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Biopesticides: Use of Rhizosphere Bacteria for Biological Control of Plant Pathogens

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ABSTRACT

The pesticides used to control pests and diseases are also implicated in ecological, environmental and human health hazards. To reduce the deleterious effects of these agrochemicals, certain antagonistic microorganisms have been characterised from rhizosphere of different crop plants that suppress various plant diseases and thus, minimise the use of pesticides. The application of these specific antagonistic microorganisms in biological control of soilborne pathogens has been studied intensively in the last two decades. These beneficial rhizosphere microorganisms inhibit the pathogenic bacteria and fungi by producing antibiotics, bacteriocins, siderophores, hydrolytic enzymes and other secondary metabolites. The efficiency of these biocontrol products can be improved by manipulation of the environment, using mixtures of beneficial organisms, physiological and genetic enhancement of the biocontrol mechanisms, manipulation of formulations and integration of biocontrol with other alternative methods that provide additive effects. These biocontrol agents could be effectively utilised in sustainable agriculture for improving growth of crop plants.

Keywords: Biological control, plant diseases, plant pathogens, rhizosphere bacteria

1. INTRODUCTION

The world will need 70 to 100 per cent more food by 2050 to feed the ever-increasing human population[1]. On the other hand, various pests and diseases cause 20 to 40 per cent economic loss annually by reducing crop yield, by deteriorating the quality produce and by contamination of food grains with toxic chemicals[2]. For the control of pests and pytopathogens in agriculture, farmers have mostly relied on the intensive application of synthetic pesticides. However, indiscriminate use of chemical pesticides to control the pathogens has generated several problems including resistance to applied pesticide, risks for health of humans and domestic animals due to the long persistence of many pesticides in soil and the entry of residual toxic chemicals in the food chain, contamination of soil and ground water and decrease in biodiversity. These problems have increased interest for development of ecofriendly microbe- based pesticides or biocontrol agents, which act differently from known chemicals[3].

Several microorganisms have been characterised to control various diseases of agricultural crops[4,5]. The analysis of sugar-beet associated bacterial and fungal communities for antagonism towards fungal plant pathogens indicated that the majority of antagonistic microorganisms suppressed only one pathogen, whereas only 4-7 per cent showed a broad antagonistic potential[6]. Suppression of disease by application of biocontrol agents is the continual manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on the plant and the physical environment around the plant. Moreover, some non-pathogenic rhizosphere bacteria may induce the physiological changes throughout the entire plants, making them more resistant to the pathogens. Thus, rhizosphere bacteria are ideal for use as biocontrol agents as they can provide the front line defense for plant roots against the attack by various plant pathogens[7,8].

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2. CHARACTERISATION OF MICROORGANISMS INVOLVED IN BIOLOGICAL CONTROL OF PATHOGENS

Several microorganisms including bacteria, viruses, fungi, protozoa and nematodes, which inhabit the soil or plant rhizosphere, have been identified to suppress the diseases of agricultural crops caused by various pathogenic bacteria and fungi[9,10]. Many rhizosphere bacteria that possess different traits for killing of pathogens are well suited to be used as biocontrol agents (Table 1). Several properties of fluorescent pseudomonads have made them as biocontrol agent of choice. These characteristics include (a) efficient colonisation of the roots, tubers, hypocotyle, etc., (b) ability to utilise a variety of organic substrates usually found in root and seed exudates, (c) their easy cultivation under laboratory conditions, (d) production of a variety of secondary metabolites and (e) their compatibility with commonly used pesticides and other biocontrol agents.

Satisfactory biocontrol was achieved with *Pseudomonas* antagonists in sugar beet[11]. Better disease biocontrol in cucumber was achieved when bacterial antagonists were applied by drenching or by coating seed with bacteria in a peat carrier. Usually *Pseudomonas* strains were found superior to *Bacillus*

Table 1. Biocontrol of pathogens by rhizosphere bacteria

Biocontrol organism	Suppressed pathogen	Disease on crops	Reference
P. fluorescens strain CHA0	*Thievaloviopsis basicola, Pythium ultimum*	Tobacco, Cucumber	20, 21
P. fluorescens strain MDU2	*Rhizoctonia solani*	Rice	22
P. fluorescens strain Pf-5	*Rhizoctonia solani, Pythium ultimum*	Cotton	23
P. fluorescens strain 3521 and B224	*Pythium ultimum*	Cotton	24
P. fluorescens strain 2-79	*G. graminis* var. *tritici*	Wheat	25
P. fluorescens strains Q29z-80	*Pythium ultimum*	Chickpea	26
Pseudomonas putida	*Rhizoctonia solani*	Wheat	27
P. putida strain 98B-27, *Serratia marcescens* strain 90-166	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	28
P. putida WCS358	*Ralstonia solanacearum*	*Eucalyptus urophylla*	29
P. aureofaciens 30-84	*G. graminis* var. *tritici*	Wheat	30
Pseudomonas aeruginosa 7NSK2	*Pythium ultimum*	Tomato	31
Pseudomonas sp.	*Erwinia carotovora*	Potato	32
Pseudomonas sp.	*Pythium ultimum, Rhizoctonia solani*	Cotton	33
Bacillus pumilus	*G. graminis* var. *tritici*	Wheat	34
Serratia plymuthica strain IC14	*Botrytis cinarea, Sclerotinia sclerotiorum*	Cucumber	35
Enterobacter aerogenes	*Phytophthora cactum*	Apple	36
Bacillus subtilis AU195	*Aspergillus flavus*	Cotton	37
B. amyloliquefaciens	*Fusarium oxysporum*	Tomato	38
B. subtilis QST713	*R. solani*	Tomato	39
B. subtilis CA32, *Trichoderma harzianum* R40I	*R. solani*	*S. melongena, Capsicum annuum*	40
Bacillus sp. BPR7	*Macrophomina phaseolina, Fusarium oxysporum, R. solani*	*Phaseolus vulgaris*	41
B. subtilis, Paenibacillus polymyxa, P. fluorescens, P. putida, *Sinorhizobium meliloti*	*F. oxysporum, F. solani, R. solani*	Alfalfa	42
Bacillus subtilis B006	*F. oxysporum* f. sp. *cucumerinum*	Cucumber	43
Bacillus sp. HCS43, *Pseudomonas* sp. HCS36	*R. solani*	Clusterbean	44

antagonists in controlling damping off disease in cucumber and sugar beet. Saikia *et al.*[12] screened 54 fluorescent *Pseudomonas* isolates obtained from broad bean rhizosphere for antagonism against *Macrophomina phaseolina* and *R. solani*, and reported that *Pseudomonas aeruginosa* strain RsB29 caused suppression of Fusarium wilt and charcoal rot of chickpea, and promoted the plant growth of broad bean. Ramette *et al.*[13] found that *Pseudomonas* populations produced the biocontrol compounds viz. 2,4-diacetyl phloroglucinol and hydrogen cyanide in the rhizosphere soil of tobacco, which resulted in suppression of root rot disease. Similarly, *P. fluorescens* strain CHA0 was found to produce several secondary metabolites, notably HCN, 2,4-diacetyl phloroglucinol, pyoluteorin and indole acetic acid[14].

Seven biocontrol strains, i.e., six Gram-negative and one Gram-positive bacteria were identified (from 216 bacterial strains isolated from 17 rhizosphere soil samples) for their ability to control tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) in stonewool[15]. *Pseudomonas fluorescens* strain WCS365 performed well in a competitive tomato root-tip colonisation assay. These biocontrol strains were identified as three *Pseudomonas putida* strains and one strain each of *Delftia tsuruhatensis*, *Pseudomonas chlororaphis*, *Pseudomonas rhodesiae* and *Paenibacillus amylolyticus*. Out of 284 isolates obtained from apple trees' rhizosphere in Iran, four *Pseudomonas fluorescens* strains (P60, P61, P96, and P97) showed reduction in the disease by dipping the crown and root of apple seedlings in greenhouse trials[16]. Soil drench method of application was found more effective than dipping the crown and root of apple seedlings. The *P. fluorescens* strain P60 in dipping method combined with soil drench exhibited greatest effect (with 70 per cent control) on reducing the crown and root rot after 6 weeks and it was found even more effective than the fungicide metalaxyl-mancozeb.

Among the 563 bacteria obtained from the roots of pea, lentil and chickpea grown in Saskatchewan[17], 26 isolates (5 per cent) suppressed the growth of *Pythium* sp. strain p88-p3, 40 isolates (7 per cent) suppressed the growth of *Fusarium avenaceum* and 53 isolates (9 per cent) suppressed the growth of *Rhizoctonia solani* CKP7. Fatty acid profile analysis and 16S rRNA sequencing of the isolates showed that 39 to 42 per cent of the isolates were the members of *Pseudomonadaceae* family and 36 to 42 per cent of the *Enterobacteriaceae* family. Fluorescent *Pseudomonas* isolates PGC1 and PGC2 showed the antifungal potential against the two most destructive

phytopathogens i.e., *Rhizoctonia solani* and *Phytophthora capsici*[18]. The production of chitinase and β-1, 3-glucanase was involved in the growth inhibition of *R. solani*. However, antifungal metabolites of a non-enzymatic nature were responsible for inhibition of *P. capsici.* Thus, multiple and diverse mechanisms are adopted by the same antagonist to suppress different phytopathogens, as evidenced in case of *R. solani* and *P. capsici*. *Bacillus subtilis* strain E1R-J isolated from wheat roots inhibited the mycelium growth *in vitro* of *Gaeumannomyces graminis* var. *tritici* (Ggt), *Coniothyrium diplodiella*, *Phomopsis* sp. and *Sclerotinia sclerotiorum*[19]. The bacterial strain reduced the take-all disease significantly caused by Ggt, under greenhouse experiment by 70.7 per cent (10^{12} cfu ml^{-1}) as compared to uninoculated control. Treatments with the bacterial strain E1R-J and the fungicide triadimefon were found to reduce take-all disease in wheat roots by 55.3 per cent and 61.9 per cent, compared to the inoculated control plants.

Ahmadzadeh and Tehrani[45] observed that antagonistic rhizobacterial isolates obtained from the rhizosphere of wheat and common bean, including isolates UTPF7, UTPF13, UTPF18, UTPF22, UTPF27 and *P. fluorescens* strain CHA0 produced HCN and protease enzyme. Some isolates also produced prolific amounts of siderophores and formed a large orange zone of up to 160 mm^2. Seventeen of 41 isolates of fluorescent pseudomonads including strain CHA0 produced different amounts of antibiotic diacetyl phloroglucinol (DAPG) ranging from 0.6 to 11.4 ng/10^8 cfu. Six of the 41 antagonistic rhizobacterial isolates showed antifungal activity against the pathogen *Rhizoctonia solani*. Sundaramoorthy and Balabaskar[46] isolated 25 native bacterial antagonists from healthy tomato rhizosphere soil in different geographical regions. The combined application of antagonistists i.e., *Pseudomonas fluorescens* and *Bacillus subtilis* was found to effectively inhibit the mycelial growth of the pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FOL) (by 40 per cent) under *in vitro* conditions, when compared to the application of individual strains of the bacterial antagonists. Under greenhouse conditions, the combined application of antagonistists EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) exhibited the highest disease reduction.

Four strains of rhizobacteria i.e., *Bacillus subtilis, Paenibacillus polymyxa, Pseudomonas fluorescens* and *Pseudomonas putida* alongwith *Sinorhizobium meliloti* were tested for their antibiosis toward damping-off disease and on the growth and yield of alfalfa crop[42]. These rhizobacterial strains strongly inhibited the growth of *Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani in vitro. In vitro,* four rhizobacterial strains produced hydrogen cyanide (HCN), siderophore and showed protease and β-1,3-glucanase activities. Under greenhouse and field conditions, seed treatment with these selected strains significantly reduced alfalfa damping-off disease incidence as compared to untreated control. *Pseudomonas fluorescens* (*Pf*1) and *Bacillus subtilis* (*Bs*1) were found as the major potential biocontrol agents against the foliar pathogen *Alternaria helianthi*[47]. These biocontrol agents showed maximum capacity in controlling the spore germination. Moreover, seed-borne infections of *A. helianthi* were controlled by seed treatment with *P. fluorescens*.

Significant growth inhibition of several pathogenic fungi *in vitro* was reported by *Brevibacillus laterosporus* strain JX-5 isolated from the poplar rhizosphere[48]. Its main antifungal component, designated as component B, reduced *Botryosphaeria dothidea* associated canker of the excised poplar branch by 70 and 90 per cent, respectively. Bioactive metabolic product was found to inhibit the growth of *Botryosphaeria dothidea* by permeating the fungal membrane, fracturing the nuclei, damaging the cell wall and eventually killing the pathogenic fungus. Abialaa and Odebodea[49] reported that two rhizobacterial isolates, out of the 19 isolates examined, showed significant chitinase enzyme activity and antifungal activity with evidence of no or low disease expression in maize seedling. The combinations of selected *Enterobacter* isolates (OSR7 and IGGR11) completely suppressed pathogenic activity of *Fusarium verticillioides* on maize seedling with evidence of no disease symptoms. Distinct variations in the microbial community of cotton rhizosphere were demonstrated between monocropped (4- and 15-year) rhizosphere soils and fallow (control) agricultural soil[50]. The monocropped soils significantly influenced cotton growth and root exudates, and also reduced soil suppressiveness to Fusarium wilt in bioassay experiments. Some disease-suppressive bacterial taxa, including *Xanthomonadaceae, Comamonadaceae, Oxalobacteraceae* and *Opitutaceae* were found associated with healthy cotton.

3. MECHANISMS INVOLVED IN BIOCONTROL OF PATHOGENIC FUNGI

The mechanisms by which antagonistic microorganisms inhibit the growth of phytopathogenic microorganisms includes: (i) antibiotic and bacteriocins production; (ii) production of siderophores; (iii) production of hydrolytic enzymes such as β-1,3-glucanase and chitinases; (iv) production of other metabolites and volatile organic compounds; (v) phytoalexins production and induction of systemic resistance[3,51]. For example, *Pseudomonas fluorescens* strains obtained from the rice rhizosphere were found to inhibit the blight fungal pathogen, *Rhizoctonia solani* by the production of chitinase, β-1, 3-glucanase, siderophores, salicyclic acid and hydrogen cyanide[22]. A significant relationship between the antagonistic potential of *P. fluoresecens* against *R. solani* and the production of β-1, 3-glucanase, salicyclic acid and hydrogen cyanide was observed. Four endophytic *Bacillus* strains, named BL1, BT5, BR8 and BF11, isolated from healthy tissues of roots, stems, leaves and fruits of tomato plants, showed growth inhibitory activity against *Botrytis cinerea*[52]. Electrospray mass spectrometry analysis showed that these strains produced heterogeneous mixture of antibiotics belonging to fengycin and surfactin for BL1 and BT5, to iturin and surfactin for BR8, to bacillomycin D, fengycin and surfactin for BF11.

3.1 Production of Antibiotics and Bacteriocins

Antibiotic production by microrganisms is one of the major mechanisms involved in growth inhibition of pathogenic fungi. These antibiotic compounds may act on plant pathogenic fungi by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia, or by exerting fungicidal effects. A large number of antibiotics including diacetyl phloroglucinol, oomycin A, phenazines, pyocyanine, pyrroles, pyoluteorin and

pyrrolnitrin etc. are produced by rhizobacteria[53], which help in the suppression of growth of pathogenic fungi (Table 2). Gurusiddaiah[25], *et al.* showed that *Pseudomonas fluorescens* strain 2-79 obtained from wheat rhizosphere, suppressed the take-all disease of wheat root caused by *Gaeumannomyces graminis* var. *tritici*. This antibiotic was found active against several fungi including *G. graminis* var. *tritici*, *Rhizoctonia solani* and *P. aristesporum. P. fluorescens strain* 2-79 RN_{10}, which acted as biocontrol agent of take-all of wheat, produced the antibiotic phenazine-1-carboxylic acid[54]. Inverse relationship between the population size of phenazine-producing *P. fluorescens* strain 2-79 RN_{10} and the number of lesions formed by *G. graminis* var. *tritici* was observed.

Table 2. Antifungal activity and antibiotics produced by fluorescent pseudomonads

Antibiotics	Producing bacteria	Target pathogen	Target disease
Phenazine-1-carboxylic acid (PCA)	*P. fluorescens* 2-79	*G. graminis* var. *tritici*	Take-all disease of wheat
	P. aureofaciens 30-84	*G. graminis* var. *tritici*	Take-all disease of wheat
2,4-Diacetyl-phloroglucinol (DAPG)	*P. fluorescens* CHA0	*G. graminins* var. *tritici*	Take-all disease of wheat
	P. fluorescens Q2-87	*Thielaviopsis basicola*	Black root- rot of tobacco
	P. fluorescens F113	*Pythium ultimum*	Damping-off of sugar beet
	P. fluorescens Pf5	*Rhizoctonia solani*	Sheath blight
Pyrrolnitrin (Prn)	*P. cepacia*	*Bipolaris maydis*	Southern maize leaf blight
	P. fluorescens Pf5	*Sclerotina homoecarpa*	Dollar spot of turf grass
Pyoluteorin (Plt)	*P. fluorescens* Pf5	*Pythium ultimum*	Damping-off of cotton
Iturin A and Surfactin	*Bacillus subtilis* RB14	*Rhizoctonia solani*	Damping-off of tomato

Pseudomonas strain F113 isolated from the rhizosphere of sugar beet, inhibited a range of plant pathogenic fungi and the antibiotic 2,4-diacetyl phloroglucinol (DAPG) was identified[55]. *P. fluorescens* strain CHA0 was isolated from rhizosphere of tobacco grown near Payerne, Switzerland, from the soil naturally suppressive to black root rot of tobacco[20]. Strain CHA0 was found to produce a variety of secondary metabolites i.e. pyoluteorin, DAPG, hydrogen cyanide, salycilic acid, pyochelin and pyoverdine, and protected various plants from diseases caused by soil borne pathogenic fungi[21]. Mutant strain CHA625 lacking the production of DAPG antibiotic showed reduced suppressive effects and the antibiotic-overproducing strains showed improved biocontrol abilities in several host-pathogen systems. The genes for the biosynthesis of many of the metabolites involved in disease suppression by fluorescent pseudomonads have been isolated and their regulation has been studied[56,57,58].

Mutation in the global regulator gene *gac*A (GacA$^-$) of *P. fluorescens* CHA0 was obtained and the mutant derivative did not produce phloroglucinol, pyoluteorin and HCN but GacA$^-$ mutant overproduced pyochelin and pyoverdine[59]. GacA$^-$ mutant failed to protect the dicotyledonous plant cress and cucumber against damping-off caused by *Pythium ultimum*. In contrast, the GacA$^-$ mutant could still protect the gramineae wheat and maize against damping-off mediated by *P. ultimum* and wheat against *G. graminis* var. *tritici*. Hammer[60], *et al.* cloned 6.2-kb DNA region, which encoded the biosynthetic pathway of the antibiotic pyrrolnitrin. This DNA region contained 4 genes, *prnABCD*, each of which was required for pyrrolnitrin production and conferred the ability to produce pyrrolnitrin heterologously in *E. coli*.

Bacillus species are particularly attractive as potential biocontrol agents because they produce stable endospores, which can survive the heat and desiccation conditions that may be faced by biocontrol agents[61-63]. For example, analysis of mutants obtained from *Bacillus cereus* strain UW85*B* showed a significant quantitative relationship between disease suppressiveness and the production of two antibiotics, zwittermicin A and kanosamine[64,65]. The purified antibiotics suppressed the disease and inhibited development of oomycetes by stunting and deforming germ tubes of germinating cysts. Another *Bacillus subtilis* strain RB14, which produced antibiotics iturin A and surfactin, was found to suppress damping-off disease of tomato seedlings caused by *Rhizoctonia solani*[66]. Li[67], *et al*. reported that four mutants of *Bacillus subtilis* strain XF-1 obtained by nitosoguanidine mutagenesis, exhibited significantly higher inhibition activity against *Plasmodiophora brassicae* and *Fusarium solani* than the wild type. Three families of cyclic lipopeptides (CLPs) fengycin, surfactin and iturin were identified from the parent and mutants. The relative contents of CLPs increased substancially in all the four mutants.

Another important class of antibiotics produced by many Gram-negative and Gram-positive bacteria is the bacteriocins. These bacteriocins are usually proteins and they are inhibitory to other related strains of the same species because of their high degree of specificity. One of the first commercial applications of biological control for root diseases has been the use of *Agrobacterium radiobacter* K84 to control the crown gall disease of dicotyledonous plants caused by *Agrobacterium tumefaciens*[68]. *P. fluorescens* strain BC8 produced a bacteriocin fluoricin-BC8 and showed inhibition of growth of virulent *P. solanacearum* strains under *in vitro* conditions[69]. The avirulent *P. solanacearum* strain having the ability to produce bacteriocin suppressed root colonisation by the virulent strain of same species, resulting in reduced incidence of bacterial wilt in tomato and in better plant growth[70]. A novel lectin-like bacteriocin related to LipA has been reported from *P. fluorescens* strain Pf-5[71].

3.2 Production of Siderophores

The utility of siderophore (pseudobactin) production by plant growth promoting rhizobacteria (PGPR) *Pseudomonas* strain B10 was first demonstrated in biocontrol of plant pathogens[72]. Antagonism *in vitro* and fluorescence by the PGPR were not observed when 10 µM ferric chloride ($FeCl_3$) was added to iron-deficient medium. Also, PGPR did not enhance plant growth when ferric iron was added to field soil

in the form of Fe-EDTA). A gene bank from plant growth promoting *Pseudomonas* sp. strain B10 was constructed[73] and complementation analysis of various mutants showed that a minimum of 12 genes arranged in four gene clusters were required for the biosynthesis of pseudobactin.

Siderophore-negative mutants were derived from *P. fluorescens* strains 3551 and B224 by chemical, Tn5 insertion and UV mutagenesis. Sid^- mutants of strain 3551 provided less biocontrol than parent strain against *Pythium ultimum* causing damping-off disease of cotton whereas Sid^- mutants of strain B224 showed less increase in growth of wheat than Sid^+ parent strain in presence of wheat pathogen *P. ultimum* var. *sporangiferum*[24]. Analysis of various siderophore-negative Tn5 mutants showed that pseudobactin (of either pyoverdine and pyochelin type) was necessary to achieve wild-type levels of protection against *Pythium*-induced damping-off [31].

A spontaneous mutant of *P. fluorescens* RS111 was isolated that was less sensitive to antagonism by other strains of fluorescent *Pseudomonas*[74,75]. This mutant, designated as RS111a, appeared to be more effective in suppression of Fusarium wilt than the parental strain. Differences in siderophore production were observed between RS111 and RS111a. On CAS medium, non-fluorescent pseudobactin mutants of RS111 produced halo zone, indicating that RS111 produces more than one siderophore. On the other hand, RS111a only produced pseudobactin, as the non-fluorescent mutants did not produce halos on chromo-azurol S (CAS) supplemented medium. Another *Pseudomonas* sp. strain NJ-101 obtained from agricultural soil produced siderophore and hydrogen cyanide, and also exhibited efficient degradation of the insecticide carbofuran[76]. The growth inhibition of *Fusarium* sp. validated the antagonistic activity of NJ-101 against the common phytopathogens.

Seven *B. megaterium* isolates were found to produce siderophore, IAA, ammonia and HCN[77]. *In vitro* screening for antagonism against *F. oxysporum* revealed significant inhibitory effects on mycelial radial growth by all the seven isolates. Production of IAA and siderophore was highest in the isolate JUMB3 (127 µg/ml and 124 per cent, respectively) and lowest in JUMB7 (35 µg/ml and 44 per cent respectively). Indigenous rhizospheric *Paenibacillus polymyxa* strain CTS-B19 and *Bacillus subtilis* strain CTS-G24 exhibited antagonistic activity against *Fusarium oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola*[78] . *In vitro* detection for fungal wall degrading enzymes revealed that both isolates produced chitinases, β-1,3-glucanases, proteases and cellulases. Siderophores and catalase activities were observed only in *Bacillus subtilis* strain CTS-G24.

3.3 Production of Hydrolytic Enzymes

P. stutzeri strain YPL-1 was found to inhibit the mycelial growth of *F. solani* and caused lysis of mycelia and germ tube[79]. The fungal growth inhibition was correlated with the production of extracellular chitinase and laminarinase enzymes. Chet *et al.*[80] cloned the chitinase encoding gene from *Serratia marcescens* and transferred it into *E. coli*. This chitinase preparation was effective in reducing disease incidence caused by *R. solani* in cotton under green house condition. The partially purified chitinase caused extensive bursting of the hyphal tips of *R. solani*. Subsequently, three different chitinase genes from *Serratia, Aeromonas* and *Trichoderma* were isolated[81]. The cloned genes were expressed in *E. coli* and subsequently introduced into *R. meliloti, P. putida* and *Trichoderma* strains. The transformants resulted in increased chitinolytic activity against *Sclerotium rolfsii* and *Rhizoctonia solani*.

Singh *et al.*[82] observed production of chitinase and β-1,3-glucanase from two chitinolytic bacterial strains, *Paenibacillus* sp. 300 and *Streptomyces* sp. 385, when grown in the presence of colloidal chitin as the sole carbon source. Suppression of Fusarium wilt of cucumber by a combination of these two bacteria might be due to the production of these hydrolytic enzymes. Downing *et al.*[83] transferred cloned *chi*A genes of *Serratia marcescens* and *cry*1Ac7 of *Bacillus thruingiensis* in the sugarcane-associated endophytic bacterium *Herbaspirillum seropodicae*. Expression of the genes in the endophytic bacterium resulted in biocontrol of sugarcane borer *Eldana saccharina*. Similarly, recombinant strains of *Rhizobium meliloti* were constructed by transfer of chitinase encoding genes and expression of chitinase was observed during symbiosis in alfalfa roots[84].

Serratia plymuthica IC14 isolated from soil around melon roots[35] was reported to possess chitinolytic and proteolytic activities and it also produced antibiotic pyrrolnitrin as well as siderophores. Foliar application of strain IC14 protected cucumber against *Botrytis cinarea* grey mold and *S. sclerotiorum* white mold disease of leaves under green house conditions reducing disease incidence by 76 and 84 per cent, respectively. Kohli *et al.*[85] obtained forty bacterial isolates from rhizosphere of sunflower and *Pseudomonas maltophila* was identified as biocontrol agent against the two root-rot pathogens *Rhizoctonia solani* and *sclerotinia sclerotiorum*. It was found to produce chitinase, which was responsible for the lysis of mycelial biomass. Recently, β-1, 3-1, 4-glucanase gene (*Bglu*1) was cloned from *Bacillus velezensis* strain ZJ20[86]. The mycelial morphology of three pathogenic fungi was destroyed by the purified β-1, 3-1, 4-glucanase. Glucanase gene from *B. velezensis* ZJ20 was highly expressed in *E. coli* BL21 and the recombinant protein was found to inhibit the growth of pathogenic fungi.

3.4 Production of Secondary Metabolites and Volatile Organic Compounds

Hydrogen cyanide (HCN) is produced by many rhizosphere bacteria and it has been found to play a significant role in biological control of the pathogens[87]. Statistically significant increase in the suppression of symptoms caused by *Mycophaerella graminicola* and *Puccinia recondita* f. sp. *tritici* on wheat seedling leaves was observed by treatment with HCN over-producing bacterial strains. To assess the role of HCN production in control of *Thielaviopsis basicola* on tobacco, Tn5-derived mutant strain CHA5 lacking HCN production was used alongwith parent strain CHA0. Mutant CHA5 showed significantly less control of tobacco root rot and did not reduce the percentage of infected root surface. Complementation of the mutant CHA5 with HCN^+ genes restored full biological control activity[88].

Bacterial and plant pathogens also cause the plant to synthesise ethylene[89] and the exogenous ethylene often increases with the severity of a fungal infection, while some ethylene synthesis inhibitors significantly decrease the severity of a fungal infection[90,91]. Plant's ethylene concentration is lowered by the inoculation of many plant growth-promoting bacteria through the action of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase[92,93]. Thus, by lowering the ACC level within a plant, the amount of ethylene that can be subsequently formed in the plant, is also reduced. Two biocontrol bacterial strains were transformed with the *Enterobacter cloacae* UW4 ACC deaminase gene and the effect of the transformed and nontransformed bacteria was assessed on the damage to cucumbers caused by *Pythium ultimum*[94]. The ACC deaminase-containing biocontrol bacterial strains were found more effective than biocontrol strains that lacked this enzyme. In addition, one ACC deaminase-transformed biocontrol strain significantly reduced the extent of soft rot of potato slices caused by the bacterial pathogen *Erwinia carotovora* in sealed plastic bags.

Four *P. fluorescens* strains UM16, UM240, UM256 and UM270 showed a high degree of antagonism against the phytopathogen *Botrytis cinerea* and protected the *M. truncatula* plants from infection of *B. cinerea* by reducing stem disease symptoms and browning of roots[95]. *P. fluorescens* strains were found to produce phenazines, HCN and ACC (1-aminocyclopropane-1-carboxylate) deaminase as well as the production of biofilm, siderophores, proteases and indole-3-acetic acid were identified in most of the strains. Sulfur-containing compounds including dimethyl disulfide were among the most abundant volatile organic compounds (VOCs) emitted by all four *Pseudomonas* strains. Only one strain UM270 was found to produce dimethylhexadecylamine, a compound with antifungal and PGP activities.

The volatile organic compounds produced by antagonistic *Paenibacillus polymyxa* strain WR-2 were identified in the presence of root exudates and organic fertilizer[96]. The growth of *F. oxysporum* f. sp. *niveum* was inhibited by 38 per cent, 36 per cent and 40 per cent in agar medium, sterilised soil and natural soil, respectively by the VOCs produced by strain WR-2. This inhibitory effect was increased to 60 per cent, 58 per cent and 64 per cent with the addition of organic fertilizer in agar medium, sterilised soil and natural soil, respectively. The VOCs produced by WR-2 completely inhibited the germination of *F. oxysporum* spores. Out of 42 identified VOCs, seven VOCs i.e., benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone and phenol were found to inhibit the growth of *F. oxysporum*.

3.5 Phytoalexins Production and Induction of Systemic Resistance

Some non-pathogenic rhizobacteria induce physiological changes in the plants due to production of phytoalexins, making them more resistant to pathogens[97]. Phytoalexins synthesis can be used as indicator of enhanced defense mechanism in bacteria-treated plants. Higher phytoalexins concentration was observed in nodules of field grown plants than that from sterile pot cultured plants inoculated with different *R. leguminosarum* strains, suggesting that a plant defense mechanism is induced under field conditions. van Peer *et al.*[98] showed involvement of induced resistance and phytoalexin accumulation in biological control of *Fusarium oxysporum* f. sp. *dianthi* carnation by treatment with *Pseudomonas* sp. strain WCS417r. Bacterisation of roots with WCS4178 decreased the number of diseased plants with cultivars 'Pallas' and 'Lena' by about 50-20 and 69-38 per cent, respectively and there was an increased accumulation of phytoalexins in stems of bacterised and inoculated plants compared with non-bacterised, fungus-infected plants. Similarly, induction and accumulation of phytoalexins in cowpea roots was observed when the plants were infested with mycorrhizal fungus *Glomus fasciculatum* and it caused resistance to Fusarium wilt disease[99]. In mycorrhizal plants, the production of phytoalexins compounds was always higher than in non-mycorrhizal plants. Thus, inoculation of the vesiclular-arbuscular mycorrhiza (VAM) fungus improved plant growth of cowpea plants and imparted tolerance to wilt disease. Goel *et al.*[100] reported that inoculation of fluorescent *Pseudomonas* strain MRS13 in chickpea caused 61.1 per cent and 35.1 per cent increased accumulation of flavonoid-like compounds in cultivars C235 and H8618, respectively in comparison to uninoculated control.

Some biocontrol strains induce a sustained change in the plant and enhance the tolerance capacity of the plant to infection by a pathogen, and this phenomenon is termed as induced systemic resistance (ISR). However, the systemic acquired resistance (SAR) is manifested in providing a subsequent resistance to a broad range of other pathogens, and this resistance is acquired typically a response to a localised infection or an attenuated pathogen[101]. Various non-pathogenic *Pseudomonas* and PGPR strains have been demonstrated to induce systemic resistance in plants, which provides protection against a broad spectrum of phytopathogenic organisms. Thus, prior inoculation of the host plant to a pathogen, avirulent or heat killed pathogens results in induction of systemic resistance that provides protection to the host plant against subsequent inoculation by the virulent pathogens[102]. Induced systemic resistance in plant against a wide spectrum of pathogens has been demonstrated in over 25 crops, including cereals, legumes, cucurbits, solanaceous plants and trees.

When *P. putida* strain 98B-27 and *Serratia marcescens* strain 90-166 were inoculated along with pathogen *F. oxysporum* f. sp. *cucumerinum*, on separate halves of roots of cucumber seedlings, both PGPR strains induced systemic resistance against Fusarium wilt. Inoculation with PGPR strains resulted in delayed disease symptoms and reduced the number of dead plants compared to non-bacterised but *F. oxysporum* f. sp. *cucumerinum*-inoculated plants[28]. SAR-associated proteins were induced by treatment with biocontrol agent *P. fluorescens* strain CHA0 and its inoculation conferred systemic resistance to a viral pathogen and also induced accumulation of salicylic acid, which plays a role in signal transduction in SAR[103,104]. The mutants of *P. fluorescens* strain CHA0 that lacked the ability to produce the siderophore pyoverdine did not induce SAR and it suggested a novel role for bacterial metabolites in disease suppression[105].

Some rhizobacterial strains induce the systemic resistance

by salicyclic acid (SA) signaling in the plants. SA is also a precursor in the production of SA-containing siderophores, such as pseudomonine in *P. fluorescens* WCS374 and pyochelin in *P. aeruginosa* 7NSK2[106]. Therefore, induced resistance by *P. aeruginosa* 7NSK2 involved three siderophores, pyoverdine, pyochelin and salicylic acid, and induced resistance was found to be iron-regulated. Triggering of ISR by the wild-type *P. aeruginosa* strain 7NSK2 depends on a combined action of pyochelin and the phenazine antibiotic pyocyanin. Moreover, the expression of SA biosynthesis genes of *P. aeruginosa* PAO1 in the non-SA-producing *P. fluorescens* strain P3 was found to improve ISR in tobacco against tobacco necrosis virus[107]. Recently, salicylic acid has been found important in providing basal defence to *Solanum tuberosum* against *Phytophthora infestans*[108].

Bacterial production of the volatile 2,3-butanediol has also been found to trigger *Bacillus*-mediated ISR in *Arabdiopsis*[39]. The signaling pathway that is activated in this case depends on ethylene but is independent of salicylic acid and jasmonic acid signaling[109,110]. The transcriptome of rhizobacteria-induced systemic resistance in *Arabdiopsis* revealed that root colonisation by *P. fluorescens* WCS417r did not lead to transcriptional changes in the leaves, whereas in the roots there is a large set of genes that are differentially transcribed[111]. A *myb72* knockout mutant of *Arabdiopsis* no longer expressed WCS417r-mediated ISR, indicating that it plays an important role in signaling in the plant.

4. BIOLOGICAL CONTROL AND GROWTH PROMOTION OF PLANTS

Bacterial strains selected initially for growth inhibition of pathogenic fungi as part of evaluating biological control activity frequently demonstrated growth promotion of the inoculated plants in the absence of target pathogen[112,113]. Similarly, rhizobacterial strains selected initially for growth promotion of plants in the absence of pathogens, showed biological control activity when challenged with the pathogens[8]. Direct growth promotion was obtained when a rhizobacterium produces metabolites that directly promote plant growth without interactions with native microflora[114]. In contrast, production of secondary metabolites such as antibiotics, siderophores and HCN, which inhibited the growth and activities of pathogens and resulted in increased plant growth, are examples of indirect growth promotion by biological control[115]. Voisard[87], *et al.* reported that *P. fluorescens* strain CHA0 increased the deformation of root hairs on tobacco in a pathogen-free gnotobiotic assay. de Freitas and Germida[27] described a similar increase in lateral root hairs and overall root length after seed treatment of wheat with several PGPR strains in gnotobiotic assay. Sindhu[116], *et al.* reported plant growth promoting effects of fluorescent *Pseudomonas* sp. on coinoculation with *Mesorhizobium* sp. *Cicer* strain under sterile and 'wilt sick' soil conditions in chickpea. The coinoculation resulted in enhanced nodulation by *Mesorhizobium* sp. and shoot dry weight was increased by 3.92 to 4.20 times in comparison to uninoculated controls.

Nine different isolates of *Pseudomonas* species containing ACC-deaminase activity were selected from a pool of 233 rhizobacterial isolates obtained from the peanut (*Arachis hypogaea* L.) rhizosphere[117]. *Pseudomonas fluorescens* strain PGPR1 was found to inhibit *Aspergillus niger* and *A. flavus* under *in vitro* conditions. Four of these isolates, *viz.* PGPR1, PGPR2, PGPR4 and PGPR7, produced siderophore and indole acetic acid (IAA). Fluorescent pseudomonad isolates, *viz.* PGPR1, PGPR2 and PGPR4, significantly enhanced the pod yield (23-26, 24-28 and 18-24 per cent, respectively), haulm yield and nodule dry weight in comparison to the control in field trials. Moreover, inoculation of the seeds with *P. fluorescens* isolates, *viz.* PGPR1, PGPR2 and PGPR4, suppressed the soil-borne fungal diseases like collar rot of peanut caused by *A. niger* and isolate PGPR4 also suppressed stem rot caused by *S. rolfsii*. Screening of 563 bacteria obtained from the roots of pea, lentil and chickpea rhizosphere showed that the growth of *Pythium* species strain p88-p3, *Fusarium avenaceum* and *Rhizoctonia solani* CKP7 was suppressed by 5, 7 and 9 per cent of the bacterial isolates, respectively[17]. Canola root elongation was promoted by seventeen isolates (3 per cent) in a growth pouch assay, and out of these, four bacterial isolates promoted the growth of lentil and one isolate also promoted the growth of pea. The quantitative composition of *Bacillus* sp. and *Pseudomonas* sp., and their antagonistic effect towards soil-borne fungi was studied after the cultivation of oats, spring vetch and *Transy phacelia* as intercrop cover plants[118]. *Bacillus* spp. were obtained approximately three times more from soil samples as compared to *Pseudomonas* sp. The antagonistic bacteria out of the enumerated genera occurred more in the soil after oats cultivation and the least in the soil after the cultivation of transy phacelia. Antagonistic *Bacillus* sp. and *Pseudomonas* sp. caused the growth inhibition of *Fusarium oxysporum*, *Haematonectria haematococca* and *Thanatephorus cucumeris*.

The combined application of antagonistists i.e., *Pseudomonas fluorescens* (EPI-5, KPI-7, ANR-2, RTM-3) and *Bacillus subtilis* (KGI-4, PYR-3 and OCM-6) effectively inhibited the mycelial growth of the pathogen *Fusarium oxysporum* f. sp. *lycopersici* under *in vitro* conditions as compared to the application of individual strains of the bacterial antagonists[46]. Tomato plants treated with EPI (Pf-5)+KGI (Bs-4)+KPI (Pf-7) strains exhibited the highest disease reduction and also showed a significant stimulatory effect on plant height. The dry weight of tomato plants were increased upto 27 per cent in comparison to the non-bacterised control under green house conditions and also increased tomato fruit weight. Similarly, combinations of two *Enterobacter* isolates OSR7 and IGGR11 having significant chitinase enzyme activity (CEA > 85 per cent) and antifungal activity against *Fusarium verticillioides*, suppressed the pathogenic activity completely on maize seedlings and their inoculation also significantly enhanced seedling height, stem girth, leaf area, nitrogen and potassium contents[49]. The bioformulation of the antagonistic *Bacillus subtilis* strain NH-100 and *Bacillus* sp. strain NH-217 showed the induction of various defense-related enzymes, suppressed the red rot disease in sugarcane and also enhanced the crop yield under field conditions[119].

However, the major disadvantages in using the PGPR as a biocontrol agent include variability of field performance and

the necessity for precautions to ensure survival and delivery of the product. Moreover, the performance of a given biocontrol agent under field conditions may be restricted to a specific location, due to the effects of soil and climatic conditions. Thus, soil edaphic factors such as temperature, soil moisture, pH, clay content, interactions of biological control microorganisms with other rhizosphere bacteria and with pathogens also affect their persistence, survival and tolerance to adverse conditions. The variable production or inactivation *in situ* of bacterial metabolites responsible for plant growth promotion also contributes to inconsistent performance of PGPR under field conditions.

5. APPROACHES TO INCREASE THE EFFICIENCY OF BIOCONTROL AGENT

Recently, various biocontrol agents have been tested for controlling plant diseases on different crops under field conditions and some of the antagonistic bacterial strains have been released as commercial biocontrol products (Table 3). Some of these biocontrol products were found to control relatively narrow spectrum of diseases on particular host crops. Moreover, it is also well established that root colonisation by the biocontrol agent is the prerequisite to suppress the plant disease. Root colonisation by inoculated bacteria could be improved by increasing the population size, survival of bacteria alongwith manipulation of soil factors that may positively affect colonisation. Bacterial characteristics such as growth rate, cell surface properties, chemotaxis to root exudates, production of secondary metabolites and tolerance to dehydration and temperature have also been found to contribute to competence of inoculated biocontrol agent in the rhizosphere. Use of green fluorescent protein (*gfp*) and *in situ* monitoring based on confocal laser scanning microscope (CLSM) could be applied for understanding of the rhizosphere competence and root colonisation[120].

Screening of mutants directly for increased or decreased ability to colonize the roots is another approach to broaden the array of traits important for colonisation. Mutants of of both phenotypes have been identified from *Pseudomonas* strains and prototrophy for amino acids and vitamins, rapid growth rate, utilisation of organic acids and lipopolysaccharide properties have been found to contribute to colonisation ability. Moreover, modification of the genes involved in the biocontrol activity of biological control agents also plays a key role in improving the rhizosphere competence as well as antifungal activity of biological control agents. For example, the biocontrol activity of *P. fluorescens* carrying PCA coding mini-Tn5 vector was enhanced by introducing *phz*H gene from *Pseudomonas chlororaphis* PCL1391[121]. On the other hand, mutation in the genes encoding for antifungal metabolite 2,4-diacety lphloroglucinol did not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugarbeets[122]. Recently, genes have been identified in biocontrol strains that can be induced or repressed by the presence of phytopathogenic fungi. *In vivo* expression technology (IVET) has been used to show that various genes, including genes encoding diacylglycerol kinase, ABC transporters and outer membrane porins in *P. putida* can be induced in the presence of pathogenic fungi *Phytopthora parasitica.*

Encapsulation of an antagonistic bacterium, *Pseudomonas*

Table 3. List of some commercially available biological control agents

Trade name	Biocontrol organism	Formulation	Target pathogen / disease	Crops tested	Company and country of origin
BlightBan A506	*P. fluorescens*	Lyophilised cells / powder (Wettable)	*Erwinia amylovora* / Fire blight Frost damage control of fruits	Pear and apples Cherry, strawberry, tomato and potato	NuFarm Americas, Burr Ridge, II.
Galtrol Diegal No gall	*Agrobacterium radiobacter*	-	*Agrobacterium tumefaciens* Crown gall	Several crops	Fruit growers chemical Co, New Zealand
Epic Kodiak	*Bacillus subtilis*	Dry powder	*Rhizoctonia solani, Fusarium, Alternaria and Aspergillus* sp. Damping off	Cotton and legumes Cotton and legumes	Gustafsan Inc. Tx. USA
Biocoat	*P. fluorescens* *WCS* 374r	Dust	*F. oxysporum* of *raphani* and *dianthi* Fusarium wilt and carnation wilt	-	S & G seeds, BV, Netherlands
Mycostop	*Streptomyces griseoverdis*	-	*A. brassicicola* Damping-off of crucifers	Crucifers	Kemira Agro, Oy, Finland
Bio-save, 10 LP and 11 LP	*P. syringae* strains ESC-10 and ESC-11	Wettable powder	*Botrytis cinerea, Mucor pyriformis, Geotrichum candidum* and *Penicillium* sp. Postharvest fungal diseases	Citrus and pome fruit	Jet Harvest Solutions, Florida
System	*Bacillus subtilis*	Dust	Seedling pathogens	Bean, barley, cotton and peanut	-
Deny	*Burkholderia cepacia*	Powder	*Pythium* sp. Damping-off	-	CCT Crop, Carlsbad, USAD

fluorescens strain LRB3W1 in alginate polymer was carried out for application on cabbage seeds[123]. Seedlings were transplanted into soil infested with *Rhizoctonia solani*, which causes damping off disease. The damping off disease in encapsulated seedlings was lower than that of untreated control after one week after treatment. Additionally, 2 weeks after germination, the seedlings were inoculated with *Fusarium oxysporum* f. sp. *conglutinans*, a pathogen of cabbage yellows. The yellows disease was less severe with bacterial encapsulation treatment compared with the untreated control. The results indicated that the bacterium survived in the alginate polymer for a prolonged period at 4 °C temperature, thus, encapsulation of cabbage seed with the biocontrol bacterium was found effective for protection against cabbage soilborne diseases.

The biocontrol performance of *Pseudomonas* strains may be improved by the introduction of antibiotic biosynthetic genes[58]. Recombinant DNA strains with greatly increased DAPG and phenazine-1-carboxamide (PCN) production have been constructed[124,125]. The production of DAPG and PCN may be placed under the control of strong promoters or of exudate-induced or rhizosphere-induced promoters to enhance the synthesis of the antibiotics[126]. Moreover, the genes responsible for the production of secondary metabolites and involved in plant growth promotion could be transferred to other rhizobacterial strains possessing good colonising and competitive ability. Moreover, biocontrol strains could be selected, which show a constant and medium-independent production of secondary metabolites. Further, it has been reported that PGPR strains show greater and more consistent disease suppression when mixtures of ecologically diverse strains with similar functions are applied. The efficacy of a biocontrol agent can be further improved by developing the better cultural practices and delivery systems that favour the establishment of biocontrol agent in the soil.

6. CONCLUSION

The interactions between the biocontrol agent, microbial population in the rhizosphere, the plant and the environment are responsible for the variability observed in disease suppression and plant growth promotion. The inconsistency in performance of these biocontrol strains is a major constraint to their wide spread use in commercial agriculture. The application of mixtures of biocontrol agents may be a more ecologically sound approach because it may result in better colonisation and better adaptation to the environmental changes occurring throughout the growing season[115]. Moreover, genetic manipulation of biocontrol bacteria with increased production of toxic compounds or lytic enzymes, improved space or nutrient competence, wider host range or enhanced tolerance to abiotic stress may lead to construction of biocontrol bacteria with improved biocontrol efficacy[127,128].

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