

Among many bacterial metabolites, bacterial volatiles have reported candidates for induced resistance against diverse plant pathogens. The representative example is a bacterial volatile 2,3-butanediol. Previous work demonstrated that 2,3-butanediol produced *Bacillus* spp. and *P. chlororaphis* induced systemic plant defences against *Pectobacterium carotovorum* subsp. *carotovorum* in Arabidopsis and tobacco (Ryu et al 2004; Han et al 2006). *P. chlororaphis* failed to elicit ISR against *P. syringae* pv. tabaci in tobacco (Han et al. 2006). Thus, the authors concluded that 2,3-butanediol was not effective against biotrophic pathogens but was against necrtrophic pathogens. However, recent reports provided controversial evidence on such effectiveness, because 2,3-butanediol protected Arabidopsis seedlings against a biotrophic pathogen *P. syringae* pv. tomato (Rudrappa et al. 2010). The 2,3-butanediol may mediate induced resistance depending on the plant species.

In applying a large-scale trial of VOCs on the open field, the weak point was the inconsistent results depending on plant species and rapid evaporation rate after treatments. The field trials with 2,3-butanediol on tobacco revealed the successful reduction of symptom development (Cortes-Barco et al. 2010ab). Our preliminary study obtained that 2,3-butanediol was rarely effective on cucumber plants of biotrophic pathogen *P. syringae* pv. lachrymans (data not shown). The further screening of VOC-mediated SAR on the same pathogen allowed the selection of two new candidates: 3-pentanol and 2-butanone. The two volatiles did not report SAR capacity on any plant species. In our laboratory, root application of 3-pentanol also elicited SAR against *Xanthomonas axonopodis* pv. vesicatoria in pepper (unpublished data). For 3-pentanol-mediated SAR, the concentration (1 mM) was relatively higher than that of 2-butanone, but a similar level to BTH.

Unexpectedly, at mid-summer in 2011, an outbreak of aphids occurred in cucumber in the Daejeon area, South Korea, resulting in the cucumber plants being severely infested, especially on the newly developing leaf tissues. We measured aphid damage by counting number of aphids (individual nymph and adult) per leaf. At 34 dps, the number significantly decreased in all treatments compared to control treatment. The control treatment showed 361 nymphs and 19 adults per leaf. Soil drench of 1 mM and 10 10 μM 3-pentanol and 0.1 μM and 10 nM 2-butanone exhibited 21, 34, 25 and 112 nymphs and 1.0, 3.0, 0.1, and 2.7 adults, respectively.

3-Pentanol and 2-butanone cause an increase in ladybirds

When we counted aphids on the cucumber leaves at each time point, the seven spotted ladybird beetles were differently detected depending on each treatment. The mean number on the cucumber leaf treated with 1 mM 3-pentanol, 0.1 μM 2-butanone, and 10 nM 2-butanone were 7.2, 7.1 and 7.0, respectively, while the control plant was detected to be 2.8. The two VOC and BTH treatments are statistically significant lower numbers compared to control treatment. Only 10 μM 3-pentanol applications did not show ($P = 0.05$) any different treatments. BTH was ineffective on the reduction of ladybird numbers compared to control. The ladybird beetle is the best known natural enemy to insect pests including aphids. In the closed system, such as greenhouse conditions and occasionally the open field, the natural enemy has often been used to control insect pests in crop plants (van Lenteren 2000). For instance, the predatory generalist ladybird beetle has provided biological control of aphids (*Marcosiphulm euphorbiae*) on roses grown in the greenhouse (Snyder et al. 2004). In this system, ladybird beetles successfully reduced aphid populations during an outbreak without altering density of a specialist parasitoid ratio, indicating minimization of ecological disruption by affecting intraguild predation.

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CHAPTER THIRTY-EIGHT

INDUCED SYSTEMIC RESISTANCE AGAINST *PSEUDOMONAS SYRINGAE* PV. *MACULICOLA* BY A LONG CHAIN BACTERIAL VOLATILE EMITTED FROM *PAENIBACILLUS POLYMYXA* IN *ARABIDOPSIS THALIANA*

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Abstract

Some strains of plant growth-promoting rhizobacteria (PGPR) elicit induced systemic resistance (ISR). Previously, volatile organic compounds (VOCs), including acetoin and 2,3-butanediol, emitted from PGPR were identified as bacterial determinants of ISR. We screened for ISR using a microtiter plate and I-plate bioassay, in which seedlings were challenged with the pathogen *Pseudomonas syringae* pv. maculicola ES4326 in the presence of bacterial volatiles, to investigate the induction of ISR signalling by the VOCs emitted from the reference strain, GB03, and test strain, *Paenibacillus polymyxa* E681 which showed strong capacity on ISR and plant growth promotion under *in vitro* and field conditions. To identify the plant signalling pathways involved, we screened *Arabidopsis* plants transformed with *PR1*::GUS and *PDF1.2*: GUS, indicators of

salicylic acid and ethylene signalling, respectively. GB03 elicited ISR via ethylene-dependent signalling, as indicated by elevated *PDF1.2* expression in exposed seedlings, while E681 increased SA-dependent signalling, as indicated by elevated *PR1* expression, suggesting that E681 and GB03 strains activate different signalling transduction pathways. The efficacy of induction was also strain-specific, with stronger protection against *P. syringae* in plants exposed to VOCs released from E681 versus plants exposed to GB03. Among more than thirty low molecular weight volatile compounds were identified, including methanethiol, isoprene and an acetic acid-butyl ester, hexadecane, and a C16 hydrocarbon was found to be released exclusively from strain E681 that can prime transcriptional levels of the *PR1* defence gene. These results provide the first evidence for the existence of a novel E681 signal molecule that can serve as a bacterial determinant in ISR against *P. syringae*.

Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of root-colonizing bacteria in the rhizosphere of many plant species that exert beneficial effects on plants, such as enhancing plant productivity and eliciting induced systemic resistance (ISR) against multiple plant pathogens (Kloepper & Metting 1992; Ryu et al. 2004a; 2006). These PGPR have been found to produce many bacterial determinants that promote plant growth and ISR (Van Loon et al. 1998; Kloepper et al. 2004). Ryu et al. (2003) demonstrated the significant growth promotion of *Arabidopsis* by the volatile organic compounds (VOCs) emission of *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a. Exposure of *Arabidopsis* to VOCs from strains GB03 and IN937a resulted in a significant reduction in the severity of diseases caused by *Erwinia carotovora* subsp. *carotovora*. The signalling pathway was found to be ethylene-dependent but also salicylic acid (SA)-independent, using *PDF1.2* and *Jin14*, indicator genes for ethylene/jasmonate and jasmonate signalling, respectively (Ryu et al. 2004b). Using the *PR-1* indicator gene, no role was found for SA signalling in volatiles from strain GB03 during ISR. An analysis of the bacterial volatiles produced by strain GB03 indicated that 2,3-butanediol elicits plant growth and ISR in a dose-dependent manner.

Here, our objective was to evaluate whether VOCs from *Paenibacillus polymyxa* strain E681, that reported plant growth promotion and induced systemic resistance in several crops (Ryu et al. 2005ab), can also promote induced resistance against *P. syringae* pv. maculicola ES4326 in

Arabidopsis. We further elucidated the mechanisms of ISR using hormonal mutant lines of *Arabidopsis*, and transgenics transformed with *PR-1a::*GUS and *PDF1.2::*GUS promoter fusions. In addition, a comparison of the VOC profiles of bacilli strains GB03, IN937a and E681 revealed that a C16 hydrocarbon, hexadecane, is produced only by strain E681. Our results led to identifying a novel hydrocarbon-based signal molecule from strain E681 as the bacterial determinant(s) involved in ISR in plants.

Materials and Methods

Bacteria and plant preparation

PGPR strains *Bacillus subtilis* GB03 and *Paenibacillus polymyxa* E681 were streaked onto tryptic soy agar (TSA, Difco Laboratories, Detroit, MI, USA) plates and incubated for 24 h in darkness at 28°C. Strain E681 was previously isolated from the roots of winter barley in the southern part of Korea. For long-term storage, bacterial cultures were maintained at -80°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) that contained 20% glycerol. *Arabidopsis thaliana* plants were prepared as previously described (Ryu et al. 2003; 2004ab).

Assessment of induced systemic resistance by bacterial volatiles

To determine if exposure of *Arabidopsis* seedlings to bacterial volatiles elicited ISR against *Pseudomonas syringae* pv. maculicola ES4326, we developed a 24-well, microtitre-based disease assay system. Twenty microliters of bacterial suspensions of PGPR strains *B. subtilis* GB03 and *P. polymyxa* E681 at OD_{600} = 1 (10^{8-9} cfu/ml) were drench-applied to two-week-old *Arabidopsis* seedlings. Seven days after PGPR inoculation, 2.5 ml of a bacterial suspension (at OD_{600} = 1) of *P. syringae* pv. maculicola ES4326 grown on King's B medium were added to each well, after which whole seedlings were soaked in the suspension for 5 min, and the bacterial suspension was removed. Then, the 24-well microtiter plates were placed in a growth chamber maintained at 21°C in 12/12 day and night conditions. Disease severity was measured four to seven days after pathogen challenge.

Results and Discussion

Bacterial volatile analysis of *P. polymyxa*

We recently reported that solid-phase micro extraction (SPME) combined with gas chromatography-mass spectrometry (GC–MS) provides a better tool for profiling volatile blends in strains of PGPR (Farag et al. 2006). Results from this analysis provided a comprehensive compositional profile of the volatiles released by strain IN937a. We used this method in the current study to profile the volatiles released by E681, and strain GB03 was included as a positive control. High levels of acetoin were consistently released from strains E681 and GB03 (74 and 200 μg/24 h, respectively) releasing only 0.1 μg /24 h of acetoin (data not shown). Marginal detection levels of 2,3-butanediol were detected in VOCs released by the E681 strain. Besides 2,3-butandiol and acetoin, other volatiles released from three bacilli strains GB03, IN937a and E681 included methanethiol, isoprene and acetic acid-butyl ester (data not shown). A comparison of the VOC profiles of strains GB03 and E681 revealed that hexadecane, a C16 hydrocarbon, is released exclusively only from strain E681. Hexadecane, which was called cetane, is an alkane hydrocarbon with the chemical formula C16H34 (MW = 226.44 daltons). The production of a volatile hexadecane at the headspace of *P. polymyxa* was the first report.

The effect of bacterial volatiles on PR-1 and PDF1.2 gene expression in plants

To compare the previous result obtained with strain GB03, we evaluated expression from *PR1* and *PDF1.2* genes GUS construct in *Arabidopsis*. A GUS assay revealed that exposure to VOCs released by strain E681 increased the expression of the *Arabidopsis PR-1* promoter 7.3-fold, but did not affect that of the *PDF1*.2 promoter. In contrast, treatment with strain GB03 stimulated the expression of the *Arabidopsis PDF1.2* promoter 27-fold relative to the control. A comparison of the VOC profiles of strains GB03, IN937a and E681 revealed that hexadecane, a C16 hydrocarbon, is released exclusively only from strain E681. Hexadecane, which was called cetane, is an alkane hydrocarbon with the chemical formula C16H34 (MW = 226.44 daltons). The production of a volatile hexadecane at the headspace of *P. polymyxa* was the first report.

Our results presented here demonstrate that the rhizobacterium *Paenibacillus polymyxa* E681 produces a volatile blend that elicits ISR

against *P. syringae* in the absence of physical contact with plants. In this current study, we further demonstrated that ISR resulting from exposure to a unique VOC released by strain E681 depends on salicylic acid signalling. Previously, an analysis of the VOCs released from three bacteria, including *Paenibacillus polymxa* from potato tubers, showed that dimethylformamide, pentadecene and hexadecane are unique volatiles generated by *P. polymxya* (De Lacy 1999). This result is in agreement with our data (not shown). A comparison with our previous data (Farag et al. 2006) revealed that hexadecane was emitted exclusively from *P. polymyxa,* but not from *B. subtilis* and *B. amyloliqefaciens*. To the best of our knowledge the only other bacterial species known to produce hexadecane is the cyanbacterium *Oscillatoria perornata* (Tellez et al. 2001). To date, microbial production of this hydrocarbon has not been extensively studied, and the plant's response to hexadecane has not been thoroughly assessed. Hexadecane is a novel candidate signal molecule that can induce *PR-1* expression. How plants perceive and respond to hexadecane has yet to be fully elucidated using large-scale gene expression techniques.

Production of cytokinin by *P. polymyxa*, or by the plant exposed to *P. polymyxa*, was proposed to be a bacterial determinant in growth promotion (Timmusk et al. 1999). Our data presented here indicate that bacterial volatiles emitted from *P. polymyxa* E681 play an important role in promoting the growth of *Arabidopsis* seedlings. Moreover, the same strain produces hexadecane from a blend of volatiles that elicit ISR against *P. syringae* pv. maculicola strain ES4326. Interestingly, the hexadecane did not show any effect on the plant growth promotion (data not shown), indicating that individual VOC from a bacterium play a different role on plant growth promotion and ISR in plants. VOCs released by strain E681 elicited only ISR and induced expression of the *PR-1* gene to a greater extent than from those released by strain GB03. The qRT-PCR results indicate that hexadecane emitted from strain E681 elicited plant defence mechanisms mostly depending on SA and JA signalling by measuring *PR1* and PDF1.2 gene expressions respectively (data not shown). Our results provide evidence that strain E681 emits a long chain C16 volatile triggering ISR response stronger than 2,3-butanediol or acetoin, suggesting the presence of unidentified molecules that need to be identified and examined in large-scale experiments.

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CHAPTER THIRTY-NINE

DIVERSITY OF THE PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM THE ROOT OF TREE PEONY (*PAEONIA OSTII*)

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Abstract

The diversity of the phosphate solubilizing bacteria in the rhizosphere and rhizoplane of tree peony (*Paeonia ostii*) plants was investigated in this study. Thirty-six phosphate-solubilizing bacteria were screened from 311 rhizosphere and rhizoplane bacterial strains of tree peony plants. The phosphate-solubilizing bacteria included 25 rhizobacteria isolates, and 11 rhizoplane bacteria isolates. *Pseudomonas* sp. FLR2 showed the highest phosphate solubilizing activity (326.2 mg/L). Phylogenetic analysis based on the partial 16S rDNA sequences indicated that the strains were clustered into three groups: 22 strains of Firmicutes (61.11%), 10 strains of Gammaproteobacteria (27.78%), and 4 strains of Actinobacteria (11.11%). The dominant phosphate-solubilizing bacteria were *Bacillus* (33.33%). Our results show that some phosphate solubilizing bacteria strains have great potential applications in ornamental plant cultivation.

Introduction

Plants only absorb an inorganic form of phosphorous in the soil, and phosphorous deficiency is a major constraint to plant growth (Mathurot et al. 2009; Sridevi & Mallaiah 2009). Unfortunately, the level of inorganic phosphorus is very low in soil (Babita et al. 2009). Phosphate solubilization by soil bacteria, that makes the P available in soil solution

for plant growth, is considered to be an important attribute of plant growth promoting rhizobacteria (PGPR). Numerous investigations document the presence of phosphate solubilizing bacteria from the rhizosphere or rhizoplane of different plants (Paul & Sundara 1971; Elliott et al. 1987; Popavath et al. 2008; Jigang et al. 2009).

The tree peony is an important ornamental plant indigenous to China, which belongs to the section Moutan in the genus *Paeonia*, Paeoniaceae. Based on the characteristics of plant growth-promoting bacteria strains, we think this is a good way to screen and apply PGPR strains on the tree peony cultivation.

Materials and Methods

Phosphate solubilizing bacteria were screened with Pikovskaya (PKO) agar from 311 rhizosphere and rhizoplane bacterial strains of tree peony plants. After 10 d of incubation at 28°C, strains that induced a clear zone around the colonies were considered to be positive. Determination of phosphate solubilizing activity by the strains was carried out following a standard method (Poonguzhali et al. 2007).

The DNA of bacterial isolates was prepared according to the procedures of Park et al. (2005). The 16S rRNA genes were amplified from genomic DNA by PCR using the primers 27F and 1492R (Volker & Vilma 1996). Amplification products were examined by agarose gel electrophoresis and purified using the PCR Purification Kit (Gold Chain BioTech Centre, Beijing) according to the manufacturer's protocol.

Target fragments were sequenced with an ABI 3100 DNA sequencer by the Chinese National Human Genome Center (Shanghai, China). The presence of possible chimeric sequences was investigated using the CHIMERA-CHECK program of the Ribosomal Database Project II (RDP II). The most similar sequences were searched within the NCBI database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST).

Results and Discussion

Thirty-six phosphate-solubilizing bacteria were screened from 311 rhizosphere and rhizoplane bacterial strains of tree peony plants (see Table 39.1 below).

Table 39.1. The 16S rDNA sequence analysis of the phosphate-solubilizing bacteria strains

Genus	**Strains**	**Closest strains** (Accession No.)	**Sequence similarity** (%)
Bacillus	FLR26	*Bacillus thuringiensis* (FJ235080)	100
	FLR3	*Bacillus thuringiensis* (KC121063)	99
	LLR2	*Bacillus thuringiensis* (AM747225)	100
	LLR10	*Bacillus thuringiensis* (JN700135)	98
	FL31	*Bacillus mycoides* (EU5867900)	97
	LR39	*Bacillus mycoides* (AY373357)	98
	FLR36	*Bacillus altitudinis* (FJ174641)	99
	FL25	*Bacillus altitudinis* (FJ976556)	97
	LLR37	*Bacillus pseudomycoides* (AM747227)	99
	LR1	*Bacillus pseudomycoides* (AF013121)	98
	FL7	*Bacillus* sp. (FJ267543)	99
	FLR13	*Bacillus* sp. (AJ002154)	100
Enterobacter	LLR6	*Enterobacter hormaechei* (EF428236)	97
	LLR11	*Enterobacter hormaechei* (EU164545)	99
Leifsonia	FLR20	*Leifsonia* sp. (AB366295)	99
	FL12	*Leifsonia* sp. (DQ901014)	99
	LLR19	*Leifsonia* sp. (AJ244675)	100

Lysinibacillus	FLR11	*Lysinibacillus sphaericus* (FJ174634)	100
Paenibacillus	LR11	*Paenibacillus* sp. (EU741009)	99
	FL22	*Paenibacillus* sp. (AJ131119)	99
	FLR33	*Paenibacillus* sp. (AF227827)	98
	LLR13	*Paenibacillus* sp. (AB043868)	100
	FLR29	*Paenibacillus taichungensis* (EU982882)	99
	LLR25	*Paenibacillus taichungensis* (EU179327)	99
	FLR35	*Paenibacillus polymyxa* (AJ320493)	97
	LLR9	*Paenibacillus polymyxa* (AF355463)	98
	FLR15	*Paenibacillus polymyxa* (AF273741)	99
Pseudomonas	FLR56	*Pseudomonas pavonaceae* (D84019)	99
	FLR18	*Pseudomonas pavonaceae* (AY054374)	100
	LLR21	*Pseudomonas* sp. (AF005994)	97
	FL17	*Pseudomonas* sp. (AF098467)	98
	FLR2	*Pseudomonas* sp. (AB00424)	98
Stenotrophomonas	LLR16	*Stenotrophomonas maltophili* (AF170732)	98
	FLR8	*Stenotrophomonas maltophili* (Y13836)	97
	LR25	*Stenotrophomonas sp.* (X95924)	99
Streptomyces	FL16	*Streptomyces aureofaciens* (AY207608)	99

Phylogenetic analysis based on the partial 16S rDNA sequences indicated that the strains were clustered into three groups: 22 strains of Firmicutes (61.11%), 10 strains of Gammaproteobacteria (27.78%) and 4 strains of Actinobacteria (11.11%). The dominant phosphate-solubilizing bacteria were *Bacillus* (33.33%).

The phosphate-solubilizing bacteria included 25 rhizobacteria isolates and 11 rhizoplane bacteria isolates. *Pseudomonas sp*. FLR2 showed the highest phosphate solubilizing activity (326.2 mg/L).

Our results show that some phosphate solubilizing bacteria strains have great potential applications in ornamental plant cultivation.

Table 39.2. Phosphate-solubilizing activity of the strains isolated from the root of tree peony plants

Strains	**Phosphate-solubilizing activity** P (mg/L)	**Strains**	**Phosphate-solubilizing activity** P (mg/L)
FL12	180.6 ± 2.8	FLR35	122.6 ± 2.7
FL16	38.5 ± 2.3	FLR36	93.5 ± 0.8
FL17	211.2 ± 1.5	FLR56	11.3 ± 4.0
FL22	12.0 ± 10.9	FLR8	41.9 ± 0.7
FL25	47.4 ± 8.6	LLR10	92.0± 3.3
FL31	98.8 ± 3.3	LLR11	69.0 ± 2.3
FL7	20.2 ± 3.9	LLR13	22.3 ± 2.8
FLR2	326.2 ±1.6	LLR19	220.6 ± 7.9
FLR11	263.5 ± 2.2	LLR2	69.3 ± 3.2
FLR13	33.9 ± 1.0	LLR21	130.8 ± 1.2
FLR15	77.8 ± 7.1	LLR25	175.2 ± 4.1
FLR18	92.1 ± 5.3	LLR37	49.2 ± 2.4
FLR20	11.9 ± 2.7	LLR16	15.0 ± 2.6
LR39	15.0 ± 7.5	LLR6	47.1 ± 6.7
FLR26	214.9 ± 8.5	LLR9	179.8 ± 3.7
FLR29	201.7 ± 5.3	LR1	38.1 ± 9.2
FLR3	250.8 ± 2.6	LR11	101.0 ± 4.6
FLR33	68.0 ± 2.8	LR25	29.7 ± 1.2

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CHAPTER FORTY

EFFICACY OF INTEGRAL® (*BACILLUS SUBTILIS* MBI 600) IN SHEATH BLIGHT MANAGEMENT AND YIELD ENHANCEMENT OF RICE

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Abstract

The use of PGPR products is gaining importance in agriculture. In this study, the efficacy of a commercial product of *Bacillus subtilis* MBI 600 (Integral®, containing 2.2×10^{10} cfu/ml) was evaluated on the management of sheath blight (ShB) caused by *Rhizoctoniasolani* and yield of rice. The *In vitro* efficacy of Integral® against *R. solani* and the suppression of ShB

lesions through detached leaf assay were studied. The growth-promoting activities of Integral® were evaluated under greenhouse conditions. Further, Integral® was tested for its compatibility with commonly used rice fungicides. Field studies were conducted using Integral® on a transplanted crop for managing ShB and yield enhancement. The mode of action of Integral® against *R. solani* was studied using scanning electron microscopy (SEM) and its colonization potential on rice seeds was monitored. Our results indicate that Integral® significantly suppressed ShB lesions in rice caused by *R. solani*. Seed bacterization with Integral® under greenhouse conditions significantly enhanced seedling germination and growth. Further, Integral® was found to produce siderophores and was compatible with rice fungicides. Under field conditions, Integral® provided significant increase in seedling height, tiller production, lowered ShB and enhanced grain yield at 2.2×10^9 cfu/ml as seed treatment + seedling dip + foliar spray. In SEM, Integral® caused the loss of structural integrity of *R.solani* hyphae and sclerotia causing lysis. Integral® has good colonization potential on seeds and even survived for six days after application. Overall, our results indicate that Integral® has good potential in managing ShB and enhancing yield in rice under the conditions evaluated.

Introduction

The application of plant growth-promoting rhizobacteria (PGPR) in controlling plant diseases is gaining significance. *Bacillus subtilis* are gram positive PGPR widely used in plant disease biocontrol. Managing soil borne diseases of rice through PGPR application is gaining momentum in Asian Countries. Among different soil borne diseases affecting rice cultivation, sheath blight caused by *Rhizoctonia solani* causes significant grain yield losses (Savary et al. 2000). In US rice growing regions of the Midsouth, sheath blight is the most devastating disease on rice (Groth & Lee 2002; Lee & Rush 1983; Marchetti 1983). In Asian countries such as India, the disease is presently managed through application of chemical fungicides.

The screening of commercial PGPR strains in rice disease biocontrol is important so as to formulate alternatives to chemical control. These commercial products can either be used as an alternative or a supplement to existing chemical control methods. Further, to be successful, the product should be highly effective against the plant pathogen under study besides exhibiting strong growth promoting effects and in contributing to yield enhancement. Further, the product should be highly compatible with the commonly used fungicides in rice. In the present study, the

commercial product of *B. subtilis* MBI 600 was commercially produced by Becker Underwood Inc. as Integral® was used for screening its efficacy against rice sheath blight and growth promotion and yield in rice. The strain MBI 600 was originally obtained from the Phytobacteriology Laboratory strain collection, Department of Entomology and Plant Pathology, Auburn University, AL, USA. Integral® contains a minimum of 2.2×10^{10} spores/ml and is packaged in 500 ml bottles.

Preliminary studies revealed that the strain MBI 600 improved seed germination, enhanced seedling growth and contained rice sheath blight pathogen *R. solani* under *in vitro* conditions using standard procedures under laboratory and greenhouse conditions. In the present study, the efficacy of Integral® in suppressing ShB lesions *in vitro*, its growth-promoting effects on rice under GH, the chemical compatibility of strain MBI 600 with commonly used chemical fungicides in rice, and field evaluation against ShB and yield enhancement were studied. The strain was also characterized for its growth promoting traits. The mode of action of MBI 600 strain in Integral® in suppressing mycelia growth and sclerotia of *R. solani* was determined through Scanning Electron Microscopy.

Materials and Methods

To check for purity, inoculum from bottles of Integral® was streaked onto TSA plates and checked for growth and purity. To confirm the identity of MBI 600 strain, the 16s rDNA sequence homology technique was used. Integral® was screened at 2.20×10^6, 2.20×10^7, 2.20×10^8 and 2.20×10^9 cfu/ml. as seed treatment on CV. Cocodrie and sown in pots under GH conditions using standard procedures. The rate of seedling emergence was recorded every day for 7 d. Root and shoot lengths and weights were recorded 15 d after sowing. The compatibility of fungicides such as propiconazole (Tilt 250 EC), validamycin (Sheathmar 3L), benomyl (Benlate 50 WP), carbendazim (Bavistin 50 WP), tricyclazole (Beam 75 WP), mancozeb (80 WP), azoxystrobin (Heritage 50% WDG), and hexaconazole (Danzole 5 EC) were tested using standard procedures. Integral® was evaluated for the production of Indole Acetic Acid (IAA), siderophores, HCN, cellulose, chitinase and phosphate solubilization capacity using standard protocols. Under GH, Integral® was evaluated at concentrations of 2.20×10^6, 2.20×10^7, 2.20×10^8 and 2.20×10^9 cfu/ml as seed treatment using standard procedures. The rate of seedling emergence up to one week and seedling growth at the end of 15 DAS were recorded. Under field conditions, Integral® was evaluated as seed treatment (ST), seedling root dip (SD) and as foliar spray (FS) at $2.20 \times$

10^8 and 2.20×10^9 cfu/ml on rice cultivar "Swarna (MTU-7029)." Seedling growth in nursery, tiller production and grain yields were compared along with efficacy in containing ShB disease. The mode of inhibition of MBI 600 strain in Integral® on mycelia and sclerotia of *R. solani* was studied using scanning electron microscopy (SEM). Antibiosis, hyperparasitism of MBI 600 on pathogen mycelia under *in vitro* conditions, live interaction on rice leaves, and antagonism on sclerotia were investigated using standard procedures. The capacity of Integral® to colonize rice seeds up to 7 d was investigated using standard protocols by using rifampicin resistant mutants of MBI 600.

Results and Discussion

BLAST analysis of the 16s rDNA sequence of the strain MBI 600 generated from 1,409 base pairs confirmed the purity and identity to the original identification of the parental strain prior to formulation in liquid. Greenhouse studies indicated that seed germination and seedling growth was enhanced with Integral®. The highest germination percentage (81% to 89%) was recorded with 2.20×10^8 and 2.20×10^9 cfu/ml of Integral® at 7 DAS, as against 61% in control. Root and shoot lengths were significantly higher with Integral® over control and were highest at 2.20×10^9 cfu/ml. Further, Integral® showed good compatibility with hexaconazole, propiconazole and validamycin (at 1000 ppm), and carbendazim and azoxystrobin (at 400 ppm), moderate compatibility with tricyclazole, and poor compatibility with benomyl and mancozeb (at 1000 ppm). Integral® was found to produce siderophores. Under GH conditions, Integral® as seed treatment (ST) + seedling dip (SD) + foliar spray (FS) at 2.20×10^9 cfu/ml provided a significant increase of seedling shoot length and root length besides suppressing ShB lesions over others. At field level, Integral® significantly reduced ShB severity, enhanced seedling growth, and tillers/plant and grain yields at a concentration of 2.2×10^9 cfu/ml as ST+SD+FS.

Scanning Electron Microscopy studies indicated that strain MBI 600 caused significant loss of structural integrity of pathogen hyphae with several deformities, as well as shrivelling, coiling and lysis in the studies relating to antibiosis and hyperparasitism (see Fig. 40.1 below). On sclerotia, Integral® caused the colonization of MBI 600, maceration and deterioration of inner sclerotial walls. Seed colonization studies indicated that the population of MBI 600 significantly decreased over time up to 6 d after seed treatment (see Fig. 40.2 below).

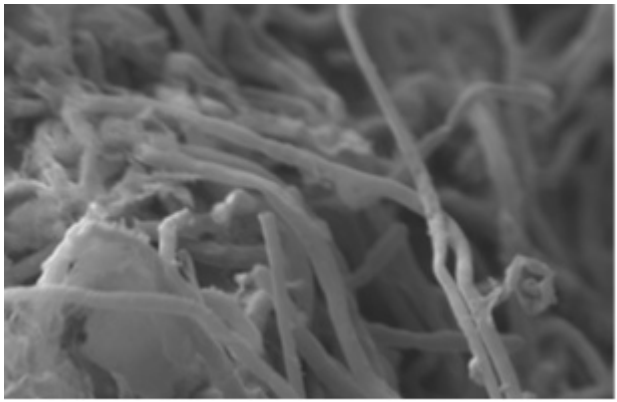

Fig. 40.1. Scanning electron photomicrographs showing the mycoparasitism and lysis of *B. subtilis* MBI 600 on *R. solani* hyphae

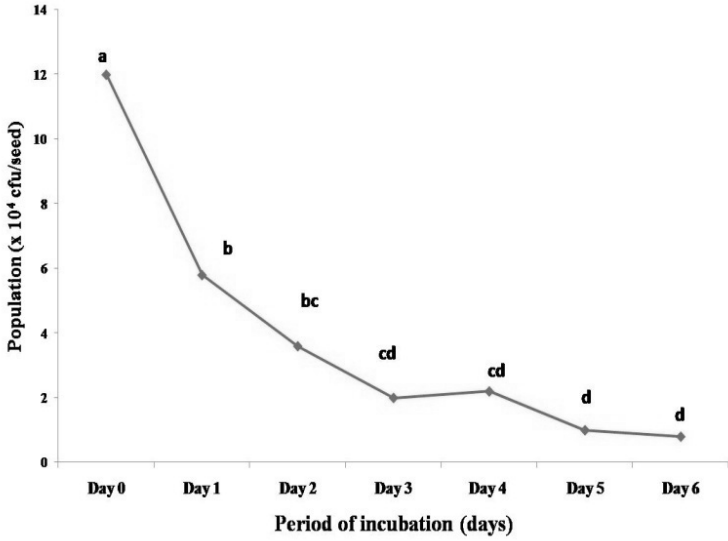

Fig. 40.2. Seed colonization potential of Integral® applied to rice seeds (CV: Cocodrie)

Overall, our results indicate that Integral® is effective in suppressing ShB disease of rice and enhanced grain yields. Further, the strain MBI 600 has good colonization potential on rice seeds.

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CHAPTER FORTY-ONE

EVALUATION OF PEANUT GENOTYPES FOR PHYSIOLOGICAL AND BIOCHEMICAL RESISTANCE TO *ASPERGILLUS FLAVUS* INFECTION

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Abstract

Aflatoxin contamination by *Aspergillus flavus* is a qualitative problem in peanut. The fungus invades during pre-harvest and post-harvest stages, causing severe economic losses. Host plant resistance against *A. flavus* invasion is an important aspect in integrated disease management. The physiological and biochemical resistance of peanut genotypes needs to be evaluated to confer resistance at field level. In our present study, ten genotypes were evaluated at field level in a factorial RCBD, with irrigation and drought as the main treatments, with 12 sub-treatments and 6 replications in *A. flavus* sick soil. Physiological resistance was

determined by evaluating kernel moisture and pod wall moisture. Biochemical resistance was evaluated by estimating seed protein at harvest using SDS PAGE. Our results indicate that kernel moisture was significantly higher in all genotypes under irrigation compared to drought. Under irrigation, genotypic differences were non-significant except for K 134 and TAG 24. Under drought, no significant differences were noticed among genotypes. The highest kernel moisture content was recorded in TCGS-341 (59.3%) followed by VG-39 (51%) under irrigation. Under drought, VG-39 (45.3%) recorded the highest kernel moisture content and the lowest was recorded by TPT-4 (32.7%). Significant differences in pod wall moisture between irrigation and drought were noticed. Under irrigation, TAG-24 showed the highest pod wall moisture (51.8%), whereas TCGS-888 showed the lowest pod wall moisture (41.2%). Under drought, the highest pod wall moisture was noticed in TAG-24 (44.7%) followed by Narayani (40.2%). Significant differences in protein profiles were observed in genotypes, and TPT-4 significantly differed from others. Our results indicate significant differences in physiological and biochemical characters in peanut genotypes. Correlation of these parameters with aflatoxin contamination under field conditions is necessary prior to determining the resistance reaction of these genotypes. We will discuss our results with the role of plant growth-promoting rhizobacteria in disease management and reducing aflatoxin contamination in peanut.

Introduction

Aflatoxin contamination of groundnut is a major problem in groundnut at both pre- and post-harvest stages. These toxins are secondary metabolites produced by the *Aspergillusflavus* group of fungi (*A. flavus* and *A. parasiticus*). These toxins are highly toxigenic having carcinogenic, hepatotoxic and teratogenic effects. Strong sources of genetic resistance are not available for this qualitative problem in groundnut. Screening of groundnut germplasm for resistance to aflatoxin contamination is a continuous process for the identification of reasonably resistant lines for further breeding programmes.

Determining the physiological and biochemical basis of resistance of various germplasm lines is important and these attributes often correlate with field resistance. Important physiological factors such as kernel moisture and pod wall moisture, and biochemical factors such as seed protein, contribute to the resistance phenomenon in groundnut. Further, germplasm evaluation at both irrigation and drought is important since

expression of these physiological and biochemical factors vary under both conditions. In the present study, certain groundnut genotypes were evaluated for their physiological and biochemical basis of disease resistance under irrigation and drought. The information generated is thus useful for deriving valid conclusions based on the correlation with aflatoxin contamination of groundnut germplasm under both irrigation and drought.

Materials and Methods

Field experiments were carried out on dry land at S.V. Agricultural College Farm, Tirupati. The experimental site was located at 13° North Latitude and 79° East Latitude with an altitude of 182.9 m above mean sea level in the tropical belt of South India.

Plant material

Ten groundnut genotypes were used in screening for aflatoxin production. They were Narayani, TPT-4, TPT-25, K-134, TAG-24, TCGS-888, TCGS-913, TCGS-341, TIR-9 and VG-39, obtained from the Regional Agricultural Research Station, Tirupati, AP, India.

Mass multiplication of *Aspergillus flavus*

The virulent strain of *A. flavus* was obtained from the Department of Plant Pathology, Regional Agricultural Research Station, Tirupati, AP, India. The fungus was multiplied by the organic-matrix method as per the modified procedure given by Will et al. (1994). Cracked Bajra seeds were soaked in water overnight, following which they were taken out and sterilized in autoclave at 121°C for 15 min. A five mm disc of *A. flavus* from actively growing 10 d culture was transferred to sterilized seed and incubated 1t 25–30°C for 15 d.

Field evaluation

The experiment was conducted during Rabi season with two main treatments and 10 sub treatments, with 6 replications with factorial RBD design. The two treatments were carried out under drought and irrigated conditions with 3 replications each in mini plots sized 1.8 × 2.5 m^2. The field was prepared by ploughing three times and then levelling by harrowing. The seeds were sown in the plots sized 1.8 × 2.5 m^2. A spacing

of 0.30 × 10 cm was adopted. The groundnut varieties were: Narayani, TPT-4, TPT-25, K-134, TAG-24, TCGS-888, TCGS-913, TCGS-341, TIR-9, VG-39 were screened in the present study. The field was prepared by ploughing thrice and then levelling by harrowing. The seeds were sown in the plots as per the layout given below along with the recommend dose of fertilizers. The mass multiplied inoculum *A. flavus* was applied 30 days after sowing (DAS) and again at 60 DAS. Initially, a slight furrow was made at one side of the row and then the pathogen was applied at 2.5 g per one meter length of the plot, after which the furrow was closed. Irrigation was given just two days before the inoculum application to allow the multiplication of the pathogen in the soil. A total of sixteen irrigations were given to the crop in the irrigation treatment at an interval of 7 to 10 d. The crop under drought treatment was given regular irrigations (7 to 10 d interval) up to 60 DAS. After 60 DAS, the intervals between irrigations were up to 18 d for imposing the stress conditions. The total number of irrigations given to the crop in this treatment was reduced to nine. The crop was harvested after 120 d and the genotypes were then evaluated for physiological determinants of resistance such as kernel moisture and pod wall moisture. The biochemical characteristics of harvested groundnut seeds, such as total seed proteins, were determined as follows.

Determination of kernel moisture and pod wall moisture

Kernel moisture and pod wall moisture were measured at the time of harvesting (Cole et al. 1993) when 5 mature pods were collected from each genotype. After that, shells were separated from kernels and the fresh weight was taken for each genotype separately. Afterwards, shells and kernels were kept separately in butter papers and oven dried. The dry weight was then taken, and the kernel moisture and pod wall moisture were calculated and expressed in percentages.

Kernel moisture = Fresh wt. of kernels - Dry wt. of kernels ×100.
Pod wall moisture = Fresh wt. of shells - Dry wt. of shells × 100.

Estimation of total proteins

The total protein content in harvested groundnut seeds was estimated as per the procedure given by Lowry et al. (1951). One gram of groundnut seeds was taken and washed thoroughly with tap water followed by distilled water and dried between folded paper. The seeds were homogenized in a mortar (0.1 M Tris-HCl, PH-8.3; 0.5 M sucrose and

0.5% β-mercaptoethanol) at 2 ml/g. The homogenate was squeezed through muslin cloth and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and an equal volume of 20% Trichloro Acetic Acid (TCA) was added to each sample and kept for 2 h at 4°C. The TCA precipitate was collected by centrifugation at 10,000 rpm for 10 min. The pellet was washed twice with 5% TCA and thrice with ice cold solvent ether. The final protein pellet was dried under vacuum and solubilised in a minimal known volume of 0.1 N-HCl solution. Then, the suspension was centrifuged at 3,000 rpm for 10 min and the supernatant was collected. Twenty microlitres of protein extract obtained from different samples were taken and to each sample 5 ml of freshly prepared alkaline copper sulphate reagent (Reagent C) were added. The samples were mixed thoroughly and the solution was allowed to stand for 10 min at room temperature, and 0.5 ml of Folin-Ciocalteau reagent was added to each sample, mixed thoroughly and incubated for 30 min. The absorbance of the sample was read at 660 nm by using spectronic-20. The amount of protein (mg/g of seed) was calculated by using bovine serum albumin (BSA) standard curve. An electrophoretic analysis of total seed proteins was made using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). The total proteins from the seeds of twelve groundnut genotypes was analyzed according to the procedure given by Laemmli (1970) using SDS-PAGE.

Results and Discussion

Estimation of total proteins and analysis by PAGE

The results of total protein content in harvested kernels are presented in Table 41.1 below. The data reveals that the total number of proteins was greater under irrigated conditions. The genotypic differences were significant under irrigated conditions, except for TPT-4, TCGS-341 and Narayani. The genotypic differences were non-significant under drought conditions, except for K-134 and VG-39. The highest number of proteins was reported in TIR-9 (192 mg/g) and the lowest in TCGS-913 (132 mg/g) under irrigated conditions. The highest number of proteins were reported in Narayani (176.4 mg/g) and the lowest in TCGS-913 (122 mg/g).

Table 41.1. Total seed proteins in different groundnut genotypes estimated at harvest

Genotype	Total seed protein (mg/g)	
	Irrigated	Drought
Narayani	163.0	176.4
TPT-4	165.3	146.4
TPT-25	173.3	162.0
K-134	152.3	135.4
TAG-24	189.0	161.0
TCGS-888	146.3	154.2
TCGS-913	132.0	122.0
TCGS-341	162.0	144.0
TIR-9	192.0	154.0
VG-39	142.0	135.0
Mean	161.7	149.0

	SEM	CD (5%)
Treatments	0.2859	0.8257
Genotypes	0.7003	2.0225
Interaction	0.9904	2.8602
CV (%)	4.4	

The seed proteins from 10 groundnut genotypes were analysed by SDS PAGE and presented in Plate 10. The total proteins are heterogeneous in size ranging in molecular weight from 12 KD to more than 100 KD. Most of the proteins were present in all the genotypes. In Tirupati-4, 4 major bands were observed. However, a significant difference was observed in protein banding profiles among all genotypes under study. A protein band with a molecular weight of 97.4 KD was present in all genotypes except in Tirupati-4. A protein band of 80 KD was observed in all genotypes and was less represented in K-134, TCGS-888 and TAG-24 and much less in Tirupati-25. The protein bands in a region between 55 KD and 70 KD molecular weight differ qualitatively among genotypes and are well expressed in K-134, less expressed in Narayani and TPT-25, and completely absent in TPT-4. A molecular weight of 43 KD and 30 KD protein was observed in almost all genotypes and well expressed in Tirupati-4. In all genotypes, a 22 KD protein band was observed but well expressed in TPT-4. A low molecular weight protein with a molecular weight of 12 KD was observed in TPT-4 only.

Kernel moisture

The data on kernel moisture are presented in Table 41.2 below. In irrigation treatment, kernel moisture was significantly higher in all the genotypes compared to drought conditions. The genotypic differences were non-significant for all genotypes except K-134 and TAG-24 under irrigated conditions. Under drought conditions no significant differences were found among genotypes, whereas TCGS-888 and K-134 show equal values. Among the genotypes tested, kernel moisture was highest in TCGS-341 (59.3%) followed by relatively in VG-39 (57.3%) and lowest in TCGS-913 (51%) under irrigated conditions. Under drought conditions, the highest kernel moisture was observed in VG-39 (45.3%) and the lowest in TPT-4 (32.7%). The interaction between genotypes and treatments was also significant.

Table 41.2. Kernel moisture and pod wall moisture in different groundnut genotypes estimated at harvest

Genotype	Kernel Moisture (%)		Pod wall Moisture (%)	
	Irrigated	Drought	Irrigated	Drought
Narayani	52.8 (45.7)	41.3 (41.3)	47.5 (43.2)	40.2 (36.3)
TPT-4	53.4 (45.3)	32.7 (35.1)	44.5 (44.0)	33.7(33.6)
TPT-25	55.3 (45.4)	35.0 (34.4)	47.6 (44.8)	32.0 (33.2)
K-134	52.0 (46.1)	34.1 (35.7)	48.5 (44.1)	27.3 (31.4)
TAG-24	54.6 (48.1)	44.9 (43.2)	51.8 (45.5)	44.7 (41.9)
TCGS-888	52.2 (42.8)	42.0 (38.6)	41.2 (39.9)	32.7 (34.9)
TCGS-913	51.0 (45.6)	34.0 (35.7)	44.3 (41.7)	30.1 (33.3)
TCGS-341	59.3 (47.3)	44.1 (35.1)	50.2 (44.1)	36.4 (35.2)
TIR-9	53.6 (51.3)	38.3 (42.0)	46.7 (42.3)	33.2 (33.3)
VG-39	57.3 (47.9)	45.3 (43.5)	49.3 (44.6)	36.7 (39.00)
Mean	54.15 (46.55)	39.17 (38.76)	47.16 (43.42)	34.7 (35.21)

Figures in parentheses are angular transformed values for kernel moisture and Pod wall moisture. Figures not in parentheses are log aflatoxin values.

	Kernel moisture		Angular Transformed Values		podwall moisture		Angular Transformed values	
	SEM	CD (5%)	SEM	CD (5%)	SEM	CD (5%)	SEM	CD (5%)
Treatment	0.57	1.76	0.34	0.96	0.65	1.85	0.39	1.1
Genotypes	1.41	4.02	0.83	2.35	1.59	4.53	0.59	2.7
Interaction	1.99	5.68	1.17	3.32	2.25	6.41	1.34	3.8

Podwall moisture

The data regarding podwall moisture in groundnut seeds during harvesting are presented in Table 41.2 above. The results show that there is significant difference in podwall moisture between irrigated and drought conditions. Under irrigated conditions, TAG-24 showed the highest podwall moisture (51.8%) among all genotypes and TCGS-888 showed the lowest pod wall moisture (41.2%). Under drought conditions podwall moisture was high in TAG-24 (44.7%), followed by Narayani (40.2%), VG-39 (36.7%) and K-134 (27.3%). There is no significant difference among all genotypes under irrigated and drought conditions. However, the genotypes TPT-4 and K-134 have equal values for pod wall moisture under irrigated conditions, and K-134 and TCGS-888 have equal values under drought conditions.

Determining physiological and biochemical resistance for groundnut germplasm is important, along with greenhouse and field evaluation. Further, these parameters need to be correlated with seed aflatoxin content. Our results are useful for further studies on physiological and biochemical characteristics that determine resistance to aflatoxin contamination. Correlation studies with aflatoxin contamination in groundnut germplasm, subject to both pre-harvest droughts under field conditions in sick soil as well as under post-harvest conditions, are our future lines of research.

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CHAPTER FORTY-TWO

EVALUATION OF BIOPESTICIDES FOR CONTROL OF *XANTHOMONAS CAMPESTRIS PV. PELARGONNI* IN GERANIUM SEEDLINGS UNDER GREENHOUSE CONDITIONS

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Abstract

Geranium production is often affected by bacterial blight disease caused by *Xanthomonas campestris pv. pelargonni* that causes huge economic

losses. The disease is also popularly known as bacterial stem rot, bacterial leaf spot, or bacterial wilt. Consistent results are not available with currently available chemical control methods, and reports on commercial biocontrol applications to manage the disease are scant. The present study focuses on the screening of certain commercial biopesticides as foliar applications in managing this dreadful disease in Geraniums. Greenhouse studies were conducted to test the efficacies of biopesticides (Actigard 50 WG @ 1.25 oz/100 gal, CG100 @ 0.8% V:V, Citrex @ 150 ml/100 L, HM-0736 @ 14.4 oz/100 gal, Kasumin @ 45 fl oz/100 gal, SP2015 @ 12 fl oz per 100 gal, Taegro @ 3.5 oz/100 gal, and Tank mix: SP2015 + CuPRO 2005 @ 8 oz + 2 lb/100 gal.) at the concentrations mentioned. Seedlings (CV: Maverick Red) were raised in Speedling trays using standard procedures, and five-week-old seedlings were transplanted into plastic pots. There were ten treatments including a pathogen and a healthy control. At 10 d after transplanting, the bacterial pathogen was spray inoculated until run-off to seedlings happened, except for the healthy control. Four days after inoculation, the evaluated products were foliar sprayed at respective concentrations. The experiment was continued for up to seven weeks and disease severity was calculated. The results indicate that the treatment of SP2015 tank mixed with CuPRO showed the lowest amount of disease. Also, Actigard and CG100 showed the lowest disease severity. All the products have shown a significant reduction in disease. Our results indicate the potentiality of these products to manage the bacterial blight of geraniums.

Introduction

Geraniums rank number one in terms of units sold among flowering potted plants, and number three in terms of wholesale value. Perhaps the most difficult problem to overcome in geranium production, and certainly the most serious disease of geraniums, is bacterial blight caused by *Xanthomonas campestris* pv. pelargonni**.** Bacterial blight—also referred to as bacterial stem rot, bacterial leaf spot or bacterial wilt—has caused millions of dollars in losses. This deadly disease is feared by growers because it is extremely difficult to control due to the way it spreads through cuttings, soil and water. Furthermore, the disease can cause crop failures in warmer environments (Kivilaan & Scheffer 1958). Bactericides have not been very effective in controlling this disease. Although research reports may indicate 80%–90% control with chemicals under experimental conditions, often less than 50% control is achieved under commercial conditions. The strict sanitation practices required to control bacterial

diseases include the destruction of infected plants as well as cleaning and disinfecting, tools, benches and flats that are used repeatedly. Irrigation should be carefully carried out in a manner that keeps foliage surfaces dry to avoid splashing. This proposal aims to achieve the control of *Xanthomonas campestris* pv. *pelargoni* in geranium seedlings with foliar applications of biopesticides. Chemicals are not considered environmentally safe by the public and the geranium growers are constantly under pressure to find alternative disease control strategies. However, few researchers have identified certain biocontrol agents for managing bacterial blight in Geranium (Flaherty et al. 2001). Hence, our research, supported by the USDA-IR4 program, evaluates various industry products for the control of bacterial blight caused by *Xanthomonas campestris* pv. pelargonni.

Materials and Methods

Efficacy experiments were carried out in a greenhouse at Auburn University. For the production of seedlings, Geranium seeds (CV. Maverick Red) were individually sown in Speedling trays in a soilless medium (Premier peat) containing 2:2:1 (v/v) vermiculite, sphagnum peat and perlite. The seeds were covered with approximately 0.5 cm of fine vermiculite and placed under intermittent mist at 24 ± 2°C in a glass greenhouse. Seedlings were removed from the mist after germination (7 d) and grown under natural light at 24°C during the day and 21°C at night until transplanting for another 4 weeks.

Five-week-old geranium seedlings were then transplanted into 10 cm × 9 cm plastic pots containing premier peat, with one seedling per pot. There were 10 treatments in this assay as listed below. A randomized complete block design with each treatment and the non-treated control consisting of 8 replications at 1 plant each was established to evaluate the efficacy. Treatment replications were separated by a buffer of 4 ft on the greenhouse bench and maintained at 26°C during the day and 22°C at night with irrigation every day. At 10 d after transplantation, a suspension of 1.0×10^6 bacterial cells of *Xanthomonas campestris* pv. pelargonni per ml was grown on Nutrient agar for 48 h and harvested for use as inoculum on geranium seedlings. Prior to the application of the bacterial pathogen inoculum the seedlings were sprayed with water for 5 min and then the inoculum was applied to the run off with a hand-held sprayer, except for the healthy control. The seedlings were irrigated each day for a week after inoculation. Four days after pathogen inoculation, the evaluated products were foliar sprayed according to the concentrations listed below with a compressed air sprayer. The products were then applied every week after

this. The experiment was continued for a total of 7 weeks. Each plant within each replicate and treatment was rated for disease severity as regards the number of bacterial lesions per plant at the termination of the experiment at 7 weeks after transplantation. The following are different treatments: (1) Non-treated healthy control, (2) Pathogen-inoculated control, (3) Actigard 50 WG @ 1.25 oz/100 gal, (4) CG100 @ 0.8% V:V, (5) Citrex @ 150 ml/100 L, (6) HM-0736 @ 14.4 oz/100 gal, (7) Kasumin @ 45 fl oz/100 gal, (8) SP2015 @ 12 fl oz per 100 gal, (9) Taegro @ 3.5 oz/100 gal, and (10) Tank mix: SP2015 + CuPRO 2005 @ 8 oz + 2 lb/100 gal.

Data analysis

The data was analyzed using ANOVA and the means were separated by a least significant difference (LSD) at $P = 0.05$.

Results and Discussion

The application of various products to geraniums resulted in no phytotoxic response. The treatment of SP2015 tank mixed with CuPRO showed the lowest amount of disease (see Table 42.1 below). Treatment with Actigard and CG100 also showed the lowest disease. Among the products tested, all showed significant disease reduction compared to non-treated pathogen control under the conditions tested. Overall, our results indicated the potentiality of these products in managing the bacterial blight of geraniums.

Table 42.1. Efficacy of various products on the severity of *Xanthomonas campestris* pv. pelargonni in geranium seedlings under greenhouse conditions

Treatment	Disease Severity[1]	Phytotoxicity
Control (Healthy)	0.4*	--
Pathogen Control	9.6	--
Actigard 50 WG @ 1.25 oz/100 gal	2.8*	No
CG100 @ 0.8% V:V	3.6*	No
Citrex @ 150 ml/100 L	4.5*	No
HM-0736 @ 14.4 oz per 100 gal	5.3*	No
Kasumin @ 45 fl oz per 100 gal	4.9*	No
SP2015 @ 12 fl oz per 100 gal	6.1*	No
Taegro @ 3.5 oz/100 gal	5.6*	No
Tank mix: SP2015 @ 8 oz + CuPRO 2005 @ 2 lb per 100 gal	1.8*	No
LSD P = 0.05	2.1	

[1] Mean number of lesions per seedling from 8 replications, one seedling per replication.

* Significantly different from pathogen control according to LSD test at P = 0.05.

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CHAPTER FORTY-THREE

EFFICACY OF VARIOUS BIOPESTICIDES IN MANAGING *PYTHIUM* ROOT ROT OF *PETUNIA*

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Abstract

Damping off in bedding plants is a major disease and is more problematic in Petunias. *Pythium* spp is a major fungal pathogen associated with the disease and causes both pre-and post-emergence damping-off. Presently, the disease is managed through the application of chemical fungicides and certain cultural control methods. However, the greenhouse production of

Petunias is still affected largely by the disease. In the present study, certain biopesticides were screened for their efficacy in managing damping-off disease of petunias under greenhouse conditions. The methodology involved raising seedlings in a soil-less peat mix under GH and transplanting them (at 3 weeks old) into plastic pots filled with same potting mix. Screened products include Adorn 4FL, Adorn+Subdue Maxx, BW240 WP, Cg100, Disarm 480 SC, FenStop, Heritage, Pageant 38 WG, and Subdue Maxx at prescribed concentrations. There were 11 treatments including a pathogen control and a healthy control in a RCBD fashion. The products were drenched around plant base at 7 days after transplanting. After 4 days following product application, seedlings were inoculated with *Pythium* at base. The seedlings were maintained in GH and the experiment was terminated after one month, and damping off and root rot disease were then assessed. Of different products, Adorn (V-10161), Adorn + Subdue Maxx, BW240 WP, Heritage and Pageant recorded absolutely no root rot (disease severity of 0) as compared to pathogen control (disease severity of 3.0). Chemicals such as Cg 100, Disarm, FenStop, Heritage and Subdue Maxx were also significantly effective in disease reduction with root rot severities in the range of 0.2 to 0.4. All the screened chemicals were found to be effective in reducing the damping-off severity in petunias. The disease severity with Cg 100 application was on a par with the healthy control (0.2 disease severity). However, the chemical significantly reduced the disease when compared to control plants treated with the pathogen (3.0 disease severity). In general, all the chemicals registered a significant enhancement of plant height, stem girth, and root and shoot weights compared to the pathogen control. Overall, preliminary screening tests indicated that all the tested products were effective in reducing root rot and damping-off incidence and severity in petunias incited by *Pythium* spp.

Introduction

Among the different diseases affecting Petunia (*Petunia* spp), damping-off, and root and crown rot are the major ones. The diseases are caused by a soil-borne fungal pathogen *Pythium* spp. Important symptoms of petunia under greenhouse conditions include pre- and post-emergence damping-off, root rot and crown rot. Prior to infection, seedlings look healthy, however, after infection the seedlings collapse near the soil line, thereby causing huge economic losses. The pathogen *Pythium* is more prevalent and problematic under humid conditions, especially when susceptible varieties are grown. Seed treatment with chemical fungicides often gives

inconsistent results and largely cultural methods are adopted in all crop growing areas to overcome the menace. The pathogen also causes the rotting of the root system and crown regions. Earlier research on damping-off of bedding plants has mostly concentrated on sanitation, environmental manipulation and fungicidal treatment to overcome the disease. The application of certain amendments to potting mix also contributes to disease reduction. For example, amending potting mixes with aluminum contributes to reduced damping off in certain bedding plants (Benson 1995). Though petunia seedlings are not sensitive to aluminum toxicity, poor seedling emergence and restricted root development of seedlings has been reported when exposed to aluminum at 100 Al in a non-limited peat, with vermiculite medium at pH 4.2 (Benson 1993). In this study, certain biopesticides were screened for their efficacy in managing the damping-off and root rot of Petunia caused by *Pythium* under greenhouse conditions.

Methodology

Petunia seedlings were produced in plastic trays containing commercial soil-less peat mix under greenhouse conditions. Three-week old seedlings were transplanted into 4 inch plastic pots (1 plant/pot) filled with the same potting mix and maintained under greenhouse conditions.

The pathogen *Pythium aphanidermatum* obtained from the culture collection of Plant Pathology laboratory, Auburn University was used in the present study. This *Pythium* sp. was highly virulent and was originally isolated from petunia roots. Mycelial plugs were produced on corn meal agar plates for use in inoculation.

A preliminary test was conducted to screen different products against the damping-off, root and crown rot of petunia under greenhouse conditions. The concentrations of products were prepared as prescribed in the evaluation protocol and applied as drench around the plant base at 7 d after transplantation. After 4 d following product application, transplanted petunias were inoculated with three 5 mm mycelial plugs of the pathogen at the base. To ensure disease incidence, an inoculum was prepared by dissolving the pathogen culture in sterile distilled water and adding it to the potting mix after 4 d. A randomized complete block design (RCBD) was used for the evaluation studies. There were 11 treatments, including healthy and pathogen control. Each treatment was replicated five times. Plants were watered with fertilizers as needed and maintained under greenhouse conditions. Seedlings were monitored for *Pythium* symptoms and for phytotoxicity symptoms, if any occurred. Plants were assessed for

damping-off and root rot disease after one month of transplanting. The experiment was terminated after one month since no phytotoxicity symptoms were recorded.

Data collection and analysis

Plant growth parameters such as plant height, caliper, and root and shoot weights were measured at one month after transplanting. Damping-off and root rot disease severities were recorded after one month. Damping-off severity was measured on a "0–5" scale where 0 = no visible symptoms, 1 = plants slightly drooped, 2 = prominent drooping of plants with browning of stem tissues at base, 3 = 50% of the plant wilting due to root rot, 4 = >50% of the plant wilting due to root rot, and 5 = death of the plant.

Root rot severity was measured on a "0–4 scale" where 0 = normal/healthy, 1 = < 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = > 75% of root rot. The data were subjected to statistical analysis and treatment means were differentiated using SAS 9.2 (PROC-ANOVA).

Results

Among the different products evaluated, root rot severity was effectively controlled by the majority of products, such as Adorn (V-10161), Adorn + Subdue Maxx, BW240 WP, Heritage and Pageant with absolutely no root rot (disease severity of 0) when compared to control plants inoculated with *Pythium* spp (disease severity of 3.0). The other chemicals, such as Cg 100, Disarm, FenStop, Heritage and Subdue Maxx, were also significantly effective in disease reduction with root rot severities in the range of 0.2 to 0.4. All the screened chemicals were found to be effective in reducing the damping-off severity in petunias. The disease severity with Cg100 application was equal to the healthy control (0.2 disease severity). However, the chemical significantly reduced the disease when compared to control plants treated with the pathogen (3.0 disease severity) (see Table 43.1 below).

Table 43.1. Efficacy of various biopesticides in managing the *Pythium* root rot of *Petunia*

Treatment	Plant growth parameters				Disease severity	
	Plant height (cm)	Stem caliper (mm)	Root weight (g)	Shoot weight (g)	Root rot	Damping-off
Healthy control	32.18^{bcd}	3.15^{c}	2.59^{d}	13.54^{cd}	1.4^{b}	0.2^{b}
Pathogen control	24.62^{d}	2.20^{d}	1.04^{e}	9.60^{e}	3.0^{a}	3.4^{a}
Adorn 4FL	45.20^{a}	4.24^{a}	3.18^{ab}	13.60^{cd}	0^{c}	0^{b}
Adorn + Subdue Maxx	45.28^{a}	4.08^{a}	3.22^{ab}	14.53^{cd}	0^{c}	0^{b}
BW240 WP	41.44^{abc}	4.24^{a}	3.42^{a}	16.75^{b}	0^{c}	0^{b}
Cg100	30.96^{cd}	3.20^{bc}	2.61^{d}	13.08^{d}	0.4^{c}	0.2^{b}
Disarm 480 SC	35.32^{abcd}	3.58^{b}	2.89^{bcd}	13.67^{cd}	0.2^{c}	0^{b}
FenStop	35.30^{abcd}	4.22^{a}	3.44^{a}	22.72^{a}	0.2^{c}	0^{b}
Heritage	38.30^{abc}	3.51^{bc}	2.83^{cd}	13.26^{cd}	0.4^{c}	0^{b}
Pageant 38 WG	42.84^{ab}	4.13^{a}	3.06^{bc}	22.79^{a}	0^{c}	0^{b}
Subdue Maxx	35.32^{abcd}	3.44^{bc}	2.82^{cd}	14.76^{c}	0.4^{c}	0^{b}

Values are means of five replications

Means followed by a common letter in the column are not significantly different at $p \leq 0.05$

Root rot severity is on a "0–4 scale" where 0= normal/healthy, 1= < 25%, 2= 26 to 50%, 3=51 to 75%, and 4= > 75% of root rotting.

Damping-off severity is on a "0–5 scale" where 0= no visible symptoms, 1= plants slightly drooped, 2= prominent drooping of plants with browning of stem tissues at base, 3= 50% of the plant wilting due to root rot, 4= >50 of the plant wilting due root rot, and 5= death of the plant.

Reduction in root rot and damping-off severities in petunias are accompanied by a concomitant increase in plant-growth attributes. In general, all the chemicals registered significant enhancement in plant height, stem girth, root weight and shoot weight compared to pathogen controls (see Table 43.1 below). Plant heights for these treatments ranged from 30.96 to 45.28 cm (as against 24.62 cm in pathogen control). The stem girths ranged from 3.20 to 4.24 mm (as against 2.20 mm in pathogen control), root weights ranged from 2.61 to 3.44 g (1.04 g in pathogen control) and shoot weights ranged from 13.08 to 22.79 g (9.60 g in pathogen control).

Conclusion

No phytotoxicity symptoms were noticed with any of the products screened in the present study. Overall, preliminary screening tests indicated that all the tested products were effective in reducing root rot and damping-off incidence and severity in petunias incited by *Pythium* spp. Due to significant disease control, the plant height, stem girth and root and shoot weights in these treatments were enhanced. Further investigations are necessary to ascertain the field efficacy of these products against root rot and damping-off diseases.

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CHAPTER FORTY-FOUR

EVALUATING PGPR STRAINS AGAINST *RHIZOCTONIA SOLANI* ON RICE (*ORYZA SATIVA*)

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Abstract

Sheath blight (ShB) of rice causes significant grain loss, to an extent of 50% under optimum disease development conditions. Reliable and effective disease management strategies are needed for managing the rice ShB disease caused by *Rhizoctonia solani* (*R. solani*). The selection of the best plant growth-promoting rhizobacteria (PGPR) strains is a vital step in ShB disease management at the field level. Of 70 bacterial strains, nine isolates exhibiting antagonistic effects against *R. solani* in preliminary tests were selected for further screening. In this experiment, nine PGPR strains (AP 301, AP 52, AP 7, AP 136, AP 295, AP 305, AP 188, AP 294 and AP 209) were screened for *in vitro* antagonistic effects on *R. solani* and for *in vivo* plant growth promotion potential. Three *R. solani* isolates collected from Arkansas, Mississippi and Texas were used throughout the experiment. *In vitro* studies indicated that all nine bacterial isolates inhibited *R. solani* mycelial growth by forming inhibition zones ranging from 0.3 to 4 mm. The most effective isolates were AP 301, AP 305 and AP 52 based on the *in vitro* mycelia and sclerotia inhibition tests against three isolates of *R. solani*.

Introduction

Sheath blight (ShB) disease of rice is a destructive disease worldwide and results in severe economic yield losses to the growers if it is not well

managed. The soil-borne fungus *Rhizoctonia solani* (*R. solani*) is the causal organism of ShB. The pathogen causes yield losses of up to 30% and reduces rice quality (Xie et al. 2008). Infection occurs when pathogen inoculum surviving in the soil comes into contact with a rice plants under conditions favourable to disease development. Infectious mycelium from previous crop debris and dormant sclerotia in the soil are two main sources of pathogen survival (Kozaka 1961; Kobayashi et al. 1997). In rice fields the pathogen survives mainly as sclerotia. These are infectious fruiting bodies formed due to the aggregation of vegetative mycelium. Sclerotia may initially be brown and over time turn to a darker brown colour. Sclerotia are irregularly shaped, small to large in size, immature to mature (due to their age), and able to survive in soil or residual plant debris for many years. After flooding rice fields, floating sclerotia come into contact with rice leaves/sheaths and infect the succeeding transplanted crop, and severely affected plants may have unfilled rice grains (Kotamraju 2010).

Various disease management options are available but none have been effective. Chemicals are widely used for disease management; however, chemical treatment is only effective if the pathogen is identified at an early stage. The ever increasing regulations and policies against chemical usage are a hindrance to developing new effective chemicals. The manufacturer has to incur high costs to develop a pesticide/fungicide and release it to the market, and returns from the product mainly depend on market competition and its effectiveness. All these factors influence the development of an alternative to chemicals. Biological control using microorganisms is effective, chemical free and sustainable.

Materials and Methods

The pathogen isolates from Texas, Mississippi and Arkansas were screened using conventional mycelium inhibition assay, sclerotia inhibition assay and detached leaf assay, respectively. Also, a seed germination test was performed to evaluate the growth promotion properties of plant growth promoting properties of rhizobacteria (PGPR). Nine PGPR strains (AP 301, AP 52, AP 7, AP 136, AP 295, AP 305, AP 188, AP 294 and AP 209) obtained from Auburn University were used as treatments throughout the experiment. A bacterial purity check was performed using a method suggested by Kotamraju (2010). Overall, there are nine treatments and five replications. The rice cultivar used in this experiment was Cocodrie supplied by the Louisiana rice research centre. All *in vivo* experiments were performed at the Dr. J. W. Kloepper lab at the department of entomology and plant pathology in Auburn, AL. The seeds were surface

sterilized prior to seeding in research pots. Each screening assay was performed separately as per the lab protocols. The data obtained were analyzed using statistical analysis software (SAS) from the SAS institute, NC, USA. The means were obtained using one way ANOVA analysis and the treatments were separated using Tukey's least significant difference.

Results and Discussion

All *in vitro* assays performed showed a difference ($P < 0.05$) to the control on mycelial growth, sclerotia inhibition and lesion height spread of *R. solani* (3 isolates) after incubation period. The three isolates were analyzed separately for percentage inhibition of mycelial growth. The total means of inhibition for the AK, MS and TX isolates were 56.8, 56.6 and 62.0, respectively. A correlation coefficient test showed that a positive correlation existed between three *R. solani* isolates. The total means of sclerotial inhibition for the AK, MS and TX isolates were 68.9, 69.4 and 69.0, respectively.

All PGPR treatments tested had a significant effect on lowering ShB lesion size when compared to the control. The total lesion spread for the AK, MS and TX isolates were 24.7, 26.3 and 26.3 respectively. The *in vivo* rice seed growth assay was evaluated under greenhouse conditions for measuring the plant growth ability of PGPR-treated seedlings. Root and shoot lengths and seedling vigour index were measured. PGPR treatments had a significant effect on treated plants. In the presence of treatments, the highest seedling vigor was found for isolate AP 305, which enhanced the plant growth by 29.24% compared with the control (see Table 44.1 below).

Table 44.1. Greenhouse screening PGPR role in rice seedling shoot length, root length and seedling vigor index improvement

			Plant growth		
Treat -ment	**Strain**	**Identification**	**Shoot length (cm)**	**Root length (cm)**	**Seedling vigour index**
T1	AP 301	*Bacillus subtilis*	24.68	9.02	3370
T2	AP52	*Bacillus subtilis* subsp. *Subtilis*	23.98	8.42	3240
T3	AP7	*Bacillus safensis*	20.98	6.1	2708
T4	AP136	*Bacillus amyloliquefaciens*	22.74	6.48	2922
T5	AP295	*Bacillus amyloliquefaciens*	24.30	7.96	3226
T6	AP305	*Bacillus amyloliquefaciens subsp. Plantarum*	24.74	10	3474
T7	AP188	*Bacillus amyloliquefaciens*	20.82	8.59	2856
T8	AP294	*Paenibacillus peoriae*	24.70	9.30	3400
T9	AP209	*Bacillus mojavensis*	22.44	6.74	2918
T10	Control		21.00	5.90	2688

Results are an average means of 5 replicates ± standard error.

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CHAPTER FORTY-FIVE

GREENHOUSE SCREENING OF PGPR ISOLATES FOR THE BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* AND COMPATIBILITY WITH CHEMICALS

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Abstract

Among various biotic stresses affecting rice, sheath blight (ShB) is a significant fungal disease causing economic crop losses. The disease is caused by a soil-based basidiomycote fungal pathogen, *Rhizoctonia solani* Kuhn. Screening and selection of elite plant growth-promoting rhizobacteria (PGPR) strains and their compatibility with conventional fungicides are vital in developing ShB integrated disease management at field level. A greenhouse experiment was conducted at the Plant Science Research Center, Auburn University, Auburn, AL, USA. Texas isolate was used for the fungicide compatibility experiment. Strain AP 301 was evaluated at concentrations of zero, 10^3, 10^6, 10^9, and 10^{11} cfu/ml in combination with azoxystrobin at zero, 396, 793, 1189, 1585, 1982 and at the recommend rate (R) 2,378 ppm. Azoxystrobin treatments were given in ascending order of 0, R/6, 2R/6, 3R/6, 4R/6, 5R/6 and the recommended rate (R). Relative lesion heights and disease severity were measured for rice plants treated with combinations of PGPR and fungicide. There was a significant difference among the treatments when compared to pathogen control. The disease incidence was lowered with increase in concentrations of treatments applied.

Introduction

Sheath blight (ShB) caused by fungi *Rhizoctonia solani* (*R. solani*) is an economically significant disease in most rice growing areas worldwide, with losses due to pathogens occurring in both quality and yield. *R. solani* is a soilborne pathogen and produces mycelium and sclerotia (Kozaka 1961; Kobayashi et al. 1997). Inoculum, when in combination with rice plants, produces initial lesions which spread over time causing the death of leaf sheaths. Under favourable conditions the pathogens spread quickly to adjacent plants and fields, thereby causing severe economic losses. The pathogen survives as sclerotia in the soil for a few years and infects the subsequent rice crop. Rice crops grown in flooded conditions are ideal for infection because the sclerotia can float and reach the base of plants, causing infection (Kotamraju 2010).

Management strategies mostly rely on chemicals. However, due to increasing chemical costs and environmental pollution an alternative is needed. Biological treatments are considered to be long-term solutions for managing ShB under sustainable conditions.

Materials and Methods

Fungi and bacteria used for experiment

The pathogen isolates from Texas were screened against PGPR in the greenhouse to evaluate the biocontrol potential. The PGPR strain (AP 301) obtained from Auburn University was used as a treatment or treatment combination with chemicals throughout the experiment. A bacterial purity check was performed using the method suggested by Kotamraju (2010). Strain AP 301 was evaluated at concentrations of zero, 10^3, 10^6, 10^9 and 10^{11} cfu/ml in combination with azoxystrobin at zero, 396, 793, 1189, 1585 and 1982 and at the recommend rate (R) 2,378 ppm. Azoxystrobin treatments were given in the ascending order of 0, R/6, 2R/6, 3R/6, 4R/6, 5R/6 and the recommended rate (R). The fungicide rate (R) for pot studies was calculated using the recommended field applied rate of 0.17 Kg ai/ha. The rice cultivar used in this experiment was Cocodrie supplied by the Louisiana rice research centre. The seeds were surface sterilized prior to seeding in research pots. The seeds were grown in 6 inch pots filled with a soil mixture of peat and field soil in a ratio of 50:50. After 30 d of germination, the pathogen sclerotia were hand inoculated at the base of the rice plants. One day after inoculation, treatments were applied as described above.

The data obtained was analyzed using statistical analysis software (SAS) from the SAS institute, NC, USA. The means were obtained using a one way anova analysis and the treatments were separated using Tukey's least significant difference.

Results and Discussion

Azoxystrobin at the recommended rate (R), when used in conjunction with any of the concentrations of strain MBI 600 (10^3, 10^6, 10^9, and 10^{11} cfu/ml, resulted in the complete reduction of ShB lesions (0% severity by RLH). In a similar way, strain MBI 600 at a concentration of 10^{11} cfu/ml when applied in conjunction with any of the concentrations of azoxystrobin under study (R/6 through R), resulted in the complete control of ShB lesions (0% ShB severity). However, MBI 600 at 10^{11} cfu/ml alone could completely inhibit ShB lesion development (see Table 45.1 below). The combined application of MBI 600 at any of the concentrations under study with azoxystrobin at the recommended rate was significantly superior (0% ShB severity) over the application of azoxystrobin alone at the recommended rate, R (4% severity by RLH).

Table 45.1. Effect of PGPR and fungicide treatments on sheath blight of rice grown under controlled conditions

Azoxystrobin (ppm)	AP 3011* 0 cfu/ml	MBI 600 @ 10^3 cfu/ml	MBI 600 @ 10^6 cfu/ml	MBI 600 @ 10^9 cfu/ml	MBI 600 @ 10^{11} cfu/ml
0	14.42^{abc}	13.55^{bcde}	15.25^{a}	5.88^{h}	0.00^{j}
R/6	14.40^{abc}	14.02^{abcd}	14.29^{abcd}	6.46^{gh}	0.00^{j}
2R/6	13.68^{bcde}	13.33^{cde}	14.85^{ab}	5.84^{h}	0.00^{j}
3R/6	14.83^{ab}	12.27^{ef}	7.26^{gh}	0.00^{j}	0.00^{j}
4R/6	6.80^{gh}	$12.89d^{ef}$	7.34^{g}	0.00^{j}	0.00^{j}
5R/6	7.20^{gh}	11.75^{f}	7.20^{gh}	0.00^{j}	0.00^{j}
R	4.20^{i}	0.00^{j}	0.00^{j}	0.00^{j}	0.00^{j}

ShB lesion size was significantly less over control (pathogen control) in other treatments involving combinations of MBI 600 @ 10^3 and azoxystrobin at different concentrations. However, a complete check of ShB lesions was seen in the following treatments that do not involve MBI 600 strain at 10^{11} cfu/ml and azoxystrobin at the recommended rate (R) as factors.

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CHAPTER FORTY-SIX

THE DUAL ROLE OF PGPR IN MANAGING SHEATH BLIGHT DISEASE AND REDUCING FERTILIZER USE IN RICE

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Abstract

Sheath blight (ShB) is a major soilborne disease causing major economic losses to rice cultivation. The disease spread is aggravated by improper use of fertilizers by growers. Most of the prevalent disease control methods are aimed against the pathogen directly and have been moderately successful. There is a need to develop an alternative control method that not only reduces ShB disease but also minimizes the use of excess fertilizers. In the experiments (in the summer and autumn), a novel ShB management strategy was evaluated by optimizing concentrations of plant growth-promoting rhizobacteria (PGPR) and rates of fertilizers. PGPR at 1×10^9 cfu/ml density was blended with different rates of nitrogen (N) and potassium (K) to evaluate their effect on ShB disease spread and their subsequent effect on rice yields. Pot culture experiments were treated with high, low and recommended rates of fertilizers to estimate the optimum dose of PGPR and fertilizer under controlled conditions. PGPR combined with N fertilizer applied at half the recommended rate showed the lowest disease lesion spread up to 2.83 mm and 2.33 mm for the summer and autumn experiments, respectively.

Introduction

Sheath blight (ShB) ranks among the most important rice diseases worldwide. It is caused by the soilborne pathogen *Rhizoctonia solani* (*R. solani*) which has multiple host plants besides rice. Under ideal disease conditions the pathogen aggressively spreads through mycelium and sclerotia within the field. Depending on the prevailing disease conditions the pathogen can incur heavy losses to the growers of up to 30% (Xie et al. 2008). Sclerotia are irregularly shaped, small to large in size, immature to mature (age of sclerotia), and able to survive in soil or residual plant debris for many years (Kozaka 1961; Kobayashi et al. 1997). Several affected plants may have unfilled rice grains (Kotamraju 2010).

Cultural, chemical and biological control strategies are available to combat ShB. Moderately resistant rice cultivars are available but the yield losses are affected by epidemics. The majority of growers rely on chemical usage to lower the results. Although chemicals are proven to be successful, environmental hazards are raising concerns. This chapter studies the compatibility of biological and chemical means in combination.

Materials and Methods

Two experiments were conducted at the Plant Science Research Center, Auburn University, Auburn, AL, USA. The pathogen isolates from Texas were screened against PGPR in the greenhouse to evaluate the biocontrol potential. A PGPR strain (AP 52) obtained from Auburn University was used as treatment or a treatment combination with fertilizer throughout the experiment. The fertilizer rates were altered in combination with PGPR to find the right amount of application in the greenhouse (see Fig. 46.1 below). A bacterial purity check was performed using a method suggested by Kotamraju (2010.) The Rice cultivar (Cocodrie) used in this experiment was supplied by the Louisiana Rice Research Center. The seeds were surface sterilized prior to seeding in research pots. The seeds were grown in 6 inch pots filled with field soil. After 30 d of germination the pathogen sclerotia was hand inoculated at the base of the rice plant. One day after inoculation, treatments were applied as described above. The pots were completely randomized on the greenhouse bench.

The data obtained was analyzed using statistical analysis software (SAS) from the SAS institute, NC, USA. The means were obtained using one way anova analysis and the treatments were separated using Tukey's least significant difference.

Results and Discussion

During the summer experiment, rice seedlings treated with bacterial strains had a significant difference ($P < 0.05$) on ShB lesions caused by *R. solani* when compared with control. Among different treatments tested against the pathogen, ShB lesion spread ranged from 0.53% to 14.25% (see Fig. 46.1 below). The lowest disease spread (0.53%) was observed with the treatment combination of PGPR-azoxystrobin-fertilizer. A positive correlation was observed between the independent variable (treatment) and experimental measurable variables. The correlation R^2 values for measurable variables RLH, ear bearing panicles, total number of grains, and grain weight per plant were 0.06, 0.03, 0.2 and 0.0005, respectively.

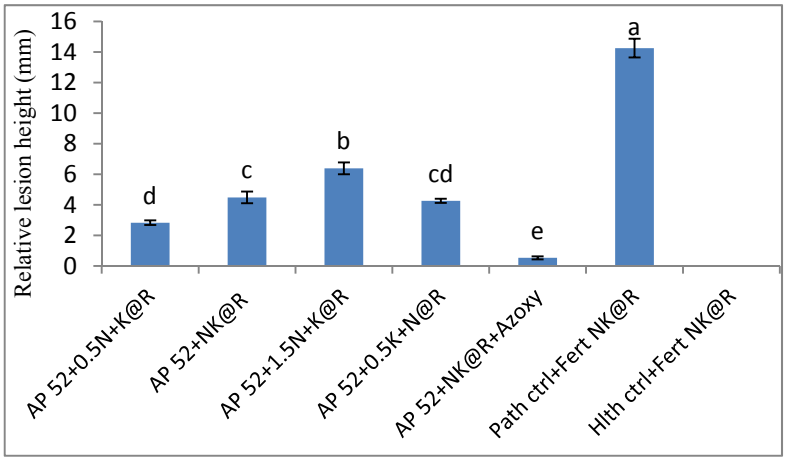

Fig. 46.1. Mean averages of relative lesion spread (%) on rice harvested during summer alongside various treatments. The error bars shown represent ± S.E.

The autumn results also showed a similar trend to that of the summer experiment. PGPR treated rice seedlings had a significant effect (P or $\alpha < 0.05$) on ShB lesions caused by *R. solani* when compared with pathogen control treatment. Among different treatments tested against the pathogen, ShB lesion spread averages ranged from 0.15% to 16.70% (see Fig. 46.2 below). Similar to the summer experiment, the lowest disease spread (0.15%) was observed with a treatment combination of PGPR-azoxystrobin-fertilizer. The total means of ear-bearing panicles, total

number of grains, and grain weight per plant for different treatments were 11.29, 111.39 and 21.23, respectively.

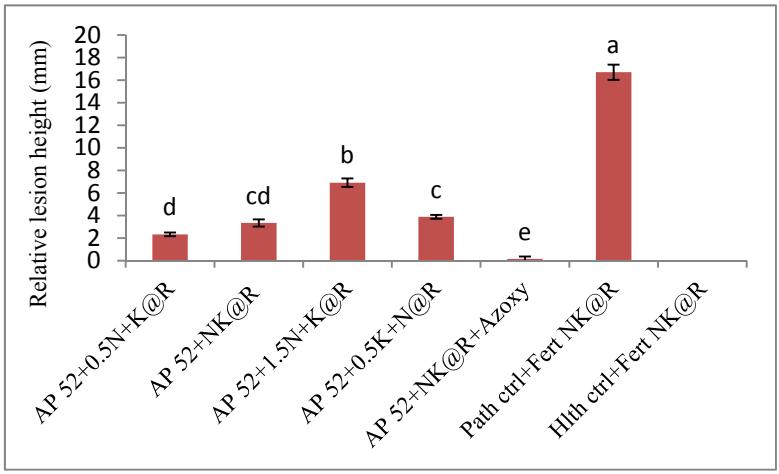

Fig. 46.2. Mean averages of relative lesion spread (%) on rice harvested during autumn, alongside various treatments. The error bars shown represent ± S.E.

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CHAPTER FORTY-SEVEN

THE ROLE OF THE FARMERS' CALL CENTRE (FCC) IN TECHNOLOGY TRANSFER TO FARMERS OF ANDHRA PRADESH, INDIA: A CASE STUDY ON RICE PESTS, DISEASES AND PGPR

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Abstract

Effective outreach methods for technology dissemination offer farmers the scope to enhance the production levels of crops. Two-way communication between farmers and agricultural scientists is one such innovation in technology transfer. The Farmers' Call Centre (FCC) was established in Andhra Pradesh through the integration of information and communication technology (ICT) and agricultural technology (AT) by the ANGR Agricultural University in 2003. The primary role of the FCC is to disseminate agricultural information on all aspects relating to plant production, protection in agricultural and horticultural crops, weather forecasting, contingency crop plans, agricultural marketing, and other allied sectors. First-hand solutions on problems encountered in crop production are being offered at our FCC. The centre works with toll free numbers of 1100/1800-425-1110. In the present study, an attempt was made to compile the database generated on farmer queries on rice pests and diseases during 2009–11. Stem borer, brown plant hopper (BPH), leaffolder, panicle mite, and hispa were perceived as the major pests. Besides these, blast and sheath blight (ShB) were major diseases causing significant yield losses. The outbreak of these pests and diseases were

analyzed seasonally (Kharif and Rabi) and monthly peaks were depicted. The district-wise outbreak of these pests and diseases was also calculated. Our results will be useful in the further identification of risk and sensitive areas in A.P. on agricultural crops in general and rice in particular with respect to major pests and diseases. Queries on the biological control of plant diseases using plant growth-promoting rhizobacteria (PGPR) are increasingly important at the FCC. Specifically, farmers were curious to learn about managing the soil-borne diseases of rice such as sheath blight and stem rot through PGPR application, as was evident from our compilation studies.

Introduction

Research advances in agriculture have to reach the farming community and gain wide applicability so as to reap the benefits. The transfer of technology (TOT) is a vital aspect in Indian agriculture that has an ultimate say on the technical advancement of a rural society. Any research advance that contributes to yield enhancement through production enhancement or amelioration of plant biotic stresses has to be sustainable in the long term, besides being economically viable and technically feasible. Technology transfer in agriculture is an uphill task and direct interaction with farmers over a technological advance is mandatory, and sometimes persuasion may be necessary for farmers to overcome conventional approaches. Direct interaction with farmers is an extension strategy that is gaining wide popularity in present-day agriculture. Though this method is expensive and requires a sizeable number of technical experts, the merits are often tangible. An innovation in this direction is to address farmers through a cost-effective approach through live communication over wire. The establishment of Farmers' Call Centres (FCCs), also called Kisan Call Centres (KCCs), based on ICT was one such innovation in India during 2004 (FACET 2012). Presently, 25 KCCs are operating in India. In a true sense, FCCs are a synthesized product of ICT and AT (Lal 2007). At present, FCCs are operating in eight states in India, working on farmers' queries and disseminating tactics related to agriculture, horticulture, livestock information and allied activities.

In Andhra Pradesh an FCC was established in 2003 and is the first of its kind, serving as a role model for several call centres run by the private and public sectors (ICAR). The FCC of A.P. is a collaborative innovation initiated by the Acharya N. G. Ranga Agricultural University and State Department of Agriculture, Andhra Pradesh. This FCC caters to the needs of farmers on agri-related issues. Working on the lines of technology

transfer through two-way communication, the FCC is equipped with a robust application method with interfaces documenting the farmers' queries as well as solutions offered to them along with telephone conversations. Multi-faceted benefits can be derived through this live, two-way communication between technical experts located at the FCC. Specifically, technology transfer is made feasible and delivered directly and on-the-spot decisions can be made by farmers. Another advantage of the FCC is that solutions to farmers' queries are being given in the local dialect (Telugu) so as to avoid any gaps in communication. These queries are maintained as a database for future records and further analysis. The basic idea is to maintain the database to assess the changing crop and pest scenarios and seasonal fluctuations in pest and disease incidence in a particular cropped area. The working team at the FCC comprises technical experts in the fields of crop production, protection, plant breeding and horticulture. Experts from the State Department of Agriculture are adept in handling queries related to agri-business, benefit-cost ratios (B/C ratio), cost effectiveness and sustainability of a package, popularity of a cultivar, information on livestock, weather forecasts, etc.

The analysis of data generated from the FCC is a worthwhile task and tangible inferences can be given on various agri-related issues. Pooled data, especially on the incidence of pests and diseases in various crops over many years, enables the prediction of risk and sensitive areas based on queries. Specifically, the feedback on farmers' problems in crop production, crop protection and production constraints will be useful in formulating research objectives designated as "need-based research." Over the years, the FCC has witnessed an overwhelming response from farmers.

Farmers' queries on biotic stresses in rice are mostly on pests such as stem borer, leaffolder, brown plant hopper (BPH), panicle mite and hispa. Besides, blast and sheath blight (ShB) are major production constraints in all crop-growing areas of AP. Queries over the telephone from farmers on these biotic stresses are on the rise and these pests continue to cause huge economic losses (Vijay et al. 2009). Severe outbreaks of blast, ShB and BPH are also not uncommon and epidemics have been reported (Thind & Sharma 2007). Though robust management practices are in vogue and are advocated to farmers through various extension strategies, alarming losses are still reported throughout A.P. (Cheralu 2008). Important reasons for this include indiscriminate use of pesticides, their losses during and after application, and ineffective farmers' outreach methods. In this chapter, we elucidated information pertaining to the prevalence of major pests and diseases of rice in A.P. Useful information was generated on a number of queries received problem-wise and area-wise for each pest and disease for

the period 2009–11. Furthermore, outbreaks in the Kharif and Rabi seasons in particular months of the year were predicted. Here, an attempt was made to understand the awareness among farmers in relevant aspects over the periods under study. Shifts and fluctuations in severity of these pests and diseases over the months were also specified. An increasing awareness among farmers on the biological control of plant diseases using plant growth-promoting rhizobacteria (PGPR) was noticed in recent years. The number of queries on PGPR-based biocontrol during the periods under report was also compiled here. We predict that the generated information would be useful in designing area-wise control measures based on correlation with soil and agro-climatic conditions prevailing in the respective zones.

Materials and Methods

Infrastructure and operation of FCC

The existing infrastructure is comprised of a two-tier system. Level-A is a prototype of any Call Centre interface and is supported by graduates and undergraduates who are well versed with local dialects. Queries on agri-related issues will then be directed to scientific experts deployed from Acharya N. G. Ranga Agricultural University and the State Department of Agriculture, Andhra Pradesh. In case queries are not readily answered, a response call facility with information on the query is available. Answering through email is also available at the FCC via www.apagris net.gov.in under the sub-heading "Your Query/Our Solution." A database pertaining to farmers' queries, along with detailed information on farmer's name, village, mandal, district, crop and nature of problem gathered for every call is maintained at the back end for future retrieval. Solutions to farmers' queries given by experts are fed into the Queries and Solutions section and will be documented on a day-to-day basis.

Farmers' queries are received directly to Level A operators and after preliminary enquiry a database on crop, problem and solutions offered by experts will be generated using the Parishkaram Call Centre Interface. In turn, the queries will be transferred to experts at Level B to experts in the fields of crop production, crop protection, horticulture and agriculture, and their allied sectors.

Data compilation on queries related to rice pests, diseases and PGPR

Total queries on important pests (stem borer, leaffolder, BPH, panicle mite and hispa) and diseases (blast and ShB) of rice were compiled during 2009–11 for both Kharif and Rabi season crops. Further, these queries were compiled district-wise in the Coastal Andhra, Telangana and Rayalaseema regions. In addition, individual queries on respective pests and diseases of rice under study were collected and compiled year-wise, region-wise and district-wise. Further, trends on peaks and declines in pests and diseases were forecasted season-wise and month-wise.

Farmers' queries on the biological control of plant diseases and PGPR applications in agricultural and horticultural crops were also compiled during 2009–11.

Results

Farmers' queries

During the period 2009–11, the total number of queries received in agriculture, horticulture and allied sectors at FCC were 51,804. Of them, 13,502 were queries on rice covering crop protection, production and market-related queries. A compilation of queries on biotic stresses in rice during the periods under study revealed that stem borer, leaffolder, hispa, BPH, panicle mite, blast and ShB were the most highly perceived pests causing alarming losses. A total of 4,078 queries were received pertaining to rice pests and the diseases under study.

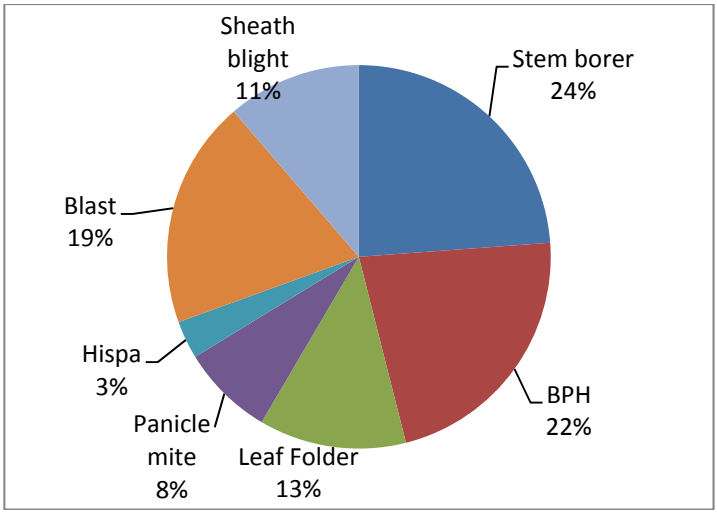

Fig. 47.2. Total queries (%) received on major pests and diseases of rice during 2009–11 at the FCC, ANGR Agricultural University, A.P., India

Rice pests

Stem borer

The total number of queries received on stem borer during 2009–11 was 971 (24% of total queries), and this was the highest among the major pests under study (see Fig. 47.2 below). Among the different districts of A.P., the highest number of queries was from Nalgonda (79), followed by East Godavari (74) and Guntur (69) (see Table 47.1 below). In general, queries on stem borer were received from all districts except Hyderabad. The monthly analysis of queries on stem borer incidence from various places across A.P. indicated that more queries were received during the months of Kharif (June to October/November) compared to Rabi (November to March) (see Fig. 47.3 below). The greatest number of queries were received during October (178), followed by September (136). In Rabi, the amount of queries was greatest during February (129), followed by March (99). The lowest amount of queries was received during the months of May and June (11 and 9, respectively) for the periods under study (see Fig. 47.3 below).

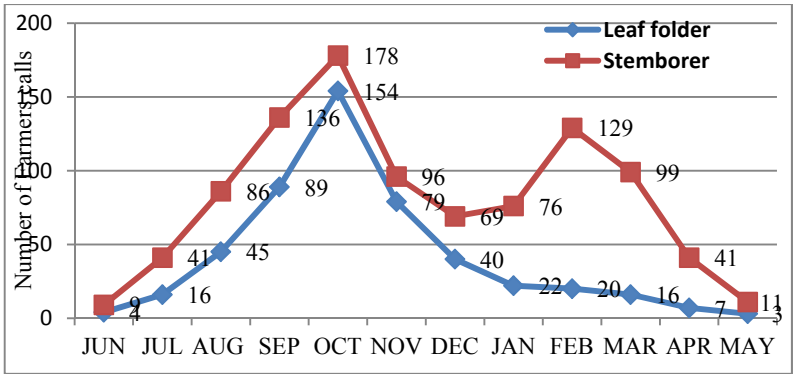

Fig. 47.3. The number of farmers' queries received (month-wise) at the FCC, ANGR Agricultural University, on rice stem borer and leaffolder during 2009–11 in AP, India

Leaffolder

The total number of queries received on leaffolder during 2009–11 was 507, and this represents 13% of the total queries (see Fig. 47.2 below). Among different districts of A.P., the greatest number of queries were from Krishna and Guntur (47), followed by Prakasam (43). In general, the queries were received from all districts in A.P. except Hyderabad (see Table 47.1 below).

Table 47.1. District-wise queries received from farmers of A.P. on rice pests and diseases during 2009–11 at the FCC, ANGR Agricultural University, AP, India

District	Stem Borer	Leaf folder	BPH	Panicle Mite	Hispa	Blast	Sheath blight
Adilabad	14	3	14	2	5	17	2
Anantapur	44	23	81	27	10	36	21
Chittoor	35	18	25	9	9	23	4
East Godavari	74	22	48	26	6	45	45
Guntur	69	47	74	25	10	81	70
Hyderabad	0	0	4	0	1	3	0
Kadapa	49	34	30	23	6	16	34
Karimnagar	59	9	45	8	5	27	15
Khammam	31	20	43	8	2	30	11
Krishna	64	47	49	39	8	61	33

Kurnool	34	15	78	31	5	32	39
Mahabubnagar	39	15	51	11	6	57	12
Medak	66	30	20	12	12	22	7
Nalgonda	79	30	39	12	4	35	22
Nellore	62	37	46	22	12	103	26
Nizamabad	55	18	21	4	7	26	8
Prakasam	27	43	42	15	4	56	15
Ranga Reddy	18	7	19	5	3	12	3
Srikakulam	13	10	6	2	1	7	11
Visakhapatnam	7	9	17	4	2	4	4
Vizianagaram	19	27	43	8	3	21	16
Warangal	65	25	74	14	3	39	20
West Godavari	48	18	38	11	5	32	43
Total	971	507	907	318	129	785	461

The monthly analysis of queries indicated that more queries were received during Kharif crop compared to Rabi (see Fig. 47.3 above). During Kharif, the greatest number of queries was received during October (154), followed by September (89) and November (79). In Rabi, more queries were received during January and February (22 and 20, respectively). The lowest number of queries was received during the months of May and June (3 and 4, respectively) for the periods under study (see Fig. 47.3 above).

Brown Plant Hopper (BPH)

The total number of queries received on BPH during 2009–11 was 907 (22% of total queries, see Fig. 47.2 above) and this was the second highest after stem borer (see Table 47.1 above). Among the different districts of A.P., the largest number of queries came from Anantapur (81). This was followed by Kurnool (78), Guntur and Warangal (74 each). In general, queries on BPH were received from all the districts; however, the lowest amount came from Hyderabad (4). The monthly analysis of queries across A.P. indicated that more queries were received during the months of October (473) followed by November (189) (Kharif) (see Fig. 47.4 below). However, the Rabi crop recorded the highest amount of queries during January and February (36 each). The lowest number of queries was received during the months of May through July for the periods under study (see Fig. 47.4 below).

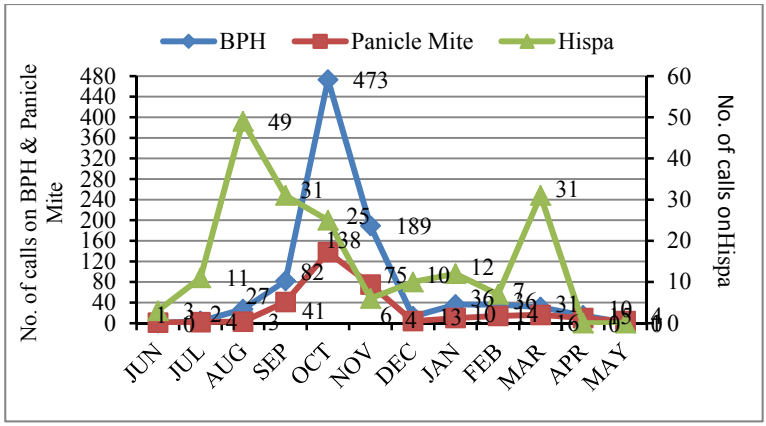

Fig. 47.4. Number of farmers' queries received (monthly) at the FCC, ANGR Agricultural University, on rice brown plant hopper (BPH), panicle mite and hispa during 2009–11 in AP, India

Panicle mite

The total number of queries received on panicle mite during 2009–11 was 318 (8% of total queries, see Fig. 47.2 above). Among the different districts of A.P., the highest number of queries came from Krishna (39), followed by Kurnool (31) (see Table 47.1 below). In general, queries were received from all districts except Hyderabad. The monthly analysis of queries indicated that more queries were received during Kharif compared to Rabi. The highest number of queries was received during October (138) followed by November (75) in Kharif. During Rabi, the number of queries was highest during February (14), followed by March (16). Queries were lowest during June and July (see Fig. 47.4 below).

Hispa

The total number of queries received on hispa during 2009–11 was 129 (3% of total queries, see Fig. 47.2 below). Among the different districts of A.P., the highest number of queries was from Nellore and Medak (12 each). In general, the pest was reported in all districts of A.P. More queries were received during Kharif and were highest in August (49) and September (31). During Rabi, queries were highest during March (31). No queries were received during April and May (see Fig. 47.4 below).

Rice diseases

(a) Blast

The total number of queries received on blast during 2009–11 was 785 (19% of total queries, see Fig. 47.2 above) and was highest among the major diseases under study. Among the different districts of A.P., the highest number of queries was from Nellore (103), followed by Guntur (81) (see Table 47.1 above). In general, the disease was reported in all districts of A.P. The monthly analysis of queries indicated that the disease was severe both during Kharif and Rabi. During Kharif, the number of incidences of the disease was highest during October (155 queries), November (83), and December (125). In Rabi, the disease was at its peak during January (121 queries) and February (140 queries). The lowest number of queries was received during the months of June and July (5 and 7, respectively) for the periods under study, and the disease was not reported during May (see Fig. 47.5 below).

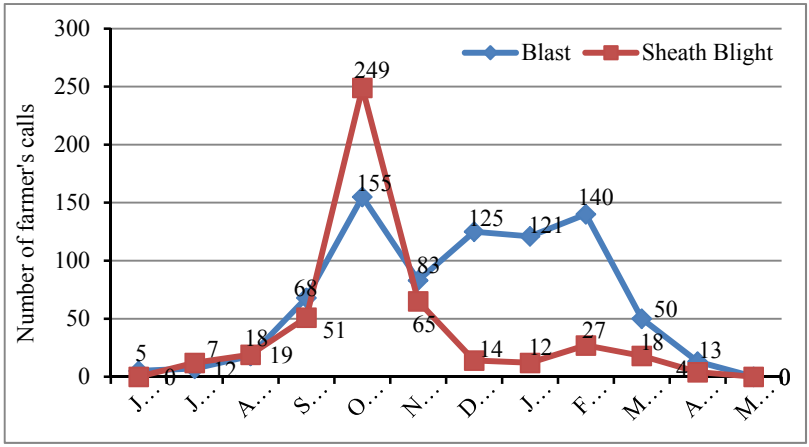

Fig. 47.5. Number of farmers' queries received (monthly) at FCC, ANGR Agricultural University on rice blast and sheath blight during 2009–11 in AP, India

(b) Sheath blight

The total number of queries received on sheath blight during 2009–11 was 461 (11% of total queries under study, see Fig. 47.2 above). Among the different districts of A.P., the highest numbers of queries were from Guntur (70), East Godavari (45) and West Godavari (43) (see Table 47.1

below). The monthly analysis of queries indicated that the disease was moe severe during Kharif compared to Rabi. During Kharif, the disease was most prevalent during October (249 queries), followed by November (65). In Rabi, the disease was at its peak during February (27 queries). The disease was not reported during May and June for the periods under study (see Fig. 47.5 below).

Queries on PGPR

During 2009–11 a total of 712 queries were received on the PGPR-based biocontrol of diseases collectively in agricultural and horticultural crops of A.P., India. Specifically, more queries were on the *Pseudomonas* application in rice, turmeric and *Rhizobium* inoculation in pulses.

Discussion

Our compiled results on farmers' queries in rice during 2009–11 at the FCC indicate that pests were rampant during Kharif compared to Rabi. The greatest number of queries on pests such as stem borer, leaffolder, BPH, and panicle mite came during October (Kharif) and January/ February (Rabi), indicating the prominence of these pests. However, queries on rice hispa were more prevalent during August and September (Kharif) and March (Rabi). The rice diseases under study followed a similar trend and queries on blast and Shb were more prevalent during October (Kharif) and January/February (Rabi). Sheath blight and blast are major diseases that affect rice production in India (Thind & Sharma 2007), and blast and sheath blight are reported as major diseases in rice. Pooled data on district-wise queries for the periods under study indicated that Nalgonda (stem borer), Krishna (leaffolder and panicle mite), Anantapur (BPH), and Nellore (hispa) have reported maximum incidence based on queries received at FCC. For rice diseases, Nellore (blast) and Guntur (sheath blight) were the districts in which the highest incidence of diseases was reported.

Arbitrary rating and indirect and negative inferences are certain ways of assessment that need to be relied upon in scientific fields in specific situations when other conclusions cannot be arrived at. Based on queries received on rice at FCC, predictions on the incidence of pests and diseases were made district-wise and month-wise. Our observations and databases generated over the periods under report are important tools for forecasting the pest and disease outbreaks in various districts of Andhra Pradesh for both Kharif and Rabi rice. For instance, farmers in the respective districts

during September/October (Kharif) and January/February (Rabi) can be advised well in advance on possible outbreaks of these pests and diseases, and this enables us to reap greater benefits of rice cultivation and save the crop from biotic stresses. Similarly, area-wise advocating of plant protection tactics based on queries and interpretations for these pests and diseases is also possible based on the endemic nature of pests and diseases, as can be perceived by observations on the number of queries over the years.

The success of sustainable agricultural practices in plant protection can also be dependent on the intensity with which the pest or disease outbreak is reported. This is more relevant in cases of a soil-borne disease, wherein epidemics were more frequent due to the rapid build-up of inoculum and resting structures (sclerotia as in stem rot and sheath blight disease of rice). Any reliable information on the possible outbreak of these soil-borne diseases in rice at different months in both Kharif and Rabi rice enables researchers and extension personnel to advocate protection tactics so as to avoid the menaces from reaching alarming levels. In this report we have provided the monthly incidences of rice pests (stem borer, leaffolder, BPH, panicle mite and hispa) and diseases (blast and sheath blight).

Canvassing of the toll-free numbers (1100/1800-425-1110) in the media is of pivotal importance so as to exploit the services of FCC to its fullest potential. Our FCC service is presently being aired on television and in agricultural magazines. Another important factor to be taken into consideration is the availability of mobile networks in rural areas, wherein farmers are keen on calling the FCC from the field itself. Our weekly observations on frequently asked questions (FAQs) also appear on television everyday and are aired on All India Radio (AIR) in different districts. Need-based research in various crops is also gaining importance in present-day agriculture. Farmers' opinions at various Zonal Research and Extension Advisory Council (ZREAC) Meetings conducted by ANGR Agricultural University are also generally taken into consideration for prioritizing the thrust areas of future research. Data generated at the FCC based on live communication with farmers can also be an important tool in prioritizing research and for diagnostic surveys by scientific personnel.

Queries to the FCC on the biological control of plant diseases in general are gaining momentum. Specifically, farmers are curious to increase their awareness on managing soil-borne diseases of agricultural and horticultural crops through biological means. Since commercial formulations of PGPR such as *Pseudomonas fluorescens* are readily available on the market, frequent queries are on the mode of action of PGPR, mode of application, optimum dose of application at field level,

and availability of biofertilizers and biopesticides. At the FCC, increased focus is on creating awareness on the PGPR-based biocontrol of plant diseases, particularly for seed- and soil-borne diseases, to discourage indiscriminate use of fungicides, resistance development by pathogens to chemicals, and in making the adopted disease management practices sustainable over the long term.

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CHAPTER FORTY-EIGHT

THE ENTOMOPATHOGENIC FUNGUS *CLADOSPORIUM* SP. AS A CANDIDATE BIOCONTROL AGENT AGAINST THE SWEET POTATO WHITEFLY, *BEMISIA TABACI*, IN SAUDI ARABIA

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Abstract

Biological control provides an environmentally harmonious and potentially stable management tactic to combat noxious pests such as *Bemisia tabaci*, notorious for its resistance to synthetic pesticides. The fungus, *Cladosporium chlorocephalum* Fersen., was identified as an entomopathogenic fungus affecting the sweet potato whitefly, *Bemisia tabaci*, in other parts of world. This study concerns a potential biocontrol agent against sweet potato whitefly in the Qassim region of Saudi Arabia. The occurrence and incidence of this fungus varies according to the plant host, geographical location and climatic season. Among the plant hosts, cabbage was the most preferred for both *B. tabaci* and *C. chlorocephalum*, while tomatoes were not suitable for harbouring *B. tabaci* population and, consequently, *C. chlorocephalum*. The incidence of *C. chlorocephalum* was higher in the autumn than at other times. Under laboratory conditions *B. tabaci* nymphs and eggs were infected by *C. chlorocephalum*; however,

nymphs were more sensitive to infection than eggs. The infection was highest under laboratory conditions than under field conditions. Ecological factors had a great impact on the dry weight and sporulation of *C. chlorocephalum*. Thus, cumulative research considered *C. chlorocephalum* to be a potential candidate as a biological control agent that could be used with other safety control methods under IPM systems.

Introduction

The sweet potato whitefly *Bemisia tabaci* Genn. (Aleyrodidae: Hemiptera) is a widespread global pest, with populations in tropical and subtropical areas within agricultural and horticultural production systems. This insect is hard to manage and causes direct and indirect damage (Brown & Bird 1992; Brown 1994; Perring 2001; Carabali et al. 2005). Moreover, the insect has a widespread host range, is multivoltine and is a highly polyphagous pest; consequently, *B. tabaci* is considered to be one of the more pestiferous insects in agro-ecosystems worldwide.

Since 1986, *B. tabaci* populations, biotypes and pest statuses have risen from relative obscurity to become one of the key insect pests threatening agriculture production worldwide (Abdel-Baky 2001; De Barro et al. 2006; Lin et al. 2007). Consequently, it is considered to be one of the worst of the top 100 invasive species worldwide (International Union for the Conservation of Nature and Natural Resources [IUCN] list).

The management of *B. tabaci* is nevertheless still based on the use of chemical pesticides. Historically, the chemical control of *B. tabaci* was the only adequate tool available for growers (Alves et al. 2008; Amjad et al. 2009). Chemical insecticides are considered to be useful and powerful tools for the control of *B. tabaci*. Unfortunately, indiscriminate use of insecticides has resulted in the development of insect resistance, secondary pests resurgence and outbreaks, and other environmental and human health issues (Roditakis et al. 2005; Naeem 2006). Therefore, insecticide has stimulated studies on IPM strategies in which biological control is considered to be a relevant measure.

Although extensive research on the biological control of whitefly has been conducted towards these insect's parasitoids and predators, several entomopathogenic fungi are recognized as important biological control agents. Entomopathogenic fungi are an essential component of whitefly control, providing a non-chemical alternative for whitefly management. Using entomopathogenic microorganisms in biological control programs is commonly referred to as microbial control, an approach that includes four strategies: classical, inoculation, inundation, and conservation (Eilenberg

et al. 2001). The infectious agents—entomopathogens—are microorganisms that invade and reproduce in an insect and spread to infect other insects.

A number of fungal species have been recognized as promising bio-control agents against *B. tabaci* worldwide (Gottel et al. 1990; Ferron et al. 1991) such as *Paecilomyces fumosoroseus*, *Beauveria bassiana*, *Verticillum lecanii* and *Aschersonia* spp. (Lacey et al. 1995). On the other hand, some authors consider *Cladosporium* spp. to be a promising biological control agent against homopterous insects (Abdel-Baky et al. 1998*;* Abdel-Baky 2000; Abdel-Baky & Abdel Salam 2003). Carvalho et al. (1972) mentioned that *Cladosporium herbarum* was associated as a controlling candidate against cashew whitefly, *Aleurodicus coccis* and other three species of whiteflies—*Bemisia* sp., *Aleurothrixus* sp. and *Dialeurodes* sp.—on various plant hosts in Venezuela (Rajas et al. 1998). Moreover, *Cladosporium aphids* infected *Alerurochiton aceris* in Finland (Hulden 1986), and *Chionaspis salicis* (L.) (Coccoidea: Diaspididdidae). Moreover, *C. cladosporioides* caused 20%–57% natural mortality of *Hemiberlesia pitysophila* under field conditions and 39% in laboratory tests in China (Pan et al. 1989). Thumar & Kopadia (1994) stated that *Cladosporium* spp. caused infections among *Aleurolobus barodensis* nymphs year round in India, while *Cladosporium* spp. occurred naturally and caused epizootic infection in the population of *Aleurocanthus spiniferus* in China (Baoyu et al. 1997). Therefore, the objectives of this study were to: (1) create a scientific search area to isolate and select entomopathogenic fungi that are more effective against *B. tabaci* and other insect pests, and more adapted to the local environment; (2) study the virulence of the fungus and determine its efficiency as a biological control agent; (3) examine the host range of insect pests to be infected under laboratory conditions; (4) study the safety of the bio-agent regarding the host plant and associated beneficial insects; (5) study the effect of some ecological factors on production and growth of the fungus contributing to mass production under laboratory conditions.

Materials and Methods

Survey of *Bemisia tabaci* and associated entomopathogenic fungi on different plant hosts and in different climates

The survey was conducted at different farms, the Experimental Station of the Agriculture & Medicine Veterinary Collage, Al-Moneria farm and some greenhouses at Al-Bekeriaha in the Qassim region during 2011–12. The survey was done through the random collection of both healthy and

infected *Bemisia tabaci* Genn. Samples of *B. tabaci* were carried out on five plant hosts (Cabbage, *Brassica oleracea*; Aubergine, *Solanum melongena*; Cucumber, *Cucumis sativus*; sweet pepper, *Capsicum annuum*; and Tomato, *Solanum lycopersicum*). Additionally, sampling of *B. tabaci* nymphs and eggs was also conducted in the four seasons.

Sampling procedure

Twenty-five plants were chosen at random to represent plants of four corners at the centre of the field or greenhouse. Five leaves from each plant (two leaves from each upper and lower third and one from the middle third of the main stem) were selected, removed and inserted into paper bags, then transferred to the laboratory for examination. The eggs and nymphs of *B. tabaci* were investigated under a stereomicroscope and counted as a total number of each insect stage/leaf. Infected eggs and/or nymphs (cadavers or healthy ones) were also counted. This procedure was applied on all five plant hosts involved in the study.

Fungal isolation and identification

The following two procedures were used in fungi isolation.
Insect cadavers showing natural external growth of fungi were collected and maintained in petri dishes contain potato dextrose agar (PDA) media. The inoculated petri dishes were kept in an incubator at 27 ±2°C and 75 ±5% R. H. until further growth of the fungi. Spores of pure cultures were inspected under a compound microscope. Insects that were precited to be infected due to their abnormal movement were surface-sterilized in a 1% sodium hydrochlorite solution (NaOCL 0.05%) for 30 seconds and washed in sterilized distilled water. Then, the insects were placed in petri dishes (25 insects/dish) on PDA media and kept in an incubator under the same temperature and RH mentioned above.

Identification of isolated fungi was done primarily by the project team work. Confirmation of the fungus identification was based on the external symptoms and the morphology of the fungi, and habit characters were used in consultation with Waterhouse & Brady (1992), Humber (1997) and the Commonwealth Mycology Institute, Kew, Surrey, England (Ellis 1971; 1976) to confirm the preliminary identification. Samples of isolates were sent the Plant Pathology Institute, Egypt to get a correct identification.

Bioassay

Each isolate of the fungus associated with the eggs and nymphs of *B. tabaci* was used to evaluate its pathogenic role on *B. tabaci* under both laboratory and field conditions. Two hundred individuals of *B. tabaci* adults, nymphs and eggs were chosen from each greenhouse or open field after being surface-sterilized in a 1% sodium hydrochlorite solution for 30 s and washed in distilled water. Each petri dish containing 25 individuals was considered as one replicate. The insects were placed in a dark colour blotter moist with the fungal suspension with one concentration (10×10^6 spores/ml) (Abdel-Baky et al. 1998). A piece of plant leaf was added to each petri dish after sterilization as a source of food. Vandenberg's (1996) and Abdel-Baky et al.'s (1998) techniques for preparation of the fungal inocula were followed. Data was collected daily, and this continued for 21 d.

Host range of *Cladosporium*

The impact and effectiveness of the entomopathogenic fungi, *Cladosporium cephalosporium*, was studied in the laboratory against a number of common insects found in the Qassim region. Among the insects used to study the host range were the cotton aphid, the cabbage aphid, the grain aphid, the sweet potato whitefly and the alfalfa leaf weevil (see Table 48.1 below).

Table 48.1. Basic information about certain insects used in host range studies

Insect species				**Host plant**
Common name	Scientific name	Family	Order	
Cotton (melon) aphids	*Aphis gosypii*	Aphididae	Hemiptera	Polyphagous
Cabbage aphids	*Brevicoryne brassicae*	Aphididae	Hemiptera	Cruciferae
Grain aphids	*Sitobion avenae*	Aphididae	Hemiptera	Grain crops
Sweet potato whitefly	*Bemisia tabaci*	Aleyrodiade	Hemiptera	Polyphagous
Alfalfa leaf weevil	*Hypera punctata*	Curculionidae	Coleoptera	Alfaalfa

The effect of certain ecological factors

The effect of the climatic seasons, temperature and light on growth and sporulation of the fungus has been investigated as follows.

Effect of climatic seasons

Distribution and incidence of the entomopathogenic fungus were studied among the four climatic seasons within a year. Samples of *B. tabaci* were taken year round, and infected and non-infected insects were counted and recorded for each season.

Effect of light on fungal sporualtion

Under lab conditions, the effect of three light regimes on fungal sporulation was studied as recommended by Wilson & Knight (1952). The light regimes used were: (1) continuous light (24 h), (2) continuous darkness, and (3) alternation of light and darkness (12/12 h).

Effect of temperatures on fungal dry weight

The effect of six temperature degrees, namely 10, 15, 20, 25, 30 and 35°C, on the fungal dry weight was also studied under lab-condition light. Potato dextrose agar media was used in this study, in accordance with Cochrane (1958).

Statistical analysis

Analysis of variance, correlation coefficient and stepwise regression models were used for data analysis (Costat 1990). Percentages of *Cladosporiun*-infected *B. tabaci* nymphs were calculated by dividing the total number of infected insect species with *Cladosporium* (summed over all samples) by the total recorded numbers of each species sampled, then multiplied by 100. Lab bioassay data were corrected using the Abbott formula, while field bioassay data were corrected using Henderson-Tilton's formula.

Results

Identification of the entomopathogenic fungi

All classification methods indicated that the five isolates belonged to the entomopathogenic fungus *Cladosporium chlorocephalum* Fersen.

Host plant and *Cladosporium chlorocephalum* infection

Population density of *B. tabaci* nymphs

Bemisia tabaci infestation varied based on the host plant type, and *B. tabaci* immature infection by *C. chlorocephalum* also varied according to the host plant (see Table 48.2 below). Cabbage ranked highest, harbouring the highest number of *B. tabaci* nymphs with an average 468.5 ± 35.07/plant leaf. In the same context, aubergine and cucumber occupied the second and third rank with an average 95.4 ± 4.99 and 84.4 ± 6.53/plant leaf, respectively. Sweet pepper occupied the fourth rank, harbouring 26.6 ± 1.89/plant leaf, and last was tomato, recording the lowest population density/plant leaf with an average 24.8 ± 2.08 nymphs (see Table 48.2 below). According to the statistical analysis, the five host plants studied differed significantly at 5% in terms of their ability to support the largest number of *B. tabaci* nymphs (see Table 48.2 below).

Occurrence of *Cladosporium chlorocephalum* associated with *B. tabaci* nymphs

By the same concept, the average number *B. tabaci* nymphs naturally infected by *C. chlorocephalum* varied depended on the host plant type and the average number of *B. tabaci* nymphs (see Figs. 48.1 and 48.2 below).

Host plants were classified into five ranks based on the average infection of *B. tabaci* nymphs. In the first rank, Cabbage harboured the highest number of *B. tabaci* infected nymphs, with an average 256.7 ± 19.4/ leaf, and this formed about 35.4% of the total infection. Aubergine, cucumber, tomato and sweet pepper were in the second rank with an average 23.94 ± 1.86, 19.9 ± 2.11, 8.3 ± 1.01 and 8.0 ± 0.79 nymph/leaf, respectively (see Table 48.2 below), which formed 11.34%, 13.88%, 11.56% and 9.88% of the total infection (see Fig. 48.2 below). Statistically, the average number of infected nymphs by *C. chlorocephalum* within the five host plants varied significantly at 5% (see Table 48.2 below).

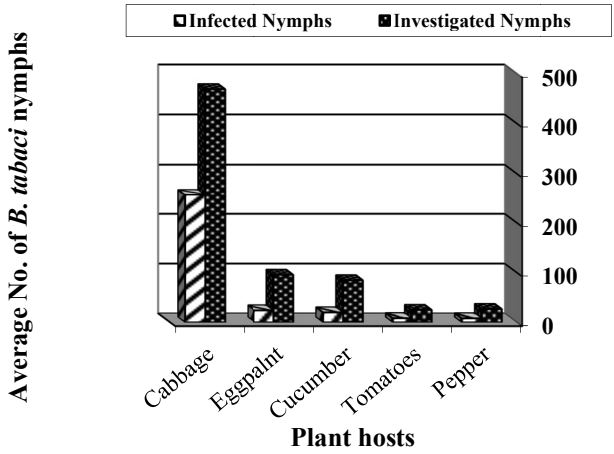

Fig. 48.1. Effect of host plant species on the occurrence of *B. tabaci* nymphs and the associated entomopathogenic fungus, *Cladosporium chlorocephalum* in the Qassim region during 2011–12

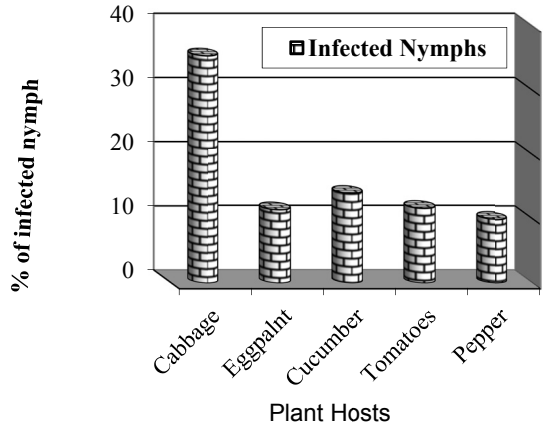

Fig. 48.2. Infection percentage of *B. tabaci* nymphs by the entomopathogenic fungus, *Cladosporium chlorocephalum*, among five host plant species in the Qassim region during 2011–12

Table 48.2. Population density of *B. tabaci* nymphs and associated infection by *Cladosporium chlorocephalum* during 2011–12

Host plant	Average No. of investigated *B. tabaci* nymphs/ leaf±SE*	Average infected No. of *B. tabaci* nymphs/ leaf±SE*	*B. tabaci* infection %
Cabbage	468.5 ± 35.07 **a**	256.7 ± 19.40 **a**	35.40
Aubergine	95.4 ± 4.99 **b**	23.9 ± 1.86 **b**	11.34
Cucumber	84.4 ± 6.53 **b**	19.9 ± 2.11 **b**	13.88
Sweet Pepper	26.6 ± 1.89 **c**	8.0 ± 0.79 **b**	9.88
Tomatoes	24.8 ± 2.08 **c**	8.3 ± 1.01 **b**	11.56
LSD 5%	46.58	25.13	
1%	62.73	33.85	
Statistical Analysis			
Treatment Sum Square (TSS)	495021.4	137773.96	
DF	4	4	
Treat. Mean square (TMS)	123755.4	34434.9	
Error Sum Square (ESS)	34077.84	117.071	
DF	45	45	
Error Mean square (EMS)	757.2854	2601.58	
F value	163.4197 with 4/45 df	132.3616 with 4/45 df	
P>F	< 0.0001	< 0.0001	

*Averages followed by same letter with in a column are not significantly different from each other at 5% LSD

Effect of climatic seasons on infection and incidence of *Cladosporium chlorocephalum* associated with *B. tabaci* populations

Climatic variations among the four seasons were studied regarding the effect of outdoor temperatures on the incidence of *C. chlorocephalum* and its ability to infect *B. tabaci* naturally (see Figs. 48.3 and 48.4 below). Data revealed that the four climatic seasons had a great impact on the incidence and infection of *C. chlorocephalum*. In the autumn, *B. tabaci* recorded a high population of 602.6 ± 30.985 nymphs/plant, and of these 195.9 ± 5.646 nymphs were infected by *C. chlorocephalum* which formed 24.35% of the total infection. Spring came next with 54.2 ± 2.851

nymphs/plant, and 8.5 ± 0.453 nymphs/plant were infected by *C. chlorocephalum* which formed 12.2% of the total infection. In spite of *B. tabaci* harboured plants with an average 16.5 ± 1.5 nymphs plant, there is no infection by *C. chlorocephalum* among *B. tabaci* populations. This means that *C. chlorocephalum* has no incidence during the summer season. In contrast, incidence of *B. tabaci* was very low during the winter at 5.0 ± 0.789 nymphs /plant, and *C. chlorocephalum* infected 0.4 ± 0.163 nymphs /plant which formed 7.4% of the total infection (see Figs. 48.3 and 48.4 below).

The statistical analysis indicates that the average number of investigated *B. tabaci* nymphs and the infected nymphs varied significantly at 5% (see Table 48.3 below). Consequently, it could be concluded from the results that the autumn was the most suitable season of the year for developing, reproduction and distribution of both *B. tabaci* and associated *C. chlorocephalum* in the open field in Qassim, Saudi Arabia.

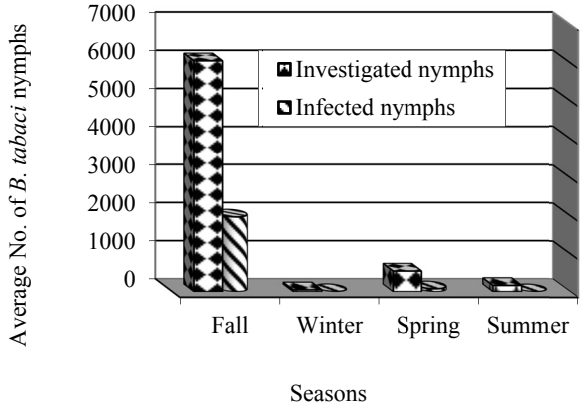

Fig. 48.3. Effect of the climatic seasons on the occurrence of *B. tabaci* nymphs and the associated entomopathogenic fungus, *Cladosporium chlorocephalum* in the Qassim region during 2011–12

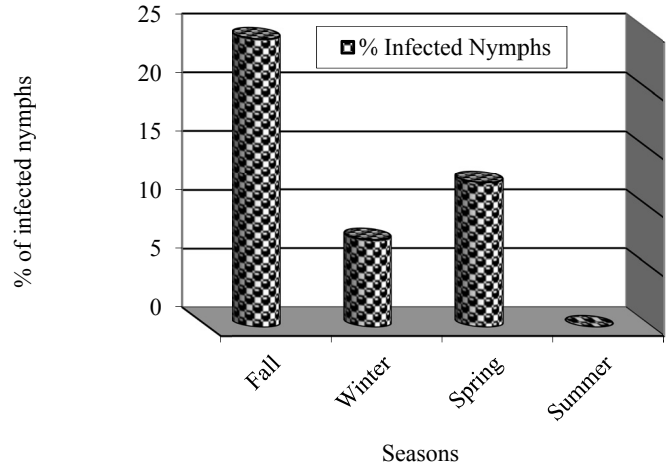

Fig. 48.4. Infection percentage of *B. tabaci* nymphs by the entomopathogenic fungus *Cladosporium chlorocephalum* among four climatic seasons in the Qassim region during 2011–12

Table 48.3. Effect of the climatic seasons on both *Bemisia tabaci* populations and their infection by *Cladosporium chlorocephalum* during 2011–12

Climatic Seasons		Average No. of investigated *B. tabaci* nymphs/plant±SE	Average infected No. of *B. tabaci* nymphs/plant±SE	*B. tabaci* infection %
Autumn		602.6 ± 30.895 a	195.9 ± 5.646 a	24.35 a
Winter		5.0 ± 0.789 c	0.4 ± 0.163 b	7.4 b
Spring		54.2 ± 2.851 b	8.5 ± 0.453 b	12.3 b
Summer		16.5 ± 1.5 c	0.0 ± 0.0 b	0.0 c
LSD	5%	38.73	8.18	
	1%	52.16	11.02	
Statistical Analysis				
Treatment Sum Square (TSS)		2603156	279634.6	
DF		3	3	
Treat. Mean square (TMS)		867718.9	93211.53	
Error Sum Square (ESS)		64755	2889.713	

DF	36	36
Error Mean square (EMS)	1798.75	80.27257
F value	482.4009 with 3⁄36 df	1161.188 with 3⁄36 df
P > F	< 0.0001	< 0.0001

*Averages followed by same letter within a column are not significantly different from each other at 5% (Duncan).

Bioassay studies

Laboratory Bioassay

Nymphs

Laboratory studies revealed that five isolates of the fungus *C. chlorocephalum* were pathogenic to *B. tabaci* immature (see Table 48.4 below). Although the initial killings of five isolates were very low, their activities were very effective (see Fig. 48.6 below).

This study also revealed that the pathogenic by five isolates of *C. chlorocephalum* varied significantly in terms of the initial kill, days after treatment, residual activity and total activity. In spite of this, isolates 1, 4 and 5 were the most virulent, causing 100% mortality of *B. tabaci* nymphs after 15 d of treatment in comparison with isolates 2 and 3 (see Figs. 48.5 and 48.6 below).

Table 48.4. Mortality of *B. tabaci* nymphs following treatment by five isolates of *Cladosporium chlorocephalum* under lab conditions *in vitro*

C. chlorocephalu Isolates	Initial Kill (0 time)	Mortality % (days after treatment)						Residual activity (RA)	Total Activity (TA)
		3	5	7	10	15	21		
Isolate 1	9.2 a	30.80 a	51.60 a	74.40 a	92.00 a	100a	100 a	74.80 a	42.00 a
Isolate 2	8.0 a	23.60 b	46.40 b	61.60 c	78.80 c	86.40 b	92.00 b	64.80 c	36.40 b
Isolate 3	3.2 a	19.20 c	40.40 c	54.80 d	74.40 c	81.20 c	85.20 c	59.20 d	31.20 c
Isolate 4	8.0 a	30.40 a	52.00 a	75.20 a	98.00 a	100 a	100 a	75.93 a	41.97 a
Isolate 5	8.80 a	25.60 b	46.00 b	64.00 b	86.00 b	97.60 a	100 a	69.87 b	39.33 b

*Averages followed by same letter within a column are not significantly different from each other at 5% (Duncan).

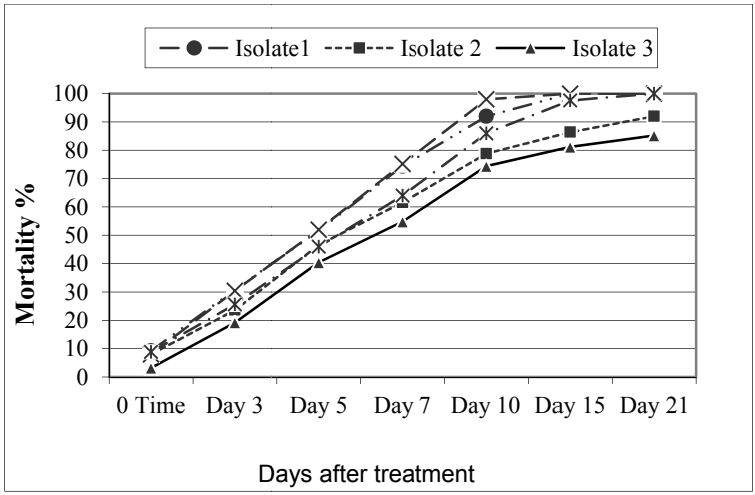

Fig. 48.5. Daily average mortality percentage of *B. tabaci* nymphs by five isolates of *Cladosporium chlorocephalum* under lab conditions in the Qassim region during 2011–12

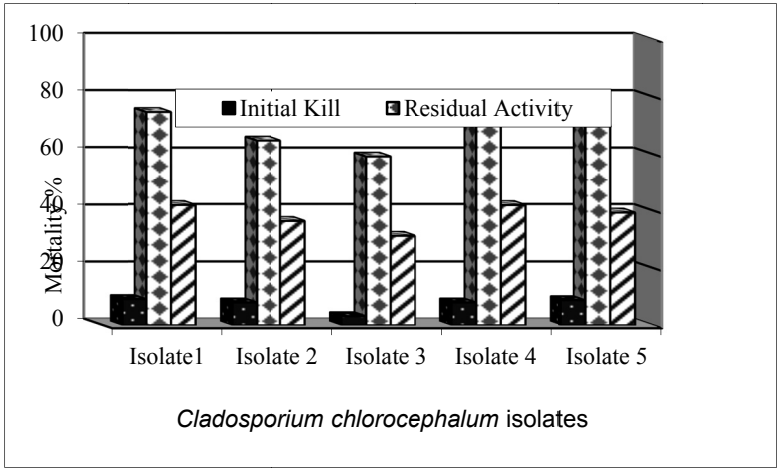

Fig. 48.6. Pathogenicity of *Cladosporium chlorocephalum* isolates against *B. tabaci* nymphs 21 d after of treatment under lab conditions

Eggs

Bemisia tabaci eggs have special circumstances being infected by *C. chlorocephalum* but with low rates. Under laboratory conditions, *C. chlorocephalum* isolates were pathogenic to *B. tabaci* eggs (see Table 48.5 below). The initial kill of five isolates was zero and this had an effect on both the residual activity and the total activity (see Fig. 48.7 below).

The virulence effect of the fungus appeared on the fifth treatment with certain isolates only, and then increased gradually until the end of the trial (see Fig. 48.8 below). Moreover, data showed that isolates of *C. chlorocephalum* caused different pathogenic efficiencies in terms of the days after treatment, residual activity and total activity. In spite of that, Isolates 1, 4 and 5 were the most efficient causing mortality ranging from 46.8–50.0% of *B. tabaci* eggs after 21 d of treatment compared with isolates 2 and 3 (see Fig. 48.7 below).

Table 48.5. Reduction percentage in *B. tabaci* eggs following treatment by five isolates of *Cladosporium chlorocephalum* under lab conditions

C chlorocephalum Isolates	Initial Kill (0 time)	Mortality % (Days after treatment)						Residual Activity (RA)	Total Activity (TA)
		3	**5**	**7**	**10**	**15**	**21**		
Isolate 1	0a	0a	2.4b	6.0b	19.6a	33.2a	49.2a	12.87 b	6.44 b
Isolate 2	0a	0a	0.8d	2.0c	9.2c	15.6c	23.2c	8.47 c	4.24 c
Isolate 3	0a	0a	1.2c	2.8c	7.6d	12.0d	21.2c	7.47 c	3.74 c
Isolate 4	0a	0a	2.8b	10.0a	16.8b	28.8b	46.8b	17.53 a	8.77 a
Isolate 5	0 a	0a	3.6a	10.8a	15.2b	29.2b	50.0a	18.13 a	9.07 a

*Averages followed by the same letter within a column are not significantly different from each other at 5% (Duncan).

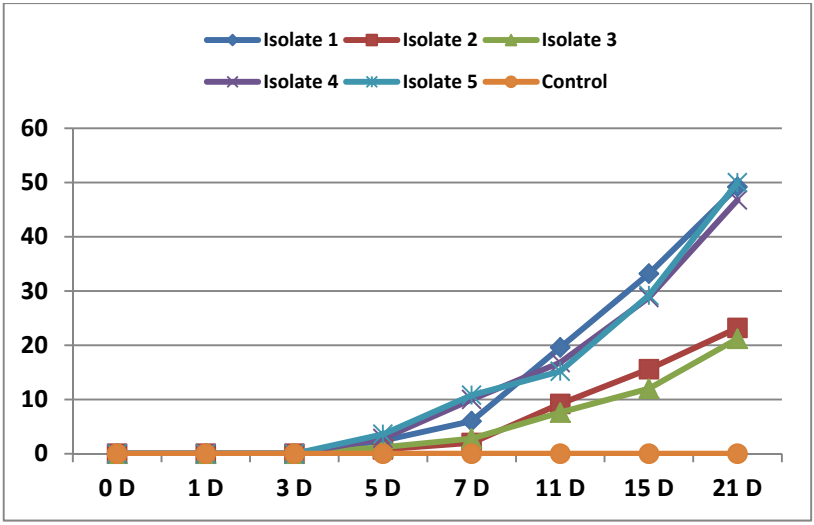

Fig. 48.7. Average daily mortality percentage of *B. tabaci* eggs by five isolates of *Cladosporium chlorocephalum* under lab conditions in the Qassim region during 2011–12

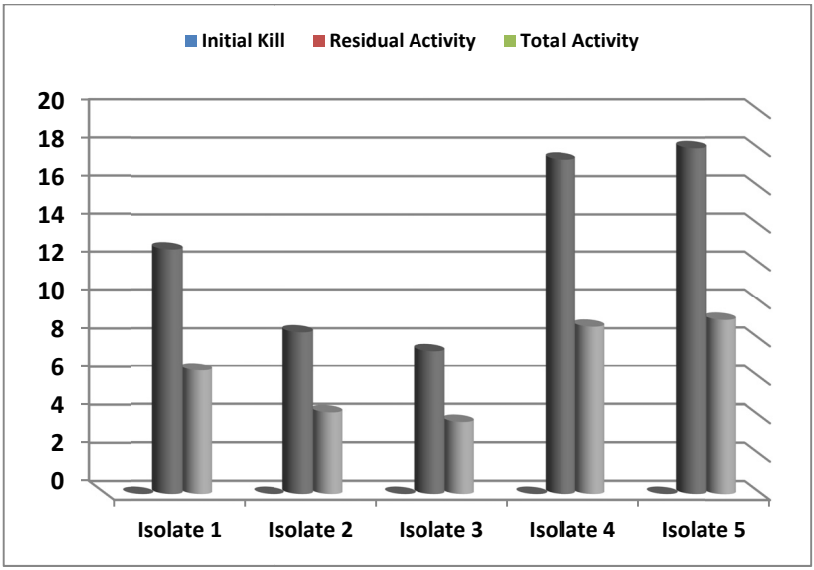

Fig. 48.8. Pathogenicity of *Cladosporium chlorocephalum* isolates against *B. tabaci* eggs after 21 d of treatment under lab condition in the Qassim region during 2011–12

Field bioassay

The data reveal that the pathogenicity of *C. chlorocephalum* isolates in the field was very low compared with lab bioassay studies (see Tables 48.6 and 48.9). Additionally, five isolates of the fungus *C. chlorocephalum* were also pathogenic to immature *B. tabaci* (eggs and nymphs) under field conditions (see Table 48.6 below), and although the initial kill was also low, the residual activity of the isolates was somewhat high (see Fig. 48.10 below). The data show that the pathogenic efficiency of *C. chlorocephalum* isolates differed in terms of the initial kill, days after treatment, residual activity and total activity. The pathogenicity of isolate 1 was the most highly significant causing 53.74% mortality after 21 d, followed by isolate 5 and 4 causing 48.52% and 47.31% after 21 d of treatment, respectively, whilst isolates 2 and 3 reduced *B. tabaci* nymphs by 39.56% and 39.16%, respectively (see Fig. 48.9 below). These results show that *C. chlorocephalum* isolates were lower by about 50% under field conditions in comparison to laboratory conditions (see Tables 48.4 and 48.6 below).

Table 48.6. Reduction percentage in *B. tabaci* nymphs following treatment by five isolates of *Cladosporium chlorocephalum* under field conditions

C chlorocephalum Isolates	Initial Kill (0 Time)	Mortality % (Days after treatment)						Residual Activity (RA)	Total Activity (TA)
		3	**5**	**7**	**10**	**15**	**21**		
Isolate 1	4.56a	13.05a	24.92a	37.88a	45.88a	52.84a	53.74a	38.052 a	21.31 a
Isolate 2	3.62a	6.91b	11.30c	20.55 b	27.38c	36.29c	39.16c	23.61 c	13.62 bc
Isolate 3	3.23a	6.59b	10.97c	18.88 b	22.13e	37.09c	39.56c	21.20 d	12.22 c
Isolate 4	2.69a	7.19b	13.38bc	21.91 b	32.51b	43.51b	47.31b	27.64 b	15.17 b
Isolate 5	3.09a	6.41b	10.35c	16.20 c	25.93d	40.42b	48.52b	24.64 c	13.87 bc

*Averages followed by same letter within a column are not significantly different from each other at 5% (Duncan).

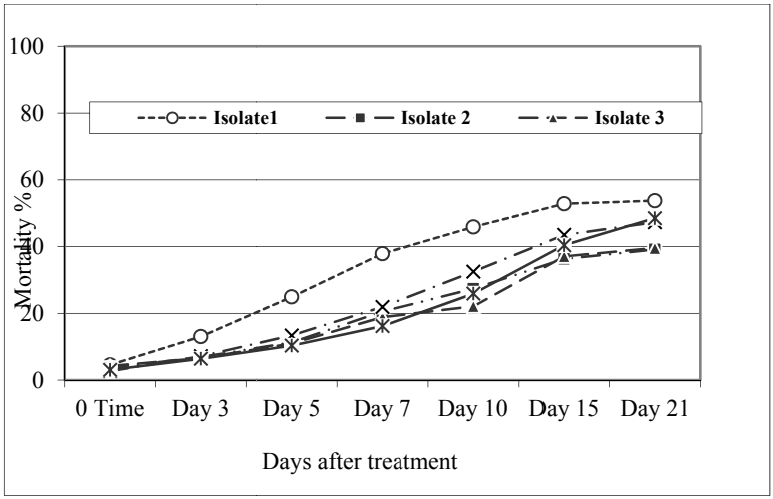

Fig. 48.9. Average daily mortality percentage of *B. tabaci* nymphs by five isolates of *Cladosporium chlorocephalum* under field conditions in the Qassim region during 2011–12

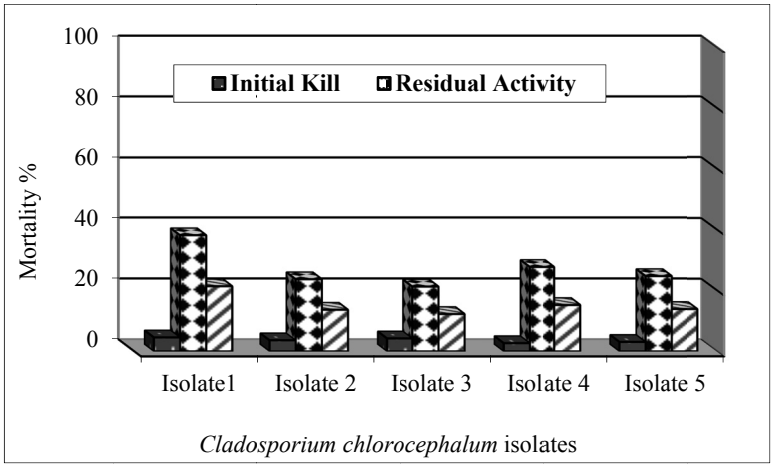

Fig. 48.10. Pathogenicity of *Cladosporium chlorocephalum* isolates against *B. tabaci* nymphs after 21 d of treatment under field conditions in the Qassim region during 2011–12

Effect of certain Ecological Factors on characters of *Cladosporium chlorocephalum*:

Effect of temperature

Data in Table 48.7 and Fig. 48.11 show that temperatures had a great impact on the growth of *C. chlorocephalum* as determined by dry weight. The lowest dry weight was obtained at 10°C with all five isolates. The dry weight increased sharply at 15°C, 20°C and 25°C, then decreased at 30°C and 35°C (see Fig. 48.11 below). Additionally, data showed that the highest dry weight occurred at 25°C. Therefore, the optimum fungal growth was at 20–25°C. These results are in agreement with our previous field results showing the prevalence of the fungus in the autumn compared to other climatic seasons.

Table 48.7. The effect of six temperature degrees on the dry weight of *Cladosporium chlorocephalum* isolates under lab conditions

***C. chlorocephalum* Isolates**	**Dry Weights**					
	10 °C	**15 °C**	**20 °C**	**25 °C**	**30 °C**	**35 °C**
Isolate 1	39.2 f	295.6 e	1814.2b	2331.1 a	516.8 c	432.6 d
Isolate 2	39.2 e	284.3 c	1238.7b	1754.3 a	350.7 c	295.3 c
Isolate 3	39.2 e	235.5 d	1105.5b	1406.0 a	383.3 c	317.8 c
Isolate 4	39.2 d	314.3 c	2653.1a	2854.7 a	453.2 b	434.2 b
Isolate 5	39.2 e	302.6 d	1408.2b	2331.1 a	444.1 c	340.3 d

* Averages followed by the same letter within a row are not significantly different from each other at 5% (Duncan).

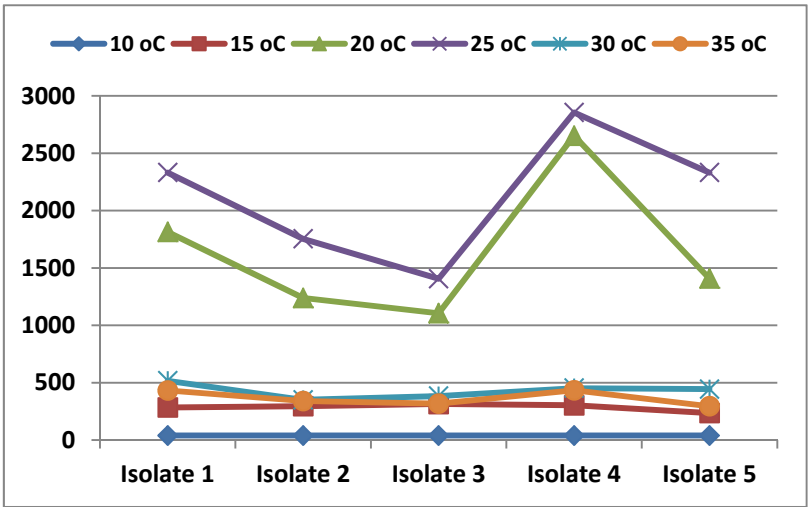

Fig. 48.11. Dry weights of *Cladosporium chlorocephalum* isolates at six temperature degrees

Effect of light durations

Light durations also had an impact on the fungal sporulation (see Table 48.8 below). Darkness and continuous light durations weren't suitable for excessive sporulation of all isolates. Meanwhile, the alternation of light and darkness (12/12 h) was the best for producing a highest yield of the fungal spores (see Table 48.8 below).

Table 48.8. The effect of three light regimes on the number of spores/ ml of five isolates of *Cladosporium chlorocephalum* under lab conditions

Isolates of *C. chlorocephalum*	No. of Spores/ ml		
	24 H Lightness	12 Light/12 Dark	24 H Darkness
Isolate 1	106×10^5 b	198×10^5 a	76×10^5 c
Isolate 2	36×10^5 b	98×10^5 a	20×10^5 b
Isolate 3	48×10^5 b	110×10^5 a	26×10^5 c
Isolate 4	96×10^5 b	114×10^5 a	68×10^5 c
Isolate 5	70×10^5 b	142×10^5 a	58×10^5 c

*Averages followed by the same letter within a row are not significantly different from each other at 5% (Duncan).

Cladosporium chlorocephalum insect host range

All insects used in this trial appeared to be susceptible to *C. chlorocephalum* (see Fig. 48.12 below). Infection among the tested insects ranged from 43.9% to 95.44% based on the insect species. *Cladosporium chlorocephalum* caused 43.9% mortality of alfalfa leaf weevil, *H. postica*, 50% of cotton aphids, *A. gossypii*, 62.5 % of grain aphid *S. avenae*, 66.7% of cabbage aphid *B. brassicae*, and 95.44% of sweet potato whitefly *B. tabaci* (see Fig. 48.12 below). These results confirm the wide host range of *C. chlorocephalum*. Therefore, this entomopathogenic fungus could be an effective tool in the biological control of harmful insect species.

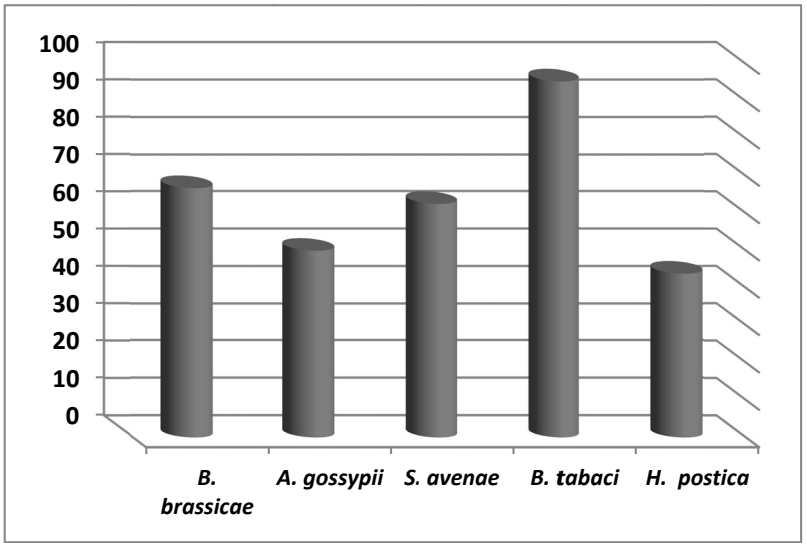

Fig. 48.12. Infection percentages among five insect species by *Cladosporium chlorocephalum* under lab conditions in the Qassim region during 2011–12

Fungal safety to plant hosts

Through a bioassay experiment at the lab we did not notice any symptoms of damage to the plants used as food for experimental insects. The scientific literature on *C. chlorocephalum* did not report any information suggesting that this entomopathogenic fungus causes plant disease symptoms.

Discussion

Microbial control (MC) agents offer alternative methods for the chemical control of whiteflies and can be more effective and selective than chemical pesticides. However, the potential significance of this entomopathogenic fungus has not been completely exploited in KSA due to one or more of the following reasons: (1) lack of information among growers, company owners and officials, (2) the extreme weather conditions during summer and winter, (3) utilization of the fungus as a biological control agent against insects is necessary for licensed commercial usage, and (4) the need for modern techniques to maximize both production and effectiveness (Mensah 2002). In spite of this, MC involves many and various microorganisms, and the entomopathogenic fungi have been used more frequently than other microbial pathogen types in biological control (Abdel-Baky & Abdel-salam 2003; Hajek & Delalibera 2010).

Differences in the fungal effectiveness among seasons, plant hosts and among the target insects are present because each pathogen has its own ecological niche, which must be totally understood to have an epizootic effect under field conditions, so this ecological basis is essential for use of the entomopathogenic fungi in IPM (Fuxa 1987).

The potential for the *Cladosporium* species to be a bio-control agent against certain harmful insect species has been evaluated by different authors around the world (Abdel-Baky et al. 1998; Abdel-Baky 2000; Gui et al. 2005; Yamoah et al. 2008; Eken & Hayat 2009).

Logically, *B. tabaci* nymphs are more sensitive to *C. chlorocephalum* than eggs since *B. tabaci* eggs have a chorion (egg shell) that is hardened from the outside, and may be a drawback for the fungal infection. This may be one of our explanations for why *C. chlorocephalum* was less virulent, causing the lowest mortality rates among eggs despite being a lab bioassay (Buchner et al. 2002). It could be concluded that *C. chlorocephalum* is based on density-dependence. By means of increasing the population density of the insects, the infection by *C. chlorocephalum* will also be increased (see Table 48.2 and Fig. 48.1 above).

Entomopathogenic fungi are affected by different environmental factors which impact the pathogen host through susceptibility, persistence, conidial dispersal, spore germination and production (Fuxa 1987). In the current study, temperatures and light showed direct effects on both fungal dry weight and number of spores/ml (mass production). In this respect, Yeo et al. (2003) reported that some entomopathogenic fungi had the ability to succeed under lab conditions and to fail under field conditions due to uncontrolled conditions as well as the interference of environmental

factors. Therefore, fungal virulence varies greatly among geographical areas and climatic seasons, plant hosts and insect species. It could be concluded that the impact of entomopathogenic fungi requires special conditions for both insects and pathogens to be successful agents (Lacey et al. 2001; Yeo et al. 2003). Therefore, successful uses of entomopathogenic fungi within IPM programs probably require ecological compatibility with other natural enemies.

The promising use of the fungal entomopathogens as bio-control agents is based not only on their efficiency against the target insect pests, but also on their safety against natural enemies and tested plants (Thungrabeab & Tongma 2007). This was confirmed by the current study, which showed no pathogenicity against certain economic plants.

Finally, this study showed that local isolates of *C. chlorocephalum* are highly pathogenic to *B. tabaci* and other sucking and chewing insect pests without harming economic plants and natural enemies. Therefore, it could be a successful candidate for the bio-control of harmful insect pests in the Qassim region. However, detailed studies should be conducted in the future to cover all factors involved in the IPM process in greenhouses and the field.

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