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It is certified that **Ms. M. Sriveni** who was accepted as an Apprentice at ICRISAT, Patancheru, India has satisfactorily completed the assigned experiments as part of the project titled "**Characterization of bacteria with potential to suppress *Fusarium solani* in plate culture**" at ICRISAT in about three months. Much of the work was of very high quality. I have no objection on submitting this work as part of her MSc degree thesis.

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Dated: December 1999

CANDIDATE'S DECLARATION

I, hereby declare that, the dissertation or part there of has not been previously submitted by me/anyone for a degree of any University

NAGARJUNANAGAR **(SRIVENI MANGINA)**
DATED:

Acknowledgement

Time in its winged flight has brought me to the submission of this project work at the end of three months. But, three months have passed out in a swift precision while pursuing the intricacies in the **"Characterization of bacteria with potential to suppress *Fusarium solani* in plate culture".**

I express my immense gratitude to Dr. K. V. Mallaiah, M,Sc., Ph.D., Professor and Head, Department of Botany and Microbiology, Nagarjuna University, Guntur for giving me this beneficial opportunity of taking up a project. My thanks are also due to Dr. M. Vijayalakshmi, Dr. V. Umamaheswara Rao, Dr. M. Raghu Ram, Dr. K. Ammani, Dr. T. Srivalli and Dr. Y. Tirupathi Rao, Lecturers in Department of Microbiology, Nagarjuna University.

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Dedicated

To

My Mom, Dad and Uncle

Contents

Summary

Black root rot caused by *Fusarium solani* is considered a minor disease of chickpea. But chickpea grown after rice has been observed during field visits to have this problem more extensively and needs confirmation. A program of acquiring microorganisms (from natural sources, such as compost) with ability to suppress *Fusarium solani* in plate culture was started at ICRISAT in 1997/98. A large number of bacteria assembled in 1998, was characterized in this study.

A total of 67 bacteria were evaluated for different traits. All the 67 grew well on nutrient agar (NA) and potato dextrose agar (PDA). Most of these grew very fast on PDA. Therefore, quarter strength (¼) PDA was used for studies on colony morphology. Thirty nine of the 67 bacteria were Gram-negative spore forming rods, fourteen were Gram-positive spore forming rods, eleven were Gram-negative (non-spore formers) and one bacterium was Gram-positive (non-spore former). Apparently, all the bacteria were aerobic. Occurrence of Gram-negative spore farming and aerobic bacteria seems a unique finding. Efforts to locate reference(s) of such bacteria in literature are continuing. Because most of the bacteria were spore formers, they survived desiccation for at least 80 days. Four of these bacteria (BCB 116, BCB 122, BCB 123 and BCB 135) suppressed *Aspergillus flavus* (causing aflatoxin of groundnut) in plate culture. One of these, BCB116, was spore former and Gram-negative. Thirty six of the 67 bacteria were studied for their interactions with five rhizobial strains of groundnut. Most of these did not affect growth of the rhizobia. This obviously adds value to the potential importance of some of these bacteria in agriculture.

Characterization of Bacteria with Potential to Suppress *Fusarium solani* in Plate Culture

Introduction

There is a growing concern in recent years, both in developed and developing countries about the use of hazardous fungicides for controlling disease of crop plant. Some chemical pesticides have already been proven to cause adverse effects on humans as well as beneficial natural enemies of pests. As a result, these have been banned for use in developed countries (Chet and Jacob 1994). This concern has lead researchers to develop safer and environmentally friendly pest-control alternatives. Biocontrol, i.e., the use of biological processes to lower inoculum density of the pathogen in order to reduce the disease producing activities, there by reducing crop loss, is a potential non-hazardous alternative. Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health. Disease suppression by bio-control agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. A direct approach in biological control of soil borne plant pathogenic fungi involves the use of antagonistic microorganisms (fungi or bacteria) and applying them by different techniques.

Bacteria associated with root rhizosphere may have beneficial effects on plant growth by providing nutrients and growth factors, or by producing antibiotics, and siderophores, which antagonize phytopathogenic fungi and bacteria. During seed

germination and plant growth, a number of soil bacteria and fungi become intimately associated with the developing rhizosphere. Many of these are soil saprophytes, doing little harm to healthy tissue. However, others cause serious diseases, being pathogenic on a variety of plants of commercial importance. The third class is bacteria living in close association with plant rhizosphere, deriving benefit from root exudates and in turn having beneficial effects on the plant. Direct beneficial effects arise when the bacteria provide the plant with useful products. The most studied case is that of bacterial fixation of atmospheric nitrogen (e.g *Azospirillum* and *Rhizobium*). Indirect beneficial effects of rhizosphere colonizing bacteria may happen by the removal of deleterious microorganisms or chemicals from the environment.

Biocontrol by microorganisms is often attributed to antibiosis. In many biocontrol systems, one or more antibiotics have been shown to play a role in disease suppression. An attractive feature of biocontrol strategies is that population of pathogens resistant to antibiotics produced by biocontrol agents is likely to develop slowly compared to chemical pesticides (Handelsman and Stabb 1996). Biocontrol can also involve suppression of the pathogen by depriving it of the nutrients. The best understood example of this mechanism is iron competition. The biocontrol agents in the atmosphere create a nutrient depleted microenvironment and thereby suppress the growth of pathogens (Handelsman and Stabb 1996). In addition to antibiosis and nutrient deprivation, certain biocontrol agents also reduce plant disease by parasitizing pathogens (Handelsman and Stabb 1996). However, colonization, or even the initial population size of the biocontrol agent has been shown to be significantly correlated

with disease suppression in only a few instances. Some biocontrol agents induce a sustained change in the plant, increasing its tolerance to infection by a pathogen, a phenomenon known as induced resistance (Handelsman and Stabb 1996). The idea that biocontrol agents might induce resistance in the host was first suggested on the basis of experiments showing that bacterial treatments protected potato tubers from subsequent infection by *Pseudomonas solanacearum* (Kempe and Sequeira 1983).

A large number of bacteria with potential to suppress diseases of crop plants were isolated at ICRISAT using a two-layer culture method (Rupela and Gopalakrishnan 1999). *Fusarium solani* known to cause black root rot of chickpea was used as test fungus in this study. Soil from termitaria, organic matter from axis of leaves of *Bilbergia spp.*, compost of certain herbs were the sources of the potential biocontrol bacteria referred to as BCBs. This thesis reports work done on 67 BCBs involving purification, long-term preservation, characterization (Gram staining, sporulation, tolerance to desiccation, biochemical tests) and potential to suppress *Aspergillus flavus*. In addition, it also reports the interaction between *Rhizobium* and the BCBs. Microorganisms that may potentially blacken the chickpea roots [a symptom caused by *Fusarium solani* (Nene et al. 1991)] were also studied.

Materials and Methods

Purification, sub culturing and long-term preservation of BCBs

Bacteria antagonistic to *F. solani* were isolated from various sources, such as compost of different herbs, using the two layer culture method. Some of the isolates when examined were mixtures and therefore it was decided to test purity of all the 67 strains. A streak plating method was used. The plates were incubated at 28±1°C for overnight and observed for isolated colonies. Purified cultures were grown on two slopes each of ¼ PDA and nutrient agar (NA) and in two tubes (5 ml autoclavable plastic tubes) each of (1 ml broth per tube) nutrient broth (NB) and quarter strength potato dextrose broth (¼ PDB). Slopes and the culture tubes were incubated at 28±1°C for over night in incubator and shaker respectively. After the growth was apparent in the culture tubes, 1 ml of glycerol (sterilized separately) was added into each tube and sealed with parafilm. In this way, a set of eight tubes (two each of ¼ PDA slopes, NA slopes, NB tubes, ¼ PDB tubes) was prepared for each strain. One set of NB and PDB tubes of each strain was stored at –13°C and the other at –75°C. The slopes were stored at about 4°C in the refrigerator.

Characterization of the BCBs

The BCBs were studied for cultural, morphological, microscopic (gram staining, spore staining) and some biochemical characteristics. Cultural and morphological characters of the BCBs were recorded during purification.

Gram staining of the BCBs

The standard staining method (Pelczar et al. 1957) developed by Christian Gram in 1884 (Ananthanarayan and Panikar 1990) was used. All the 67 BCBs were stained, observed under low power (10x, 40x) objective and then under oil immersion (100x) lens. Strains with interesting characters were photographed. The reference strains used were *Escherichia coli* (obtained from the Holy-Cross Degree College, BHEL, Hyderabad, A.P.) for Gram-negative and *Bacillus megaterium* (obtained from M.L. Prasad, Sasya Bioremedies, M.G.Road, Vijayawada, A.P.) for Gram-positive.

Tolerance of the BCBs to desiccation

Two strains per plate were grown on petri dishes containing ¼ PDA. After 24 hrs of incubation, 33 plates having 63 BCBs were placed in a desiccator having silica gel at base. The cultures were periodically tested for viability by streaking them on plates having ¼ PDA. After incubation at 28±1°c for 24 hrs the plates were observed for growth. Silica grains were replaced as and when their color changed from blue (dehydrated) to light pink (hydrated).

Sporulation studies with the BCBs

Morphologically spores can be seen and recognized under microscope as they differ physiologically from vegetative cells. Spores are generally heat resistant at boiling point of water or slightly above. If cultures are heated for 10-15 minutes above their maximum growth temperatures, the vegetative cells will be killed and the spores, if present, will remain alive. If such a heated culture produced a growth when transferred

to a suitable sterile cultured media, this was an evidence of the presence of spores that survived heating (William and Richard 1965).

Spore staining. This exercise was aimed to detect the spore forming bacteria among the BCBs. *Escherichia coli* and *Bacillus megaterium* were used as reference strains. All the 67 cultures were stained using the method described in the Manual of the Microbiological Methods (Pelczar et al. 1957). The stained smear of each BCB was observed under low power (10x, 40x) objective and then under oil immersion (100x) lens. Interesting cases were photographed.

Boiling the BCBS. The aim of this experiment was to confirm spore-forming ability of the BCBs and compare the results with those of spore staining. Reference strains were *Escherichia coli* and *Bacillus megaterium*. The inoculum for this test was taken from the 80 days old plates of the desiccation studies described above. A loop full of inoculum of a strain was transferred into a tube containing 10 ml of distilled water. The tubes were placed in boiling water for about 15 minutes, allowed to cool and a loop full of the contents was streaked on plates containing ¼ PDA. The plates and the tubes were incubated at 28°C for overnight and observed for growth. The test was repeated for strains where results of spore staining and of boiling were different. This time the inoculum was taken from the cultures stored at -13°C (the mother source).

Biochemical characterization of BCBs

NEFERM kit developed by LACHEMA (a.s. 62133 BRNO, Karasek 28, Czech Republic) and marketed by Medispan Ltd. (Anna Nagar, Chennai, India) was used for

the studies. The test is designed to identify Gram-negative non-fermenting bacteria up to species level. In this kit dehydrated media are deposited in wells of a micro-titration plate. On each plate there are 12 tests involving glucose, arginine, indole, esculin, citrate, phosphatase, urea, mannitol, xylose, maltose, lactose, nitrate.

Twenty-four hour age test strains were suspended in distilled water. The suspensions were well mixed. Density of the suspensions was made equal to the number 2 Mc Farland's barium sulfate standard. Thoroughly mixed 0.1 ml of the suspension was inoculated into each well of the labeled row. After inoculation, 2 drops of mineral oil were overlaid in column no. 2 (arginine); column no.3 (indole) and column no. 7 (urease).

The plates were incubated for 48 hrs at 30°C. After the incubation period, reagents were added into relevant wells; 1 drop of reagent IND in column no.3 (indole) 1 drop of reagent PHS in column no. 6 (phosphatase) and 1 drop of reagents of NIT in column no 12 (nitrate). The results of the test in columns 1-11 were recorded by matching the colors in the wells with the color chart given in the LACHEMA manual. Into all wells with negative reaction in column 12, zinc powder was added and the color reaction was observed (red color negative; colorless : positive).

Evaluation of BCBs with the potential to suppress *Aspergillus flavus*

Aspergillus flavus, a post harvest disease in groundnut, produces a neurotoxin, aflatoxin in infected seeds, which when ingested by humans and animals, causes severe disorders. BCBs initially isolated as antagonistic to *Fusarium solani* were also tested

for their potential to suppress growth of *Aspergillus flavus*. Dual plate method (Morton and Stroube 1955) was used for the purpose. A spore suspension of *Aspergillus flavus* was prepared in sterile water. The fungus was placed at the center of a plate having Glucose Casamino-acids Yeast-extract (GCY). Four BCBs were spotted such that they were equidistant (3.5 cms) from the center (Fig. 9). Because most of the BCBS were fast growers, these were spotted 24 hrs after fungal inoculation. Each strain had two replications. Plates were incubated at 28±1°C and observed for inhibition zone between bacterial and fungal growth at 5, 7 and 10 days after incubation.

Characterizing interactions between BCBs and rhizobia

All the rhizobial strains used in the study were obtained from the microbial culture collection at ICRISAT. The method to study interactions was standardized using four rhizobial strains (IC59 of chickpea, IC3195 and IC4059 of pigeonpea, and IC 7001 of groundnut, Fig. 11). Five rhizobial strains of groundnut (IC 6006, 7001, 7017, 7029, 7114) were studied for interactions with 35 BCBs. Yeast extract manitol agar (YEMA) was used as the culture medium in plates. On a given plate, four or five rhizobial strains were streaked (5 cm long) in different rows, 1 cm apart (Fig. 11). One BCB strain per plate was streaked on a 5 cm long line perpendicular to the rhizobial cultures at a distance of 5 mm or 10 mm depending on the spreading ability (judged in previous observations) of the BCBs. Rhizobia were inoculated first and incubated for 36 hr before inoculation of a BCB strain. The plates were further incubated for 24 hr and observed for interaction distance and spreading capacity. The distance (in mm) covered by a BCB strain towards rhizobial streak, was called "interaction distance", width (in

mm) of the growth of a BCB strain on the opposite side of the rhizobial streaks was recorded as "spreading capacity" of the BCB strain.

Evaluating new isolates potentially causing black root rot of chickpea

In an earlier pot experiment (Rasheeduddin 1999), none of the 19 BCBs suppressed *Fusarium solani* in pot culture conditions in greenhouse, even though all suppressed growth of the fungus in Dual plate test . Initial symptoms of the disease were noticed even in the pots having autoclaved soil. On inquiry we learnt that Koch's postulates were not tested for the isolate of *F. solani* used for isolation of the BCBs. Twelve microorganisms were isolated from black roots of chickpea in the non-inoculated control pots of the study. Of the 12, three were fungi (PF1, GF1, and GF2) and remaining were bacteria (PB1, PB3, PB4, PB5, PB6, PB7, PB8, PB9, GB1, GB2). These formed the twelve inoculation treatments of the experiment with objective to examine if they formed the disease symptoms on plants grown in sterilized red soils. Sterilized and unsterilized non-inoculated red soil served as the two control treatments. Unsterilized soils from the Punjab Agricultural University (PAU) was used as positive control. The disease symptoms of the black root rot have always been observed in this source. Thus the experiment had 16 treatments. Each treatment was replicated five times.

Plastic pots (15 cm diameter), plastic mesh (to hold soil from falling from holes at base), saucers (to cover the surface of soil), polyethylene beads (to minimize contamination of roots by microorganisms in air), plastic tubes (to water pots

aseptically) and their caps were sterilized by dipping them in 5% Clorox for 48 hours and washed with sterilized deionized water. Plastic mesh was placed at the inside base of every pot before filling with soil. Each pot was kept on the plastic saucer to avoid falling of water drops from the pots on the floor of the glasshouse.

Each strain was first grown in ¼ PDB for overnight and 30 ml of the broth was inoculated into pre-sterilized peat packets of (40 g). The peat packets were incubated for 3 days to allow multiplication of the bacteria. Fungi were grown on ¼ PDA plates to form a lawn. On the day of inoculation into pots, the fungal growth was scrapped and suspended in sterile water (250 ml flasks) and blended aseptically (using test tube blender, Tissumizer Mark II, Tekmark Company, Japan) to break the mycelium into small bits. The resultant suspension was used for inoculating relevant pots.

Red soil was used for growing plants. The soil was twice autoclaved in well-washed gunny bags. Two-kg soil was added per pot. In one treatment soil from PAU, Ludhiana, was mixed with the autoclaved red soil at a rate of 200 gm of PAU soil per 1800 gm of red soil (10%). Five pots of control treatment had 2 kg of unsterilized red soil.

During inoculation, 35 g of the peat with bacterial culture was added per pot and was mixed thoroughly with top 5-7 cm soil. In case of fungal inoculation, 10 ml of the fungal suspension was added to the top 5-7 cm soil and was thoroughly mixed. Each pot after inoculation was watered with 300 ml sterilized water and left undisturbed for

7 days to allow the bacterial and fungal cultures to establish in the soil. Sowing of chickpea variety ICCV 91019 known to have black roots when grown in soil from PAU was done 8 days after inoculation. Chickpea seeds were surface sterilized using mercuric chloride (0.1%) for 2 minutes followed by rinsing with sterilized distilled water.

Five seeds were dibbled sown per pot. Five ml of rhizobial inoculum (a mixture of strains IC59, IC53 and IC76) was added below each seed followed by the addition of 1 ml of bacterial/fungal inoculum into respective treatment pots. In case of control pots and pots with PAU soil, no organism other than rhizobial strains was introduced. Autoclaved ¼ strength Arnon solution (Arnon 1938) and was used for watering the plants. Watering was done when the top 3-5 cm soil was dry.

A sterilized plastic tube (150 mm long and 15 mm diameter) was inserted into each pot for aseptic watering and each tube had a lid at the top. Thinning of chickpea plants was done within a week after emergence to maintain four plants per pot. Soil surface in each pot was covered with 2-3 cm layer of sterilized plastic beads to avoid cross contamination among pots.

Temperature in the glasshouse ranged between 30°C and 35°C during daytime and 28°C and 30°C during night. Evaporative coolers were used to lower the temperature from the ambient which ranged 38°C to 44°C. One set of replications was harvested at day 32 to assess the progress of disease symptoms. The rest of the

replications were harvested at day 58 and the roots were observed for the disease symptoms.

Results

Purification, sub culturing and longterm preservation of BCBs

Purification of BCBs was done using ¼PDA. Simultaneously, the cultural characters and morphology of the purified isolates were also studied. Four of the 67 BCBs (20, 46, 62 and 117) contained two types of colonies which were further purified and named as 20a, 20b, 46a, 46b, 62a, 62b, 117a and 117b. Except four BCBs (26, 110, 152, 153) all the isolates grew on ¼PDA.

Growth of most strains on full strength PDA was very fast such that observations for colony morphology were difficult. Therefore ¼PDA was used in most of the studies. Some common features about most BCBs on were: fast growth, spreading tendency (majority), circular colonies with smooth margins, raised and sticky surfaces, and dull white colonies (Table 1). The colonies of BCB 25 and BCB 113 were non-spreading, non-sticky and milky white in color. The colonies of BCB 124, 125, 127, 129 and 151 were light yellow in color. Yellow pigment in the agar medium was seen around colonies of BCB 122 and 123.

Characterization of the BCBs

Gram staining of the BCBs

All the 67 BCBs were stained out of which, 51 BCBs were Gram-negative and 16 were Gram-positive rods (Fig. 1 to Fig. 5). BCB 14 had Gram-positive rods in pairs

generally aligned in parallel rows (Fig. 1). Out of the 51 Gram-negatives, BCB 25 and 113 were pleomorphic (Fig. 2). BCB 115 was Gram-negative with broad cells arranged in long chains (Fig. 4). BCB 135 was unique with spindle shaped rods and lightly stained (Fig. 5). See Table 2 for morphology of all the cultures as observed under microscope.

Tolerance of BCBs to desiccation

The experiment was started on 7th July 1999 and was still continuing (at the time of writing this thesis). Till 23rd September 1999, all the BCBs, except BCB 135, tolerated desiccation. BCB 64 and BCB 104 had fungal contamination and therefore discontinued, from further studies after observations on 1st September 1999 (85 days after start).

Sporulation studies with the BCBs

The results of staining and boiling revealed that 54 of the 67 BCBs formed spore, 11 were non-spore formers (Table 3). BCB 115 and 136 had dissimilarity in characters when evaluated from the set of materials in desiccation versus boiling studies. Therefore, these two strains need further studies. Most of the sporulation +ve strains had abundance of spores when observed under microscope (Fig. 6) while in some vegetation cells were also present prominently (Fig. 7). No spore was visible in non-sporulating strains when observed under microscope (Fig. 8).

Biochemical characterization of the BCBs

Forty two strains were characterized biochemically using NEFERM kit involving 12 tests. Five of the 12 were on sugars (glucose, mannitol, xylose, maltose and lactose), three involved enzymes (phosphatase, urease, and nitrate reductase), and the rest involved Indol, Esculine and Arginine tests (Table 4). Eleven of the 42 strains were able to use glucose as carbon source (glu +ve), 12 were mannose +ve, 13 were xyclose +ve, 8 were maltose +ve, and 6 were lactose +ve. Two (BCB 25 and BCB 124) were +ve for all the five sugars. Most strains (28 of 42) were +ve for phosphatase and nitrate reductase (37 of 42). All except BCB 118 were urease –ve. BCB 118 was –ve for phosphatase and nitrate reductase. All were Indole –ve and all except BCB 20 were Arginine –ve. Most (36 of 42) were +ve for Esculine test and 34 of 42 were +ve for Simmon's citrate test.

Evaluation of the BCBs for potential to suppress *Aspergillus flavus*

Dual plate method was used for evaluating the 67 isolates. After inoculation of BCBs, observations were made on day 5, 7 and 10. On day 5, eight BCBs (75, 111, 114, 116, 117a, 122, 123 and 135) had clear inhibition zones (Table 5). But when observed on day 7, only four of these (117a, 122, 123 and135) showed inhibition zones (Fig. 9 and 10). These four showed the inhibition zone even on day 10. Another five BCBs (104, 113, 124, 125 and 127) neither suppressed fungal growth nor allowed fungal growth over themselves. Rest of the 53 BCBs were overgrown by the fungus. BCB 151 did not grow in both the replicate plates.

Characterizing interaction between BCBs and rhizobia

Only five rhizobial strains of groundnut and 36 BCBs were studied. Interaction between the different BCBs and rhizobial strains, measured as interaction distance (see Materials and Methods). Spreading capacities of the BCBs was also recorded (see Materials and Methods). In most of the cases, the BCBs did not interfere with the rhizobial growth. Growth of some BCBs (116, 122, 123, 135) remained away from rhizobial growth. It was prominent in plates where BCB 25 and IC 6006, BCB 25 and IC 7017, BCB 63 and IC 7017, occurred together (Table 6).

Discussion

Purification, sub-culturing and long-term preservation of BCBs

Whole of the collection was purified once again by streaking on ¼ PDA plates. Because most of the BCBs were fast growers and unless sufficient cycles of purification were undertaken, there was a possibility of association of some slow growers. Only 4 of the 67 isolates had two types of colonies. The other cultures were pure. All the cultures were preserved in 50% glycerol at -13°C and -75°C. Glycerol prevents dehydration of the cultures. The sub optimal low temperatures will have greatly reduced metabolic activity and growth and lengthened their survival. This will be evaluated in due course.

Tolerance of BCBs to desiccation

The protoplasm of a microbial cell contains 90% water and all the biochemical reactions occur in aqueous state. Hence water is very important for their growth. Microorganisms when applied to crops/soil are expected to face desiccation. Therefore the BCBs were tested for their survival in artificially created low moisture condition in a desiccator. In nature, organisms variously adapt to overcome desiccation. The adaptation may be

through spore formation or production of an extracellular polysaccharide (Robertson and Firestone 1992).

The vegetative cell, in some bacteria, during unfavorable conditions such as lack of nutrients, scarcity of water (desiccated conditions), accumulation of toxins, produce resistant bodies known as endospores. These are more resistant to desiccation than vegetative cells because of less water content and minimal metabolic activities. Sporulation ability was the reason behind the survival of most of the BCBs after 80 days of desiccation. BCB 135, which could not stand desiccation, was found to be a non-spore former. It was further confirmed by sporulation experiments. The studies suggested that we should use one strain per plate in such studies. This should eliminate the scope of contamination between strains.

Gram reaction and sporulation

The principle under lying this technique is the difference in the cell wall composition of Gram-positive and Gram-negative bacteria. Gram negative bacteria have more lipid content (extra lipid bilayer) and less peptidoglycan. The reverse is true with Gram-positive bacteria. In this staining procedure all the bacteria take crystal violet and appear blue. Iodine acts as mordant and fixes crystal violet as crystal violet iodine complex (CVI) in the cell wall. During alcohol treatment the lipid layer gets dissolved resulting in the increased porosity and leakage of the CVI complex of the Gram-negative cells. Due to less lipid content and dehydration by alcohol, the membrane pores of the Gram-positive cells shrink retaining the complex. On further staining with safranine, Gram-negative cells appear pink but Gram-positive cells retain crystal violet

and appear blue (Pelczar et al. 1986). Of the 67 strains processed for Gram staining, majority (51) were Gram-negative and 16 were Gram-positive.

A few genera of bacteria such as *Bacillus* (Gram-positive, aerobe) and *Clostridium* (Gram positive, anaerobe) have the ability to produce resistant survival forms termed as endospores or spores. These are resistant to heat, drying, radiation and various chemical disinfectants. Spore formation takes place in the vegetative cells during unfavorable conditions such as lack of nutrients in the medium, unusual growth conditions or accumulation of toxins.

Once the endospore is formed the vegetative portion of the bacterium is degraded and the dormant endospore is released. It contains all the essential constituents for the survival, surrounded by a series of protective coats including a cortex, a spore coat and sometimes an exosporium. Cortex of an endospore consists of dipicolinic acid associated with calcium ions. The components of cortex and the dehydrated state of the endospore contribute to its resistance (Pelczar et al. 1986).

Spores can be detected morphologically by staining and physiologically by boiling. Morphologically spores are visualized under the microscope as highly refractive, ellipsoidal or spherical bodies. In stained preparations they can be well distinguished from vegetative cells (Fig. 6 and 7). Endospores need heat treatment during staining. Once stained they are difficult to decolorize. Thus endospores retain green color of malachite green where as vegetative cells easily decolorize on washing. Spores are heat resistant up to the boiling point of water or slightly above and thus

differ physiologically from vegetative cells. Heat kills bacteria by precipitating proteins (enzymes). The remarkable heat resistance of spores may be attributed to several factors like low water content inside the spores, calcium ions in the spore coat and spore proteins suspended in lipids than in water (Pelczar et al. 1986).

The reported Gram-negative endospore forming bacteria are *Desulfotomaculum* which is anaerobic and *Oscillospira* which presumably is also anaerobic (Laskin and Lechevalier, 1977). But all the BCBs studied so far are aerobic. Thirty nine of the 67 strains were Gram-negative spore formers. Literature search did not reveal any report on aerobic Gram-negative spore forming bacteria. Further search of literature is needed to ensure if these are a unique group of bacteria. Spore staining observations suggested the presence of both spores as well as vegetative structures in the same strain. It may be due to the staining of dead cells or due to the presence of off-type. Further studies should verify this.

Biochemical characterization of BCBs

Replications varied significantly when the test was performed with BCB 123 suspended in distilled water and physiological saline (0.85% NaCl). Dissimilarity across replicates disappeared when the bacterial strain was suspended in saline. Thus problem was due to the use of water. The biochemical tests reported in the Table 4 were with water as suspension medium. Therefore, all these tests will be repeated.

Evaluation of BCBs for potential to suppress *Aspergillus flavus*

"Dual plate method" was used in which the test fungus was grown at the center of a culture plate and the test bacteria placed at its periphery equidistant from the fungus. Metabolites produced by the different bacteria diffuse in the medium. If these are antagonistic to the test fungus, will restrict its growth in the vicinity of the bacterial colony. The reverse may be true in some cases when a fungus may restrict growth of bacteria. In the absence of antagonistic metabolite from a bacterial colony, fungi generally overgrew the bacterial colony.

Initial tests using ¼ PDA revealed that fungus did not grow well on it while BCBs grew well. On Glucose Casaminoacid Yeast-extract (GCY) both BCBs and *Aspergillus flavus* grew well. Actively growing mycelium was observed as a preferred source of inoculum in place of spores. Spores were difficult to transfer exclusively at the center of a plate without dropping some at other spot on the plate during the transfer.

Out of the 67 BCBs, 8 BCBs (75, 111, 114, 116, 117a, 122, 123 and 135) were antagonistic in the earlier stages of incubation and only four BCBs (117a, 122, 123 and 135) after 5 more days (total 10 days) of incubation. Perhaps the metabolites were restricted around the bacterial colony initially and inhibitory for the fungal growth. But due to prolonged incubation, the metabolite may be diluted and ineffective due to diffusion. Adaptation of fungus to the metabolites on prolonged incubation or short life of the metabolites may be other likely reasons.

Characterizing interactions between rhizobia and BCBs

Several BCBs were antagonistic to disease-causing fungi. The deleterious effect of the BCBs may not be confined to pathogenic fungi. They may adversely affect beneficial organisms living in the soil such as *Rhizobium*. As per the results of this experiment, there was no obvious harmful affect of BCBs on the five rhizobial strains of groundnut used in the study. Since the experiment was performed in artificial conditions, different from the natural root rhizosphere and the soil environment, the results of these studies can at best be indicative.

Evaluating the potential microorganisms resulting in black roots of chickpea

Black root rot of chickpea is a minor disease, which can occur at any stage; affected plants turn yellow and wilt. The dead plants are seen scattered in the field. The root system is rotten; most of the finer roots are shed, and the remaining roots turn black. (Nene et al. 1987). In recent experience, even severely affected plants (almost all the roots including nodules turned black) produced some seeds both in greenhouse and field conditions (O P Rupela, ICRISAT, personal communication). Possibility of these symptoms due to a fungus other than *E. solani* cannot be ruled out. More studies will be needed to sort it out.

In this experiment, we expected the manifestation of the disease in treatment pots with PAU soil. To our surprise, there was no full-fledge development of symptoms in any of the pots. Thus we did not achieve our objective of confirming if any of the isolates were responsible for the black root rot of chickpea. There may be several reasons. This disease occurs most commonly in high moisture (Nene and Reddy

1987) and at moderately high temperatures (25-30°C) (Nene et al. 1991). Even though the experimental temperatures were congenial for the fungus to cause disease, pots perhaps lacked luxurious watering resulting in the lack of proper symptoms.

Since the soil from PAU was in bags for a long time (at least six months), it might have had only the spores and not the mycelium. PAU treated pots contained only 10% PAU soil and 90% of red soil (Alfisol). So, the load of fungus might be too low to cause the disease. Absence of symptoms perhaps indicates to likely management option (perhaps by fallowing during dry season) to contain the disease.

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Table 1. Cultural and morphological characters of the bacteria with potential to suppress the fungus causing black root rot of chickpea.

Strain No.	Size (mm) in 24 hrs.	Shape	Margin	Elevation	Surface texture	Optical features	Consistency	Color
BCB 007	2-3	C	N	F	D	T	D	Co
BCB 014	2-3	C	S	Rc	M	O	St	Dw
BCB 020a	1-2	I	I	F	D	O	St	Dw
BCB 020b	2-3	C	S	Rc	Sh	O	St	Dw
BCB 025	1-2	C	S	Rf	D	O	D	Mw
BCB 029	2-3	C	S	Rc	S,Sh	O	St	Dw
BCB 031	2-3	C	S	Rc	S,Sh	O	St	Wi
BCB 034	>3	C	I	Ra	D	O	St	Dw
BCB 042	2-3	C	R	Ra	D	O	St	Dw
BCB 043	2-3	C	S	Rc	S	O	St	Dw
BCB 044	1-2	I	S	U	Wr	O	St	Co
BCB 045	2-3	C	S	Rc	D	O	St	Dw
BCB 046a	2-3	C	S	Rc	S,Sh	O	St	Wi
BCB 046b	1-2	C	I	G	D	O	D	Dw
BCB 047	2-3	C	S	Rd	S,Sh	O	St	Dw
BCB 051	2-3	C	S	Ra	S,Sh	O	St	Co
BCB 054	1-2	C	S	Rc	Wr	T	Ns	Wi
BCB 055	2-3	C	S	Ra	D	O	St	Co
BCB 056	1-2	I	I	F	D	O	St	Wi
BCB 058	2-3	C	S	Rc	S,W	T	St	Wi
BCB 060	2-3	Ov	S	Rc	Fl	T	St	Dw
BCB 061	>3	I	S	Sr	S	T	St	Co
BCB 062a	>3	C	S	Rc	S	O	St	Dw
BCB 062b	2-3	C	I	F	S,D	T	NS	Dw
BCB 063	1-2	I	I	F	D	O	St	W
BCB 064	2-3	C	S	Ra	Sh	O	St	Dw
BCB 065	>3	C	I	Rc	D	O	D	Dw
BCB 066	2-3	C	S	U	Wr	O	St	Dw
BCB 068	2-3	C	S	Ra	S,Sh	O	St	Dw
BCB 069	2-3	I	S	Ra	S,Sh	O	St	Dw
BCB 072	2-3	I	S	Ra	Sh	T	St	Co
BCB 075	>3	C	S	Ra	S,Sh	T	St	Dw
BCB 083	>3	I	I	Ra	S,Sh	O	St	Dw
BCB 085	>3	C	H	Rc	D	O	St	Dw
BCB 087	2-3	C	S	Rc	M	O	St	Dw

Table 1. Continued

Strain No.	Size (mm) in 24 hrs.	Shape	Margin	Elevation	Surface texture	Optical features	Consistency	Color
BCB 089	2-3	C	S	Rc	S,Sh	O	St	Dw
BCB 094	>3	I	N	Rf	S,Sh	O	St	Co
BCB 097	2-3	C	S	Rd	S,M	O	St	Co
BCB 098	>3	I	S	Sr	D	O	St	Co
BCB 099	2-3	C	S	F	M	O	St	DW
BCB 100	2-3	C	S	Sr	D	O	St	DW
BCB 101	2-3	C	S	Rc	S	O	St	Co
BCB 102	2-3	C	I	Sr	D	O	St	Co
BCB 103	>3	C	S	Ra	S,M	O	St	DW
BCB 104	2-3	I	I	F	S,Sh	T	St	DW
BCB 106	1-2	C	S	Ra	S,Sh	O	St	DW
BCB 107	2-3	I	I	F	D	O	St	DW
BCB 108	2-3	C	S	Ra	Sh	O	St	Co
BCB 109	2-3	C	S	Rc	Sh	O	St	DW
BCB 111	2-3	I	S	Sr	M	T	St	DW
BCB 113	<1	C	S	Rc	Sh	O	Ns	Wi
BCB 114	2-3	C	S	R	Sh	O	St	Co
BCB 115	2-3	C	Ro	F	D	O	Ns	DW
BCB 116	2-3	C	Rh	U	S	O	St	DW
BCB 117a	1-2	C	S	U	S	O	St	DW
BCB 117b	2-3	C	Rh	Ra	S	O	St	DW
BCB 118	>3	C	S	U	Sh	T	St	DW
BCB 122	1-2	C	S	Sr	S	T	Ns	Yp
BCB 123	1-2	C	S	Sr	S	T	Ns	Yp
BCB 124	1-2	C	S	Ra	S	T	V	Y
BCB 125	1-2	C	S	Ra	S	T	V	Y
BCB 127	1-2	C	S	Ra	S	T	V	Y
BCB 129	2-3	C	S	Ra	S	T	St	Y
BCB 130	2-3	C	S	Sr	S	T	St	Y
BCB 135	<1	C	S	F	S	O	Ns	Dw
BCB 136	2-3	C	S	Ra	Sh	O	St	Dw
BCB 151	2-3	C	S	Ra	Sh	T	St	Y

C = Circular
Co = Colorless
D = Dry
Dw = Dull white
F = Flat
Fl = Fluidy
H = Hairy
G = Granular
I = Irregular
M = Mucoid
Mw = Milkwhite
N = Notched
Ns = Non Sticky
O = Opaque
Ov = Oval
R = Radiating
Ra = Raised
Rc = Raised convex
Rd = Raised droplike
Rf = Raisedflat
Rh = Rhizoidal
Ro = Rough
S = Smooth
Sh = Shiny
St = Sticky
Sr = Slightly raised
T = Transparent
U = Umbonate
V = Viscous
W = Wet
Wi = White
Wr = Wrinkled
Y = Yellowish colonies
Yp = Yellow pigment

Table 2. Gram reaction of the bacteria with potential to suppress the fungus causing black root rot of chickpea.

Strain No.	Grams reaction	Size[1]	Shape
BCB 007	-	L	Rods
BCB 014	+	M	Rods in pairs alligned in parellel rows[2]
BCB 020a	-	M	Thin rods
BCB 020b	-	M	Thin rods
BCB 025	-	S,M	Pleomorphic rods[3]
BCB 029	-	M	Rods
BCB 031	-	M	Rods
BCB 034	-	M	Rods in bundles[4]
BCB 042	-	M	Rods
BCB 043	+	M	Rods
BCB 044	+	M	Rods
BCB 045	-	M	Rods
BCB 046a	+	M	Rods in pairs and chains
BCB 046b	-	M	Rods
BCB 047	-	M	Rods
BCB 051	-	M	Rods
BCB 054	-	M	Rods
BCB 055	-	M	Rods in chains[4]
BCB 056	-	M	Rods
BCB 058	-	S	Rods
BCB 060	-	M	Rods
BCB 061	-	M	Rods in chains, pairs and single[4]
BCB 062a	+	M	Rods in pairs, chains and single
BCB 062b	+	M	Rods in pairs and single.
BCB 063	-	M	Rods
BCB 064	-	M	Rods
BCB 065	+	M	Rods in chains, pairs and singles
BCB 066	+	M	Rods in pairs and single
BCB 068	+	M	Rods in pairs
BCB 069	+	M	Rods in pairs
BCB 072	-	M	Rods
BCB 075	-	L	Rods in chains and single
BCB 083	+	M	Rods
BCB 085	+	M	Rods
BCB 087	-	M	Rods in chains, pairs and singles

Table 2. Continued

Strain No.	Grams reaction	Size	Shape
BCB 089	+	M	Rods in pairs
BCB 094	+	M	Rods
BCB 097		M	Rods
BCB 098		M	Rods
BCB 099		S	Rods
BCB 100		M	Rods
BCB 101		M	Rods
BCB 102		M	Rods
BCB 103		M	Rods
BCB 104		M	Rods in single and chains
BCB 106		M	Rods
BCB 107		M	Rods in chains,pairs and single
BCB 108		S	Rods
BCB 109		M	Rods
BCB 111		L	Rods
BCB 113		M	Pleomorphic rods[3]
BCB 114		M	Rods
BCB 115		M	Rods in longchains[5]
BCB 116		M	Rods
BCB 117a		M	Rods
BCB 117b		M	Rods
BCB 118		S	Rods
BCB 122		S	Thin rods
BCB 123		S	Thin rods
BCB 124		S	Thin rods
BCB 125		S	Thin rods
BCB 127		S	Thin rods
BCB 129		S	Rods
BCB 130		S	Rods
BCB 135		S	Thin rods with septa[6]
BCB 136		M	Rods
BCB 151		S	Rods

1. L = Long, M = Medium, S = Short.
2. Characteristic similar to those in Figure 1.
3. Characteristic similar to those in Figure 2.
4. Characteristic similar to those in Figure 3.
5. Characteristic similar to those in Figure 4.
6. Characteristic similar to those in Figure 5.

Table 3. Presence of spores after 60 days on ¼ PDA and viability of different bacteria After boiling for 15 minutes.

Strain No.	Sporulation	Growth after boiling	Strain No.	Sporulation	Growth after boiling
BCB 007	+	+	BCB 089	+	+
BCB 014	+	+	BCB 094	+	+
BCB 020a	+	+	BCB 097	+	+
BCB 020b	+	+	BCB 098	+	+
BCB 025	-	-	BCB 099	+	+
BCB 029	+	+	BCB 100	+	+
BCB 031	+	+	BCB 101	+	+
BCB 034	+	+	BCB 102	+	+
BCB 042	+	+	BCB 103	+	+
BCB 043	+	+	BCB 104	+	+
BCB 044	+	+	BCB 106	+	+
BCB 045	+	+	BCB 107[2]	+	+
BCB 046a	+	+	BCB 108	+	+
BCB 046b	+	+	BCB 109	+	+
BCB 047	+	+	BCB 111	+	+
BCB 051	+	+	BCB 113	+	+
BCB 054	+	+	BCB 114	+	+
BCB 055	+	+	BCB 115	+	-
BCB 056	+	+	BCB 116	+	+
BCB 058	+	+	BCB 117a	+	+
BCB 060[1]	+	+	BCB 117b	+	+
BCB 061	+	+	BCB 118	-	-
BCB 062a	+	+	BCB 122[3]	-	-
BCB 062b	+	+	BCB 123	-	-
BCB 063	+	+	BCB 124	-	-
BCB 064	+	+	BCB 125	-	-
BCB 065	+	+	BCB 127	-	-
BCB 066	+	+	BCB 129	-	-
BCB 068	+	+	BCB 130	-	-
BCB 069	+	+	BCB 135	-	-
BCB 072	+	+	BCB 136	-	+
BCB 075	+	+	BCB 151	-	-
BCB 083	+	+			
BCB 085	+	+			
BCB 087	+	+			

1. Microscopic print of the strain is in Figure 6.
2. Microscopic print of the strain is in Figure 7.
3. Microscopic print of the strain is in Figure 8.

Table 4. Biochemical characterization of the bacteria with potential to suppress the fungus causing black root rot of chickpea.

Strain No.	Glu	Man	Xyl	Mlt	Lac	Arg	Ind	Esl	Sci	Phs	Ure	Nit
BCB 014								+	-	-	-	+
BCB 020a								+	+	+	-	+
BCB 020b	+	+						+	-	-	-	+
BCB 025	+	+	+					+	+	-	-	+
BCB 029	+		+					+	+	-	-	+
BCB 031			+					+	+	-	-	+
BCB 043								-	-	-	-	-
BCB 044								+	+	-	-	+
BCB 045								+	+	+	-	+
BCB 046a		+						+	+	-	-	+
BCB 046b		+	+					+	+	+	-	+
BCB 047			+					+	+	+	-	+
BCB 058			+					+	+	+	-	-
BCB 061								+	+	+	-	-
BCB 064								+	+	+	-	+
BCB 065								+	+	+	-	+
BCB 066								+	-	+	-	-
BCB 069								+	-	+	-	+
BCB 083								+	+	+	-	+
BCB 085								+	+	+	-	+
BCB 087								+	+	+	-	+
BCB 089								+	+	+	-	+
BCB 098								+	+	+	-	+
BCB 099								+	+	+	-	+
BCB 100								+	+	+	-	+
BCB 103								+	+	-	-	+
BCB 104								-	+	-	-	+
BCB 106								+	-	+	-	-
BCB 107								-	+	-	-	+
BCB 108								+	+	+	-	+
BCB 109		+						+	+	+	-	+
BCB 111		+						+	+	+	-	+
BCB 113								+	+	+	-	+
BCB 114								+	+	+	-	+
BCB 115								-	-	-	-	+
BCB 116								+	-	+	-	+
BCB 117a								+	+	+	-	+
BCB 117b								+	+	+	-	+
BCB 118	+	+	+					+	+	-	+	+
BCB 124	+	+	+	+				-	+	+	-	+
BCB 125	+	+	+	+				-	+	+	-	+
BCB 136								+	+	-	-	+

Glu = Glucose, Arg = Arginine, Ind = Indole, Esl = Esculine, Ssl = Simmon's Citrate, Phs = Phosphatase, Ure = Urease, Man = Manitol, Xyl = Xylose , Mlt = Maltose, Lac = Lactose, Nit = Nitrate reductase.

Table 5. Ability of bacterial strains (with potential to suppress the fungus causing black root rot of chickpea) to suppress *Aspergillus flavus* causing aflatoxin of groundnut

Strain No.	Suppression ability[1]			Strain No.	Suppression ability		
	Day 5	Day 7	Day 10		Day 5	Day 7	Day 10
BCB 007	+			BCB 094	-	-	-
BCB 014	-			BCB 097	-	-	-
BCB 020a	-			BCB 098	-	-	-
BCB 020b	-			BCB 099	-	-	-
BCB 025	-			BCB 100	-	-	-
BCB 029	-			BCB 101	+	-	-
BCB 031	-			BCB 102	-	-	-
BCB 034	+			BCB 103	-	-	-
BCB 042	+			BCB 104	+	+	+
BCB 043	-			BCB 106	-	-	-
BCB 044	-			BCB 107	-	-	-
BCB 045	-			BCB 108	-	-	-
BCB 046a	-			BCB 109	-	-	-
BCB 046b	-			BCB 111	+++	-	-
BCB 047	+			BCB 113	+	+	+
BCB 051	+			BCB 114	+++	-	-
BCB 054	-			BCB 115	-	-	-
BCB 055	+			BCB 116	+++	+++	+++ (4mm)
BCB 056	-			BCB 117a	+++	+	-
BCB 058	-			BCB 117b	-	-	-
BCB 060	+						
BCB 061	-			BCB 118	-	-	-
BCB 062a	-			BCB 122	+++	+++	+++ (7mm)
BCB 062b	-			BCB 123	+++	+++	+++ (5mm)
BCB 063	-			BCB 124	+	+	+
BCB 064	-			BCB 125	+	+	+
BCB 065	-						
BCB 066	-			BCB 127	+	+	+
BCB 068	-			BCB 129	+	-	-
				BCB 130	+	-	-
BCB 069				BCB 135	+++	+++	+++ (5mm)
BCB 072				BCB 136	-	-	-
BCB 075							
BCB 083				BCB 151	NG[2]	NG	NG
BCB 085							
BCB 087							
BCB 089							

1. - =Fungus grew over the bacterial colony, + = No inhibition zone but bacterial colony was not covered by the fungus, ++ = Inhibition zone <1 mm, +++ = Inhibition zone > 2mm, 2. NG = No growth of the bacterial strain.

Table 6. Characterizing interactions between rhizobia nodulating groundnut and the BCBs.

Bacterial strain/ rhizobial strain	Spreading capacity of BCBs (mm)		Interaction distance (mm)	
	Mean	Range	Mean	Range
BCB 14/IC 6006[1]	12.0	12-12	15	14-16
BCB 14/IC 7001			19.5	18-21
BCB 14/IC 7017			13	12-14
BCB 14/IC 7029			14.5	13-16
BCB 14/IC 7114			14	14-14
BCB 19/IC 6006[2]	14.0	13-15	5.2	1-10
BCB 19/IC 7001			28.5	4-53
BCB 19/IC 7017			8.5	4-13
BCB 19/IC 7029			6	1-11
BCB 19/IC 7114			4.5	1-8
BCB 20a/IC 6006[1]	13.5	12-15	12	12-12
BC B 20a/IC 7001			7	6-8
BCB 20a/IC 7017			8.5	7-10
BCB 20a/IC 7029			14.5	9-20
BCB 20a/IC 7114			13.5	12-15
BCB 20b/IC 6006[1]	14.0	13-15	15.5	12-19
BCB 20b/IC 7001			11	7-15
BCB 20b/IC 7017			8.5	8-9
BCB 20b/IC 7029			25	15-35
BCB 20b/IC 7114			11	10 -12
BCB 21/IC 6006[1]	13.0	11-15	12	12[3]
BCB 21/IC 7001			12	9-15
BCB 21/IC 7017			11.5	11-12
BCB 21/IC 7029			17	12-22
BCB 21/IC 7114			11.5	11-12
BCB 25/IC 6006[1]	4.5	4-5	2	2-2[4]
BCB 25/IC 7001			5	5-5
BCB 25/IC 7017			2	2-2[4]
BCB 25/IC 7029			3	3-3
BCB 25/IC 7114			4.5	4-5
BCB 29/IC 6006[2]	5.5	5-6	5.5	4-7
BCB 29/IC 7001			4	4-4
BCB 29/IC 7017			4	4-4
BCB 29/IC 7029			4	4-4
BCB 29/IC 7114			10.5	9-12

Table 6. Continued

Bacterial strain/ rhizobial strain	Spreading capacity of BCBs (mm)		Interaction distance (mm)	
	Mean	Range	Mean	Range
BCB 31/IC 6006[2]	20.0	18-22	12.5	10-15
BCB 31/IC 7001			31.5	8-55
BCB 31/IC 7017			8.5	7-10
BCB 31/IC 7029			13.5	12-15
BCB 31/IC 7114			9	8-10
BCB 44/IC 6006[2]	21.0	20-22	13	8-18
BCB 44/IC 7001			35	15-55
BCB 44/IC 7017			15.5	6-25
BCB 44/IC 7029			14	8-20
BCB 44/IC 7114			16.5	8-25
BCB 45/IC 6006[1]	16.5	15-18	14	13-15
BCB 45/IC 7001			60	60-60
BCB 45/IC 7017			15.5	13-18
BCB 45/IC 7029			21.5	20-23
BCB 45/IC 7114			16.5	15-18
BCB 46a/IC 6006[1]	15.0	15-15	16	15-17
BCB 46a/IC 7001			60	60-60
BCB 46a/IC 7017			26	20-32
BCB 46a/IC 7029			28.5	27-30
BCB 46a/IC 7114			21	12-30
BCB 46b/IC 6006[1]	14.5	14-15	10	6-14
BCB 46b/IC 7001			14	13-15
BCB 46b/IC 7017			12.5	12-13
BCB 46b/IC 7029			21.5	15-27
BCB 46b/IC 7114			11.5	11-12
BCB 47/IC 6006[2]	5.5	4-7	8.5	7-10
BCB 47/IC 7001			7	6-8
BCB 47/IC 7017			5.5	4-7
BCB 47/IC 7029			3	3-3
BCB 47/IC 7114			4	4-4
BCB 54/IC 6006[2]	10.0	8-12	11	7-15
BCB 54/IC 7001			35	15-55
BCB 54/IC 7017			10.5	4-17
BCB 54/IC 7029			13.5	7-20
BCB 54/IC 7114			8.5	8-9

Table 6. Continued

Bacterial strain/ rhizobial strain	Spreading capacity of BCBs (mm)		Interaction distance (mm)	
	Mean	Range	Mean	Range
BCB 56/IC 6006[1]	14.0	13-15	12.5	12-13
BCB 56/IC 7001			13.5	12-15
BCB 56/IC 7017			13.5	13-14
BCB 56/IC 7029			15	12-18
BCB 56/IC 7114			12	12-12
BCB 58/IC 6006[2]	9.0	8-10	6	6-6
BCB 58/IC 7001			4.5	3-6
BCB 58/IC 7017			5.5	3-7
BCB 58/IC 7029			6.5	6-7
BCB 58/IC 7114			5	3-7
BCB 63/IC 6006[1]	18.5	17-20	22.5	17-28
BCB 63/IC 7001			11	9-13
BCB 63/IC 7017			10.5	6-15[5]
BCB 63/IC 7029			13.5	7-20
BCB 63/IC 7114			17	14-20
BCB 65/IC 6006[2]	13.5	12-15	8.5	7-10
BCB 65/IC 7001			12	9-15
BCB 65/IC 7017			8	8-8
BCB 65/IC 7029			26	7-45
BCB 65/IC 7114			11	10-12
BCB 66/IC 6006[1]	15.0	15-15	12.5	12-13
BCB 66/IC 7001			10.5	8-12
BCB 66/IC 7017			11	7-14
BCB 66/IC 7029			17.5	16-19
BCB 66/IC 7114			16.5	14-18
BCB 69/IC 6006[1]	10.5	10-11	13.5	12-15
BCB69/IC 7001			10.5	8-12
BCB 69/IC 7017			6.5	16-7
BCB 69/IC 7029			8	8-8
BCB 69/IC 7114			9	8-10
BCB 83/IC 6006[1]	16.5	15-18	21.5	8-25
BCB 83/IC 7001			12.5	12-13
BCB 83/IC 7017			13	13-13
BCB 83/IC 7029			50	40-60
BCB 83/IC 7114			20	20-20

Table 6. Continued

Bacterial strain/ rhizobial strain	Spreading capacity of BCBs (mm)		Interaction distance (mm)	
	Mean	Range	Mean	Range
BCB 87/IC 6006[1]	16.0	15-17	19	18-20
BCB 87/IC 7001			60	60-60
BCB 87/IC 7017			11.5	10-13
BCB 87/IC 7029			36	35-37
BCB 87/IC 7114			12	10-14
BCB 89/IC 6006[2]	14.5	9-20	16.5	8-25
BCB 89/IC 7001			31	7-25
BCB 89/IC 7017			31.5	8-55
BCB 89/IC 7029			32	9-55
BCB 89/IC 7114			17.5	5-30
BCB 98/IC 6006[1]	6.0	5-7	10.5	9-12
BCB 98/IC 7001			14.5	13-16
BCB 98/IC 7017			14.5	13-16
BCB 98/IC 7029			10.5	10-11
BCB 98/IC 7114			13.5	13-14
BCB 99/IC 6006[1]	17.5	17-18	15.5	14-17
BCB 99/IC 7001			43.5	27-60
BCB 99/IC 7017			20.5	13-27
BCB 99/IC 7029			24.5	23-25
BCB 99/IC 7114			19.5	14-25
BCB 100/IC 6006[2]	14.0	13-15	11.5	10-12
BCB 100/IC 7001			12	9-15
BCB 100/IC 7017			10	7-13
BCB 100/IC 7029			27.5	25-30
BCB 100/IC 7114			12.8	8-17
BCB 104/IC 6006[1]	14.0	13-15	8	3-13
BCB 104/IC 7001			36	12-60
BCB 104/IC 7017			13.5	13-14
BCB 104/IC 7029			45	30-60
BCB 104/IC 7114			22.5	20-25
BCB 106/IC 6006[2]	19.0	18-20	7.5	7-8
BCB 106/IC 7001			8.5	5-12
BCB 106/IC 7017			4	1-7
BCB 106/IC 7029			47	34-60
BCB 106/IC 7114			9.5	7-12

Table 6. Continued

Bacterial strain/ Rhizobial strain	Spreading capacity of bacterial strain(mm)		Interaction distance(mm)	
	Mean	Range	Mean	Range
BCB 107/IC 6006[1]	8.5	7-10	11.5	7-16
BCB 107/IC 7001			10	10-10
BCB 107/IC 7017			10.5	9-12
BCB 107/IC 7029			11	10-12
BCB 107/IC 7114			14	13-15
BCB 108/IC 6006[2]	5.5	4-7	12.5	11-14
BCB 108/IC 7001			3.5	3-14
BCB 108/IC 7017			6	4-8
BCB 108/IC 7029			33	6-60
BCB 108/IC 7114			8	4-12
BCB 109/IC 6006[1]	10.0	8-12	9	8-10
BCB 109/IC 7001			9	7-11
BCB 109/IC 7017			8	8-8
BCB 109/IC 7029			9	8-10
BCB 109/IC 7114			9	9-9
BCB 115/IC 6006[1]	8.0	8-8	7.5	7-8
BCB 115/IC 7001			6.5	6-7
BCB 115/IC 7017			5	4-6
BCB 115/IC 7029			6	6-6
BCB 115/IC 7114			7.5	7-8
BCB 116/IC 6006[2]	7.5	7-8	6.5	5-8
BCB 116/IC 7001			3.5	3-4
BCB 116/IC 7017			5.5	4-7
BCB 116/IC 7029			6	5-7
BCB 116/IC 7114			5.5	4-7
BCB 117a/IC 6006[1]	8.0	7-9	10	9-11
BCB 117a/IC 7001			7.5	5-10
BCB 117a/IC 7017			8.5	7-10
BCB 117a/IC 7029			7.5	6-9
BCB 117a/IC 7114			9	8-10
BCB 117b/IC 6006[1]	7.5	7-8	8	4-12
BCB 117b/IC 7001			8	3-13
BCB 117b/IC 7017			4.5	4-5
BCB 117b/IC 7029			7.5	5-10
BCB 117b/IC 7114			6	4-8

Table 6. Continued

Bacterial strain/ rhizobial strain	Spreading capacity of BCBs (mm)		Interaction distance (mm)	
	Mean	Range	Mean	Range
BCB 118/IC 6006[2]	4.5	4-5	21	18-24
BCB 118/IC 7001			12	4-20
BCB 118/IC 7017			3.5	3-4
BCB 118/IC 7029			8	4-12
BCB 118/IC 7114			21.5	20-23

1. = At streaking, the distance between vertical streak (the BCB strain) and horizontal streak (rhizobia) was 10 mm.
2. = At streaking, the distance between vertical streak (the BCB strain) and horizontal streak (rhizobia) was 5.5 mm
3. = A contaminant was present along with the rhizobial strain.
4. = Growth of the BCB strain was away from the rhizobial strain by 3 mm.
5. = Growth of the BCB strain was away from the rhizobial strain by 4 mm.

Figure 1: Gram-positive rods in pairs, aligned in rows, strain BCB 14.

Figure 2: Gram-negative, pleomorphic bacteria, strain BCB 25.

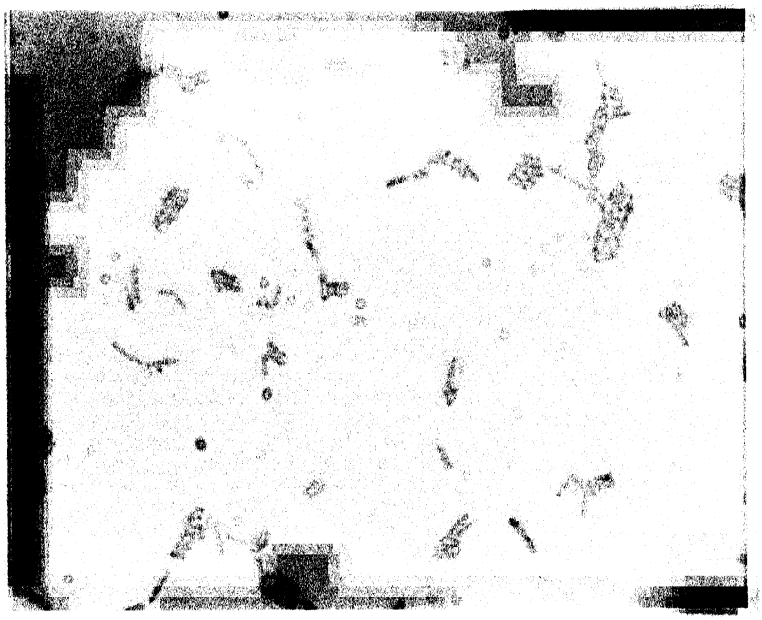

Figure 3: Gram-negative rods arranged in chains, bundles, pairs and singles. The cells in bundles were generally bigger than those in chain. Also, these cells have unstained center which may be spores (strain BCB 61).

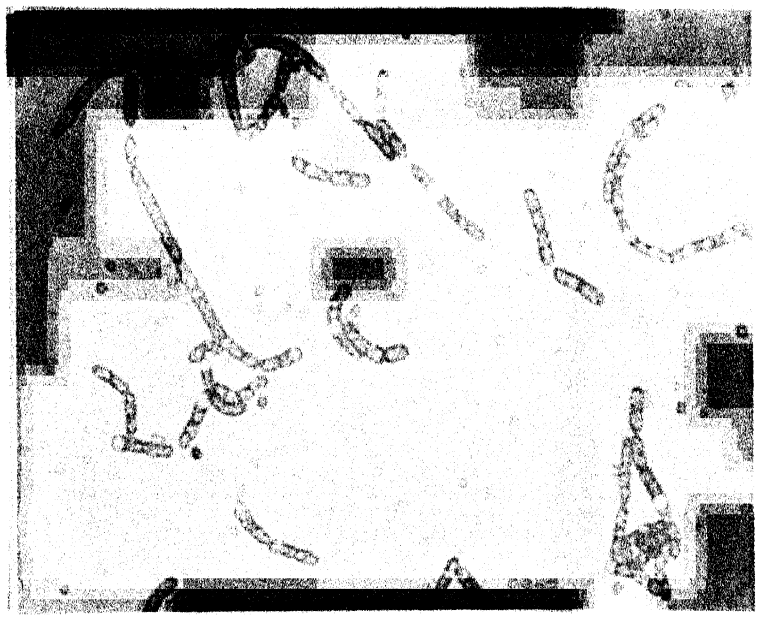

Figure 4: Broad rods in long chains. Some cells in chains are unstained and may be spores (strain BCB 115).

Figure 5: Small rods in pairs. Some cells are spindle shaped, particularly those with unstained centers which may be spores (strain BCB 135).

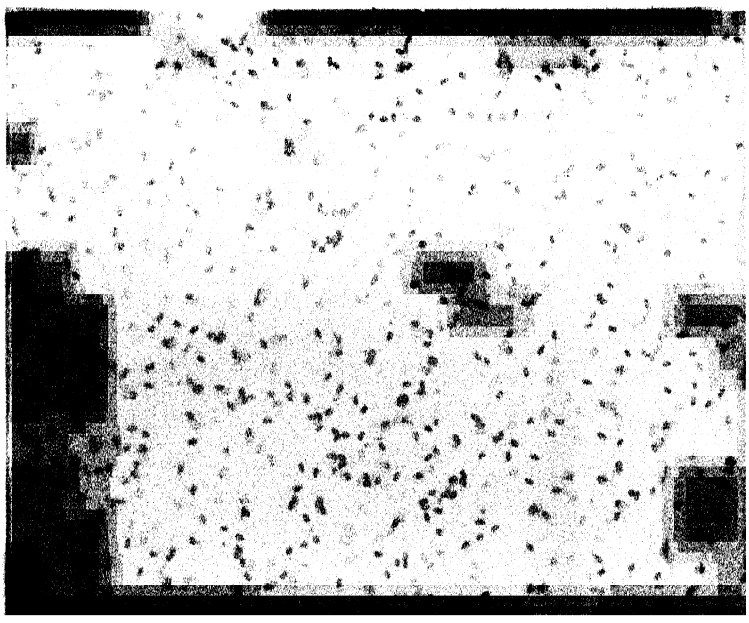

Figure 6: Green colored spores, pink colored vegetative cells. Some vegetative cells have spore (green) inside the vegetative cells (pink). A large number of spores are very lightly stained (strain BCB 60).

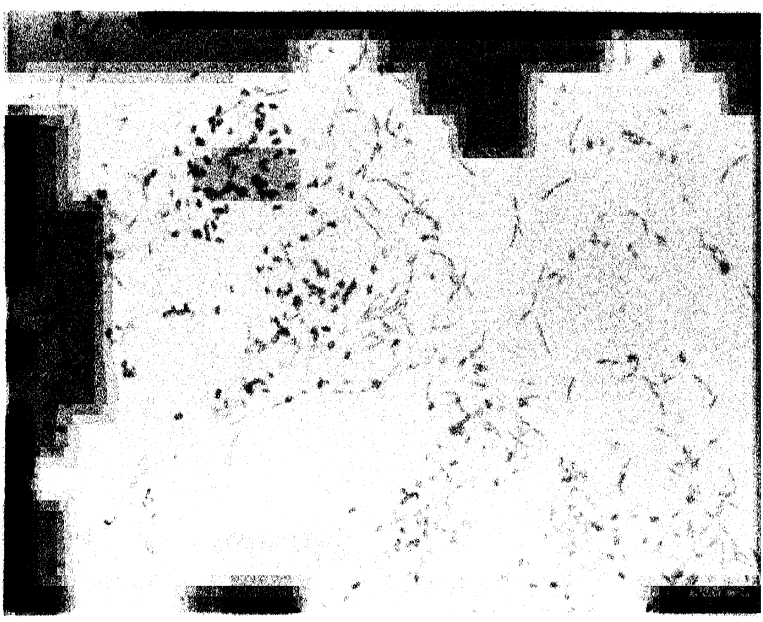

Figure 7: Spore-staining: green spores and pink vegetative cells. The vegetative cells are about twice as long as the spores. Very light pink colored small vegetative cells (small rods) were also visible (strain BCB 107).

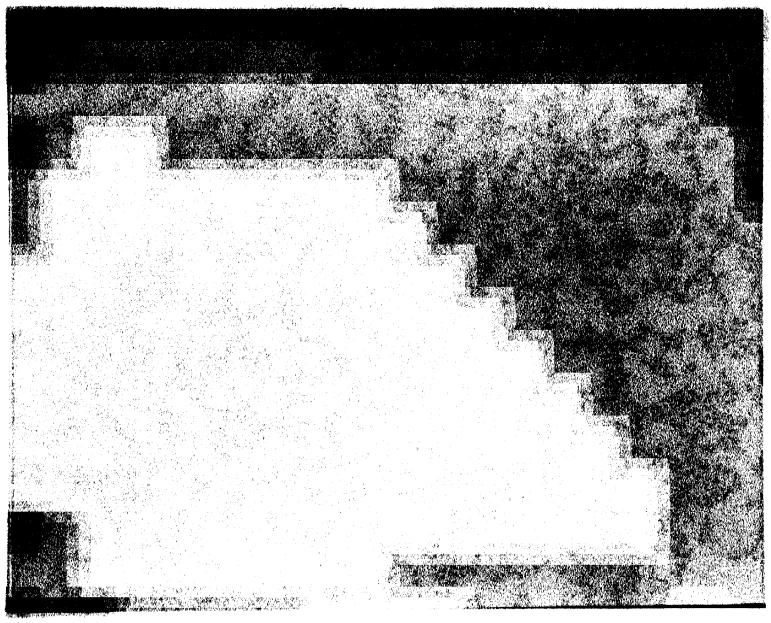

Figure 8: Spore-staining: vegetative cells only, without spores (small rods), strain BCB 122.

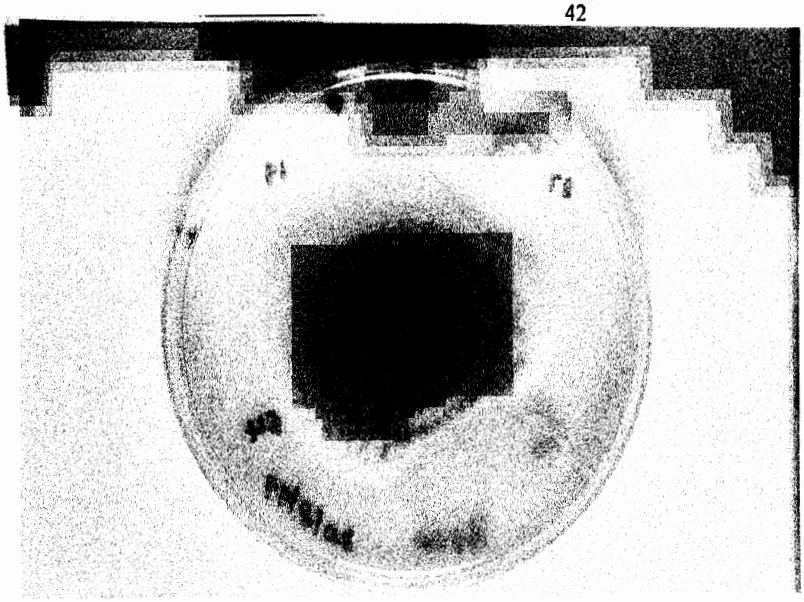

Figure 9: Dual plate test: ***A. flavus*** **is in the middle; BCB 135 (right of the arrow on top) has a clear inhibition zone; BCB 130 (top left) did not suppress the fungal growth; BCB 151 (bottom left) did not grow; BCB 136 (bottom right) had a mild inhibition zone but affected the growth of fungi adversely as indicated by flattening of growth of the outermost concentric ring of the fungus.**

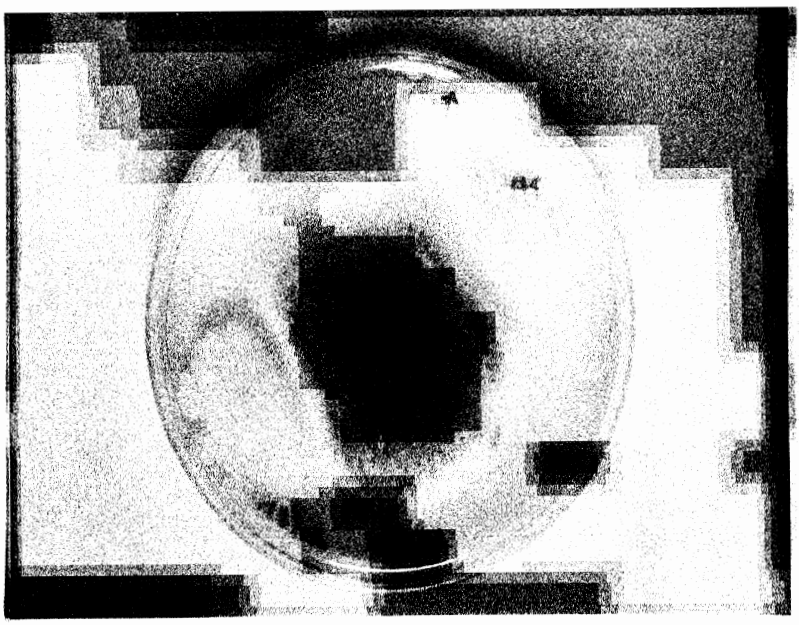

Figure 10: Dual plate test: ***A. flavus*** **is in the middle; BCB 61 (top left of arrow), BCB 111 (top right), and BCB 114 (bottom left) showing inhibition zones and BCB 118 was over-grown by the fungus.**

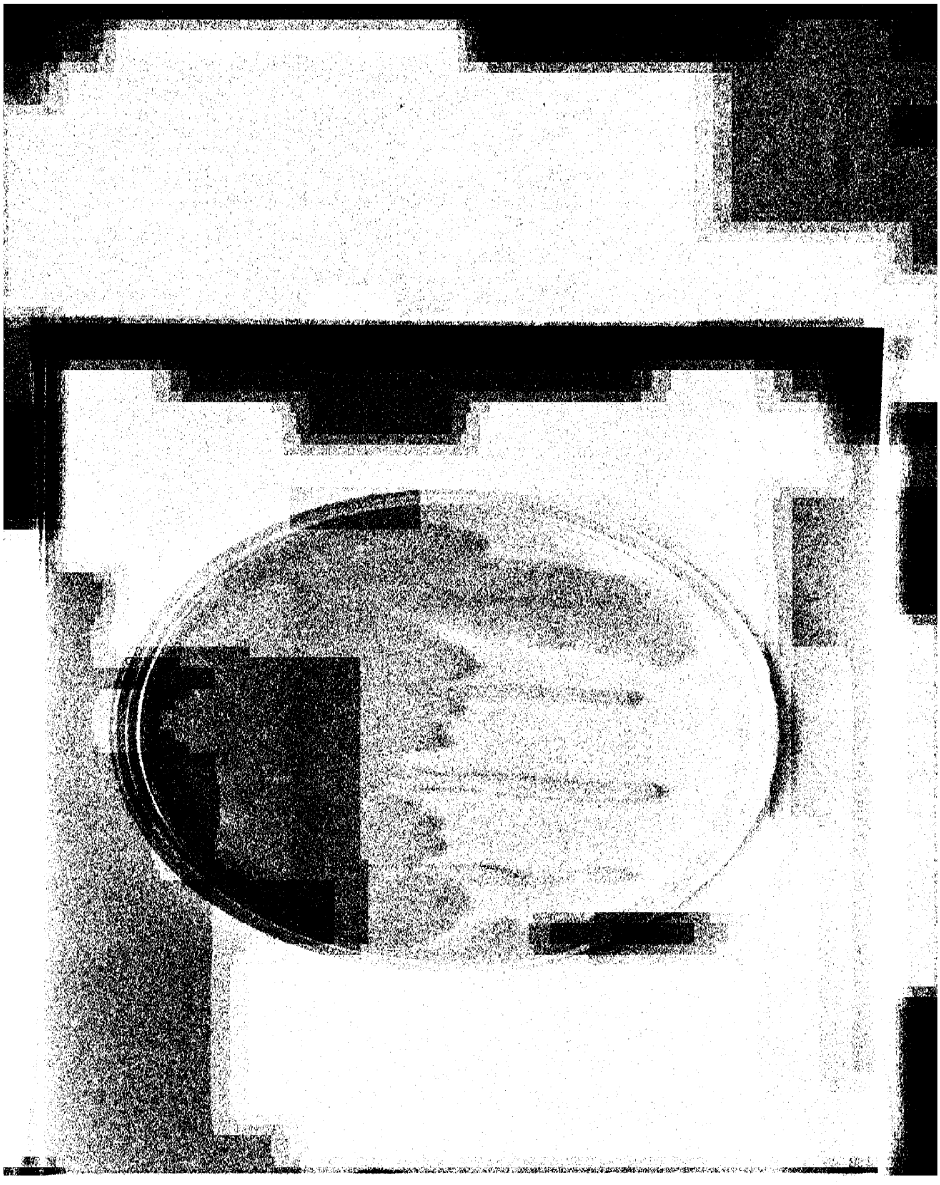

Figure 11: Rhizobia–BCB interaction: BCB 111 on vertisol streak, rhizobial strains (from top right) IC 94, IC 3195, IC 4059, IC 7001 in horizontal streaks. BCB 111 growing completely over IC 94, to small extent on IC 3195 and IC 7001 but its growth front remained away by about 0.1 mm from IC 4059.