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BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* DAMPING-OFF OF BEAN BY *BACILLUS* spp.

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ABSTRACT

Six bacilli strains, five *B. subtilis* and one *B. cereus*, isolated from the rhizosphere of healthy bean, pea, strawberry and peppermint plants grown in heavily infested fields with root rot pathogens were experimented for biological control activity against *Rhizoctonia solani*. *In vitro* studies showed that all the members significantly inhibited (35-48 %, depending upon strain) the mycelial growth of *R. solani*. A clear lytic area appeared in the *R. solani* hyphae that subjected to *Bacillus* metabolites. The production of antifungal metabolites by *Bacillus* candidates reached its maximum (58-71 %) at the end of the stationary phase (18-39 hours) of the tested bacilli. The extraordinary multiplicating bioagents ($3.1\text{-}4.3 \times 10^7$ cfu ml^{-1}) were more efficient in restricting mycelial growth of the pathogen. Mannitol broth deemed the optimum for antifungal production. Inhibition of the fungal mycelial growth positively correlated with increased concentrations of the *Bacillus* cell-free culture filtrate. In greenhouse experiment, treatment of bean seeds with any strain resulted in significant reduction in pre- and post-emergence damping-off and consequently increased survived plants. The two different formulations used, powder and suspension, proved to be important in efficacy of the bacilli. Plant vigorousness was almost higher using suspension. Positive correlation was recorded between inoculum size of the bioagent and the number of survived plants; the greatest effects (powder, 58-71 % and suspension, 68-92 %) were obtained at concentration of 10 ml or 10 g / kg seeds. Up to 20 % of bacilli mixed inocula were superior in the control of *R. solani* disease incidence in comparison with the mono bacterial ones. *Bacillus* strains did successfully survive up to 60 days either in soil or in their prepared formulations. The suspension formulation was more efficient in the establishment and surviving the bacterial cells and thus is strongly recommended for application in future biocontrol programs.

Keywords: *B. subtilis*, *B. cereus*, growth, *R. solani*, antibiosis, interactions, root rot disease, bean, biological control agents, powder, suspension.

INTRODUCTION

The fungus *Rhizoctonia* spp. is among the soil-borne pathogens causing economic yield losses for a variety of plants including beans. *Rhizoctonia solani* Kühn is the major cause of pre- and post- emergence damping-off and root rot diseases of different important legume crops. The damage caused by such diseases extends to quality and quantity of the crop beside a decrease in plant population which affects atmospheric nitrogen fixation (Estevez de Jensen *et al.*, 2004). The use of biological control agents has drawn attention and has been considered as a promising alternative to chemical pesticides. Many applications of fungi and bacteria

have been attempted (Estevez de Jensen *et al.*, 2004). The Gram-positive bacteria, like *Bacillus* spp., have been studied intensively as biological control agents with regard to production of antimicrobial metabolites (Silosah *et al.*, 1994). In general, the mechanisms of antagonism by these microorganisms include natural antimicrobial agents as phytoalexin (Chappll and Nable, 1987), holding elements needed for phytopathogens (Kloepper *et al.*, 1988), detoxification of mycotoxins that play an essential role in disease development (Toyoda *et al.*, 1988) and antibiotic production (Hammer *et al.*, 1993). Several formulations and application methods of the antagonists were examined and described in the literature, among them the powder and suspension preparations (Estevez de Jensen *et al.*, 2002).

The aim of the present research was to isolate *Bacillus* species from rhizosphere of bean, screening *in vitro* and *in vivo* for antagonism aginst *R. solani* damping-off of the host. This is an attempt to control the incidence of such disease. Suspension and powder formulations of the isolated bacilli were compared. The effectiveness of bacilli interactions for the pathogen biocontrol was studied as well.

MATERIALS AND METHODS

Microorganisms

Pathogens

Isolation and identification of *Rhizoctonia* spp.

Samples of bean (*Phaseolus vulgaris* L.) plants showing typical symptoms of root rot disease were collected from different bean fields in El-Fayoum, El-Sharkia and El-Munofya, Egypt. The method described by Abou-Zeid *et al.* (2002) was used. The infected roots were washed with tap water, cut into small pieces, air dried and surface sterilized with 2 % sodium hypochlorite solution for 3 minutes. This was followed by washings several times with sterilized distilled water and dried between two sterilized filter papers. Small pieces were transferred into sterilized Petri dishes containing peptone dextrose agar plus rose bengal and streptomycin medium (Johnson *et al.*, 1960). Plates were then incubated at 25°C and examined periodically. The developed colonies were purified using the hyphal tip technique (Hawker, 1960). The purified isolated fungi were identified according to cultural morphological and microscopical characteristics according to (Sneh *et al.*, 1992). Fungal strains were maintained on nutrient agar slants and kept in a refrigerator at 5 °C for further studies.

Pathgenicity of *R. solani*

Pathogenicity test was carried out using three isolates of *R. solani* Kühn (R1, R2 and R3) to evaluate their pathogenic ability under greenhouse conditions at the Integrated Control Research Dept., Plant Pathology Research Institute, ARC, Giza, Egypt. The most virulent strain was used for further studies.

The *R. solani* inoculum was prepared in sterilized bottles, each contained 250 g of corn sand meal medium, CSM (Abd-El-Moity, 1985). Bottles were inoculated with equal agar disks (5 mm diameter) which were removed from the edge of an actively growing culture of each strain grown

on gliotoxin fermentation agar medium, GFA (Brian and Hemming, 1945). After incubation at 25 °C for 15 days, number of propagules / g of each isolate were determined. Plastic pots (25 cm diameter) each contained 2.7 kg of sterilized soil were infested with the prepared inoculum (*ca.* 5 x 10^6 cfu g^{-1}) at the rate of 10 g of CSM / kg soil. Soil infestation was carried out 5 days before seed sowing. Pots without infestations were used as control. Bean seeds cv. Paulista were sown in all pots at the rate of 5 seeds pot^{-1} and 5 pots were used as replicates for each isolate. Percentages of pre- and post-emergence damping-off were calculated after 15 and 30 days of sowing, respectively, while healthy survived plants were recorded after 45 days of sowing.

Isolation and identification of biocontrol agents

Isolation of bacilli was carried out from the rhizosphere of healthy bean (*Phaseolus vulgaris* L.), pea (*Pisum sativium* L.), strawberry (*Fragaria* spp.) or peppermint (*Mentha* spp.) plants, grown in heavily infested fields with root rot pathogens distributed in Governorates as previously mentioned. The method described by Abd-El-Moity (1976) was used. Identification was performed according to Bergy's Manual of Determinative Bacteriology (1994) and confirmation using the ELISA (Enzyme Linked Immuno Surbant Assay) test at the Department of Plant Pathology, Faculty of Agriculture, Ain-Shams University, Cairo.

Laboratory studies

Antagonistic effect of *Bacillus* spp. against *R. solani*

Screening of bacilli strains for their antagonistic ability against *R. solani* was carried out using the dual culture plate method (Siddiqui *et al.*, 2001). A loopful of 48 h old culture of either *Bacillus* spp. was streaked on one side of Petri plate containing nutrient glucose agar, NGA (Dowson, 1957). On the other plate side, a disk (5 mm-diameter) from GFA culture of *R. solani* was transferred. Plates only inoculated with *R. solani* were served as a control. Plates were incubated at 25 °C and inhibition of mycelial growth by *Bacillus* spp. was recorded. Bacilli bioagents that produced a clear zone of inhibition or caused lysis of fungal mycelium were considered effective. The percentage of reduction in the mycelial growth of the pathogenic fungus was calculated as follows: % of reduction of linear growth = 100 - [G2 / G1 X 100] where, G1 is the growth (mm) of the pathogenic fungus in control plates and G2 is the corresponding in treated plates.

The malformation in the mycelial growth of *R. solani* was examined as follows (Abd-El-Monieum, 1996): a sterilized microscopic glass slide was covered by a thin film of diluted NGA medium under sterilized conditions. Diluted medium (1 ml NGA+9 ml distilled water) was used to reduce the fungal growth to facilitate its microscopic examination. The antagonist was inoculated at one side whereas the pathogen was inoculated on the other side of the slide. Inoculated slides were placed in sterilized Petri dishes contained filter papers saturated with 10 ml of sterilized distilled water. All plates were incubated at 25 °C for 3 days. Light microscope with fixed camera was used to examine and photograph any malformation.

Screening of bacilli strains for antibiotic resistance

The minimum inhibitory concentration (MIC) of certain antibiotics (Streptomycin, Chloramphenicol and Penicillin) against bacilli members was detected. A stock solution of either antibiotic was prepared in sterilized distilled water and added to flasks containing warm sterilized strength NGA medium to give a final concentration of 2-22 ppm (streptomycin), 2-12 ppm (chloramphenicol) or 2-500 ppm (penicillin). Media with the different concentrations were poured into sterilized Petri dishes and after solidification the plates were inoculated with bacterial cultures. After incubation at 30 °C for 48 h, the developed colonies were picked up and maintained on NGA medium containing the same concentration of each antibiotic.

Bacilli growth in various culture media

Three synthetic media, *i.e.* nutrient glucose broth, NG (Dowson, 1957), mannitol broth, MB (Fahy and Persley, 1983) and modified bacitracin fermentation, MEF (Anker *et al.*, 1949) were used to evaluate the growth patterens of the antagonistic bacterial strains. Two hundred ml of each medium were transferred to 500 ml Erlenmeyer conical flasks, autoclaved and then inoculated with 5 ml of a bacterial suspension of either bioagent. After incubation on a rotary shaker (120 rpm) at 30 °C, numbers were determined at intervals (0, 24, 48, and 72 h) using the dilution plate method and nutrient agar medium.

Growth curves for recognizing the stationary phase of the antagonistic *Bacillus* spp. were monitored. A loopful of 24 h culture of either bacilli candidate was inoculated into 5 ml of MB medium and incubated at 30 °C for 24 h. Cultures were transferred to 500 ml conical flasks containing 200 ml of the same medium. Flasks were incubated on a rotary shaker (120 rpm) at 30 °C. Viable counts were determined every 3 hours using the dilution plate method.

Factors affecting the antibiosis of bacilli bioagents towards *R. solani* Incubation period

Bacterial strains were grown in 500 ml Erlenmeyer flasks containing 200 ml of MB medium and incubated on a rotary shaker (120 rpm) at 30 °C. At either enumeration period, cultures were centrifuged for 20 min at 6000 rpm to separate the bacterial cells. The supernatant of each culture was filtered through Millipore (filtration membrane pore size 0.2 μm) to obtain cell-free filtrate which was used to estimate its fungitoxicity. This filtrate was added to warm sterilized strength NGA medium to give a final concentration of 10 % (v/v). Media with different *Bacillus* spp. culture filtrates were then poured into sterilized Petri dishes at a rate of 10 ml / plate. Plates contained only NGA medium without culture filtrate were considered as control. Plates were inoculated with equal agar disks 5 mm-diameter obtained from the periphery of 4 day old *R. solani* culture grown on GFA medium. Plates were incubated at 25 °C and examined periodically. Percentage of reduction in mycelial growth of the pathogenic fungus due to the antagonistic effect of bacteria was calculated.

Culture media

Two growth-stimulating media *i.e.* NG and MB were selected and examined for the antifungal activity of *Bacillus* spp. The previously described procedure was adopted, except that the incubation period was 33 to 39 h.

Culture filtrate concentration

Plates containing media with culture filtrate concentrations of 20, 30 and 40 % (v/v) were prepared, inoculated with *R. solani*, incubated and percentage of reduction in mycelial growth of the pathogenic fungus was calculated.

Greenhouse studies

Evaluation of two *Bacillus* spp. formulations in controlling *R. solani* root rot disease of bean

Plastic pots (25 cm diameter), each contained 2.7 kg soil, were infested with *R. solani* (5×10^6 cfu / g) at the rate of 10 g / kg soil. The *Bacillus* spp. were formulated as powder or suspension using the method described by Abd-El-Moity (1985). Bacteria were grown for 2 days at 30 °C in MB medium. For powder preparation, the culture was mixed with sterilized talc powder (1:1 v/w), then dried at room temperature. For suspension formula, the culture was mixed with sterilized water at the rate of 1:1 (v/v). Each formulation was adjusted to contain *ca.* 3×10^7 cfu g^{-1} or ml^{-1} and used for seed treatment. Bean (*Phaseolus vulgaris* L.) seeds cv. Paulista were mixed with either powder (5 g / kg seeds) or suspension (5 ml / kg seeds) preparations using Arabic glue as an adhesive material. Six seeds were sown in each pot and four pots were used as replicates for each treatment. Pots sown with non-treated seeds were served as a control treatment. Pots were irrigated periodically and examined at intervals and percentages of pre-, post-emergence damping-off and survived plants were calculated after 15, 30 and 60 days, respectively. Pots were arranged in the greenhouse in a complete randomized design. After 60 days, the survived plants were counted, up rooted and used for dry weight of plant shoots and roots determination.

Effect of *Bacillus* spp. inocula sizes

This experiment was carried out as described above, except that different doses of either powder (2.5, 5 and 10 g / kg seeds) or suspension (2.5, 5, and 10 ml / kg seeds) formulations were tested.

Effect of various *Bacillus* spp. composite inocula on controlling *R. solani*

This experiment was carried out according to a compatibility *in vitro* experiment, where each of two compatible isolates were chosen to prepare powder or suspension formula. The bacilli interactions were evaluated for controlling *R. solani* as previously described, except that the dose applied was 10 g or 10 ml /kg seeds.

Survival of *Bacillus* spp. strains in the prepared formulations

One gram (powder) or 1 ml (suspension) from either formulated product was used for serial decimal dilutions, among those one ml was used to inoculate NGA medium. Plates were incubated at 30 °C for 24 h then developed colonies were enumerated.

Survival of *Bacillus* spp. strains in soil

The antibiotic-marking of *Bacillus* spp. strains was used to facilitate their recovery from soil (Kondoh *et al.*, 2001). Since the lowest MIC values for all tested *Bacillus* strains were obtained when chloramphenicol was used, this antibiotic was applied in this experiment. One gram was sampled from rhizosphere of each treatment at 0, 15, 30, 45 and 60 days of sowing and suspended in 99 ml of sterilized distilled water and then shaked for 30 min at 140 rpm at room temperature. Such suspension was subjected to serial dilutions up to 10^{-6}. Colony counts were determined where NGA medium contained the tested antibiotic at a concentration lower than the MIC of each microorganism.

Statistical analysis

Statistical analysis was conducted using the general linear model (GLM) procedure of Statistical Analysis Systems (SAS). Significance was evaluated at P= 0.05 using Duncan's multiple range test (SAS, 1996). Correlations among various parameters were calculated by correlation coefficients (r).

RESULTS AND DISCUSSION

Microorganisms

Pathogens

Isolation and identification of *Rhizoctonia* spp.

Rhizoctonia solani was the most frequently isolated fungus. Three isolates (R1 from El-Sharkia, R2 from El-Fayoum and R3 from El-Monofia Goveronorates) were obtained.

Pathogenicity of *R. solani*

All *R. solani* strains were pathogenic and caused pre- and post-damping-off for bean plants. The percentage of diseased plants reached 60-86.7 %, depending upon strain (Fig., 1). *R. solani* no. 2 was the most suppressive and caused 58.3 % pre-emergence damping-off followed by no. 3 which caused 40 %. Regarding post-emergence damping-off, strain no. 1 came in the first rank followed by no. 3.

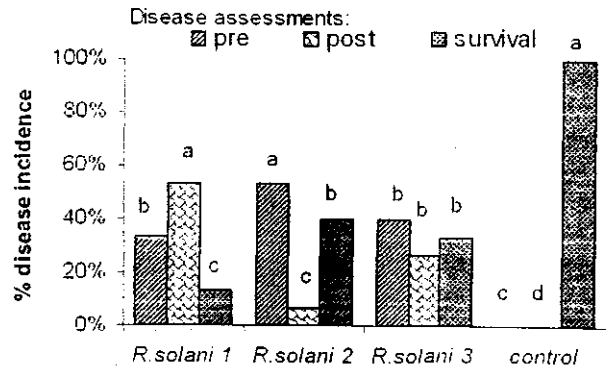

Fig. 1: Pathogenicity of *R. solani* expressed as pre- and post-emergence damping-off disease incidence as well as survived bean plants. Bars with same letters for the same disease assessment were not significantly (P=0.05) different.

Similar trend was found when fungal strains were compared according to the survived bean plants (no.2> no3> no.1).The observed percentages of pathogenicity were comparable to those obtained by Asaka and Shoda (1996) who reported 85.2 % of diseased tomato plants.

Isolation and identification of biocontrol agents

A total of 20 spore-forming bacteria were isolated from the rhizosphere of bean plants grown in heavily infested fields. Results indicated that 6 of them had an antagonistic effect against *R. solani*. These antifungal-substances producing rhizobacteria were identified. The phenotypic features of the cultures are presented in Table (1). Two different species were identified: *B. subtilis* (5 strains which have given the numbers B1 to B5) and one *B. cereus* (number B6). Adopting ELISA test, the bacilli candidates were authenticated as what has been mentioned in Table (1).

Laboratory studies

Antagonistic effect of *Bacillus* spp. against *R. solani*

All *Bacillus* spp. candidates showed antibiosis towards *R. solani* (Fig., 2a). They retarded colony growth of the pathogen by producing clear inhibitory zones (Fig., 2b). The highest reduction in mycelial growth (48 %) was obtained when *B. subtilis* (B4) was used, whereas the least effect (34.1 %) was noticed with *B. cereus* (B6). Effects of other tested *Bacillus subtilis* strains ranged between 35–40 %. The antagonistic microorganisms which cause reduction in colony growth of the pathogen by producing a clear zone may released toxic metabolites into the medium (Akhtar, 1982), while those causing reduction by growing over the pathogen did so by fast growth and better saprophytic activity, with which the pathogen could not compete, resulting in reduced growth of the pathogen (Iqbal and Akhtar, 1987). Production of antifungal metabolites bacillomycin L, bacilysin, fengymycin, iturin-A and subsporin by *B. subtilis* was reported by Yu *et al.* (2002). These products are diffused in the medium and prevent other organisms to grow close or near *B. subtilis* colonies.

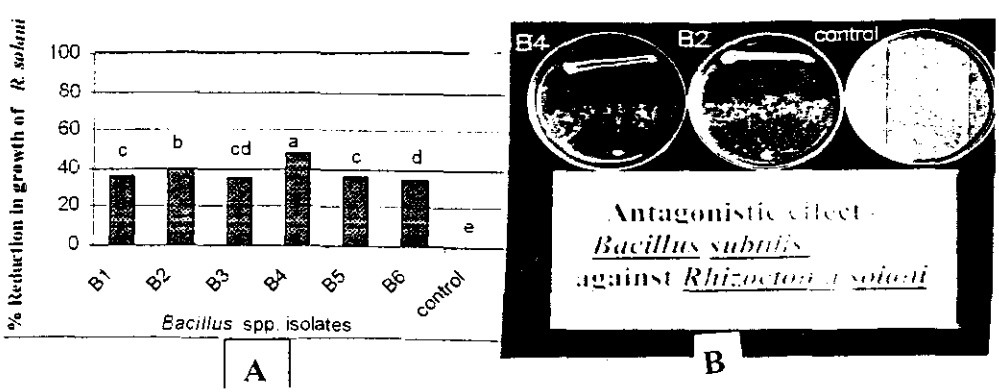

Fig. 2: Antagonistic effect of *Bacillus* spp. on linear growth of *R. solani* (A), bars with same letters are not significantly different from each other at *P*=0.05. (B) shows that mycelial growth was retarded.

Variation in antagonistic effect among *Bacillus* species is due to variation of potentiality of these species in production of antibiotic substances (Montealegre *et al.*, 2003). The observed reduction of mycelial growth in the present study by the five *B. subtilis* isolates (35.5-48 %) was comparable to those obtained by Hassanein *et al.* (2000) who reported *ca.* 36 % reduction by an *B. subtilis* strain.

Table 1: Morphological, biochemical and physiological characteristics of the isolated antagonistic bacilli

Biochemical and cultural tests	Characteristics of isolates					
	B1	B2	B3	B4	B5	B6
Gram stain	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	-
Voges-Proskauer test	+	+	+	+	+	+
Acid from:						
D-glucose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	-
D-Xylose	+	+	+	+	+	-
D-Mannitol	+	+	+	+	+	-
Gas from glucose	-	-	-	-	-	-
Hydrolysis of:						
Casein	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+
Starch	+	+	+	+	+	+
Nitrate reduced to nitrite	+	+	+	+	+	+
Growth at pH (nutrient broth):						
6.8	+	+	+	+	+	+
5.7	+	+	+	+	+	+
Growth in NaCl:						
2 %	+	+	+	+	+	-
5 %	+	+	+	+	+	-
7 %	+	+	+	+	+	-
10 %	-	-	-	-	-	-
Growth at:						
5 °C	-	-	-	-	-	-
10° C	-	-	-	-	-	-
30 °C	+	+	+	+	+	+
40 °C	+	+	+	+	+	-
50 °C	+	+	+	+	+	-
55 °C	-	-	-	-	-	-
65 °C	-	-	-	-	-	-
Proposed species	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. cereus*

(+), positive result; (-), negative result.

Fig. (3) shows a clear lytic area in the *R. solani* hyphae due to the effect of *B. subtilis*. Dark brown color in periphery of *R. solani* mycelia opposite to *B. subtilis* was observed. This color was diluted and became lighter to direction of colony center. Microscopic observation of the brown

zone revealed that affected cells were more deep color with cytoplasmic leakage that could be observed up to the hyphal septum, resulting in malformation of the hyphae. The present data could be explained in the light of fact that the secretion of antibiotics or secretion of a group of enzymes which dissolved the cell wall of the fungus caused complete destruction for the affected mycelia. This malformation probably dues to the antibiotic substances interfering with normal growth process (Ferreira *et al.*, 1991) or secreted volatile metabolites with fungicide properties (Fiddaman and Rossall, 1993).

Fig. 3: Normal mycelium of *R. solani* with clear cell wall (A) and malformation and lysis due to the effect of *B. subtilis* after 3 days (B).

Screening of bacilli strains for antibiotic resistance

The antibiotics tested demonstrated activities against all *Bacillus* spp. (Table, 2). A noteworthy point is that the six *Bacillus* spp. exhibited differing sensitivity to the antibiotics. Isolates B2 and B4 were the most resistant. The lowest MIC values were obtained when chloramphenicol was used (8-12 ppm). MIC values of streptomycin ranged between 16 and 22 ppm. Penicillin-G showed the highest MIC values which ranged between 50 and 500 ppm.

Table 2: Screening of the *Bacillus* spp. cultures for their resistance against three different antibiotics

	Minimum inhibitory concentrations (µg/ml) of antibiotics after 24 h incubation		
Species	Streptomycin	Chloramphenicol	Penicillin-G
B. subtilis B1	18	8.0	400
B. subtilis B2	22	12	500
B. subtilis B3	18	8.0	150
B. subtilis B4	20	12	500
B. subtilis B5	20	8.0	50
B. cereus B6	16	8.0	400

Bacilli growth in various culture media

Mannitol broth and nutrient glucose media proved to be the most effective (Table, 3). The highest cell counts of 2.3- 4.3 x 10^7 cfu / ml were recorded by *B. subtilis* after 48 h incubation, while *B. cereus* exhibited lower growth of only 1.7 x 10^7 cfu / ml.

The growth curves were characterized by a logarithmic phase lasting about 6- 15 hours, depending upon strain (Fig., 4). The maximum cell density of 6.3 x 10^7 – 9.7 x 10^8 cfu / ml, was reached 18- 39 hours after inoculation.

Factors affecting the antibiosis of bacilli bioagents towards *R. solani*

Incubation period

The antifungal activity was detected after 9 hours in culture during the log phase. At 40 h, near the end of the stationary phase and beginning of the death phase, the concentration of antifungal compounds reached its highest level. However, the concentration of the compounds was high at 24 h, when the culture was in its stationary phase (Fig., 4).

Table 3: Effect of media on stimulation the growth of the isolated antagonistic *Bacillus* spp. cultures after different incubation periods (hours)

	Mannitol broth	Modified bacitracin	Nutrient glucose	Mannitol broth	Modified bacitracin	Nutrient glucose	Mannitol broth	Modified bacitracin	Nutrient glucose
	24 h			48 h			72 h		
Antagonistic isolates	Cell number (x 10^7 cfu/ml) on nutrient agar								
B. subtilis B1	2.6	1.8	2.1	2.3	2	2.4	2.6	2.1	2.4
B. subtilis B2	2	1.7	1.6	4.3	1.6	2.2	2.5	2.1	2.6
B. subtilis B3	1.9	1.7	1.4	2.6	1.7	1.4	3.2	1.8	2.5
B. subtilis B4	1.9	1.2	2.3	3.1	1.7	2.9	3	1.9	3.1
B. subtilis B5	2.2	1.7	1.3	2.6	1.6	2.1	4.3	2.2	2.5
B. cereus B6	2.9	1.4	1.8	1.7	1.5	2.9	1.3	1.9	2.7

Initial counts of all strains were *ca.* 10^7 cfu/ml

In this respect, the formation of secondary metabolites by microorganisms in synthetic culture media and the quantity and composition of these metabolites depends strongly on the culture conditions and the growth phase of the culture (Krebs *et al.*, 1998), which is also true in the case of *B. subtilis*. The lipopolypeptides formed by *B. subtilis* are released into the medium only at the time of endogenous spore formation during the stationary phase of the culture (Loeffler *et al.*, 1990).

Culture media

The addition of culture filtrates of *Bacillus* spp. to *R. solani*, resulted in significant reduction in mycelial growth of the fungus compared with the control treatment (Fig., 5). MB medium was more favourable (11.8–30 % reduction) compared with NG medium (6.0–15 % reduction). *B. subtilis* B2 was the most effective in reducing *R. solani* growth and highest percentage of reduction in mycelial growth was obtained either when MB (30 %) or NG (15 %) media were used.

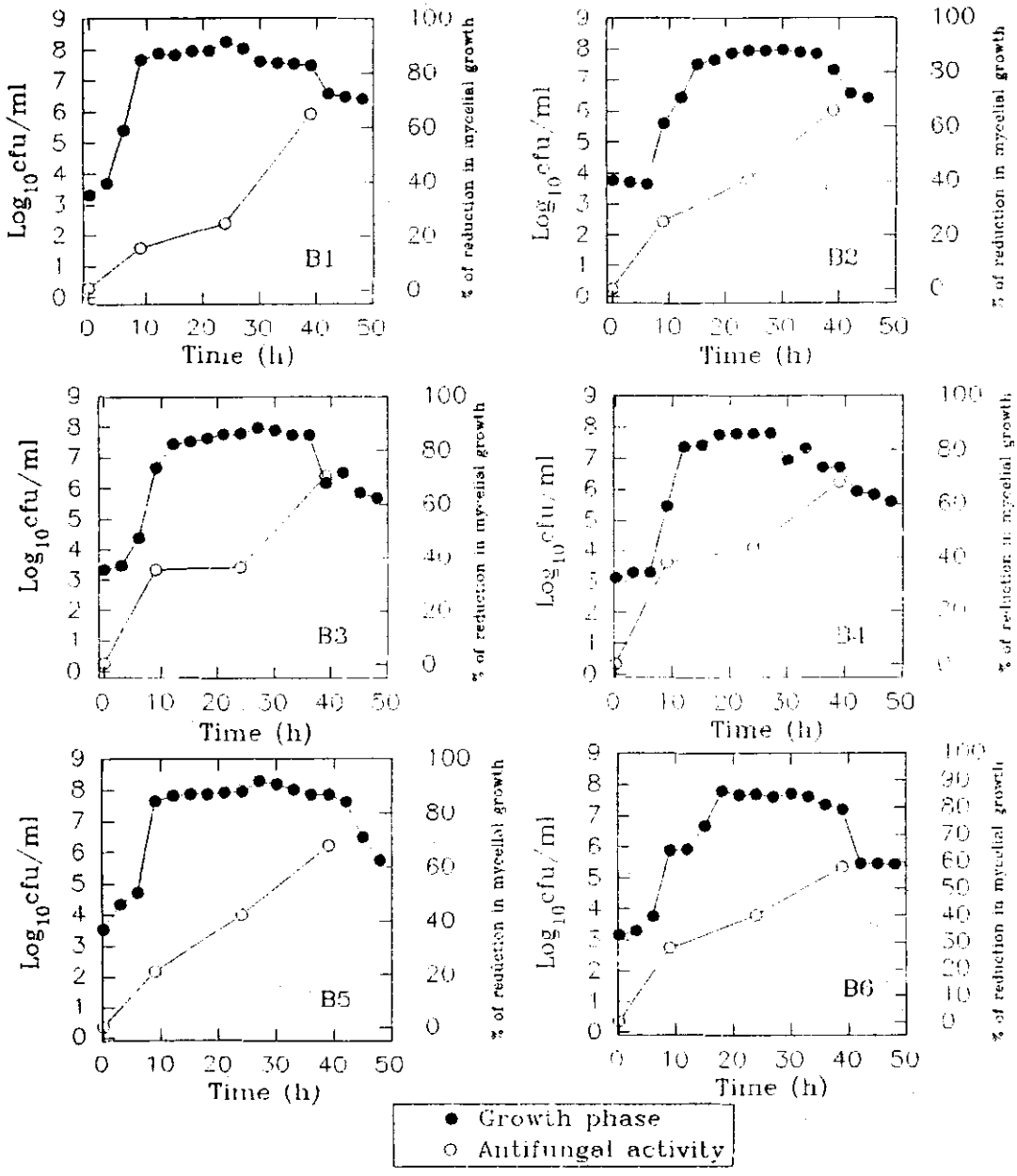

Fig. 4: Relations among growth phase of bacilli strains grown in mannitol broth medium and the antifungal activity against *R. solani*. B1 to B5 are *B. subtilis* and B6 is *B. cereus*.

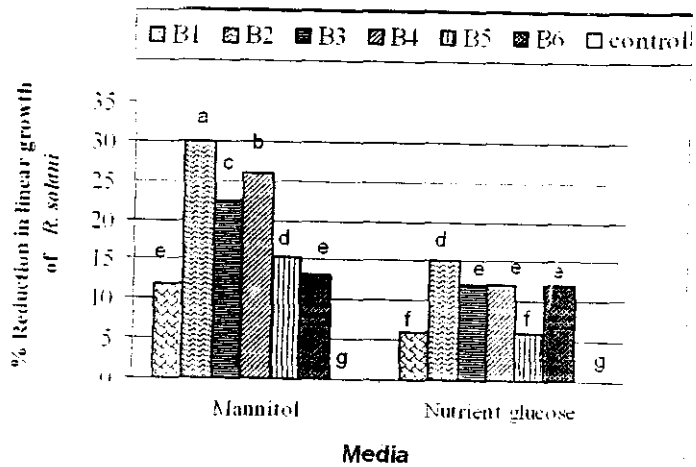

Fig. 5: Effect of culture media on antifungal metabolites produced by *Bacillus* spp. Bars with same letters are not significantly (P=0.05) different.

The lowest recorded effect of *Bacillus* spp. was correlated to the used medium. When MB medium was used, strain B1 showed the lowest effect, whereas, in case of NG medium, strains B1 and B5 showed the lowest effect. Kilian *et al.* (2000) found that production of antibiotic metabolites by *B. subtilis* depends on the culture medium. The observed higher effectiveness of MB medium over NG in the present study is might be due to the differences in carbon and nitrogen sources. These variations may lead to direct effects on the type and quantities of the antifungal substances which produced by *B. subtilis* and consequently affect the pathogen. On the other hand, there was a relationship between growth and antifungal metabolite production by the isolated bacteria. Strains exhibiting the highest growth such as B2 (4.3×10^7) and B4 (3.1×10^7) were more efficient in the reduction of mycelial growth of *R. solani*. Moreover, mannitol medium, which was the most suitable for growth of *Bacillus*, was also the optimum for antifungal production. Other investigators, however, found that the optimum medium for growth of *B. subtilis* was not the most favourable for antifungal production (Ezzat *et al.*, 2001). Such contradiction might be due to the difference in bacterial strains or other growth conditions.

Culture filtrate concentration

The addition of culture filtrates of any *Bacillus* strain at any concentration led to significant reduction in mycelial growth of *R. solani* (Table, 4). The MB medium was the most effective for all strains, except B5 where it did prefer NG medium. With 20 % culture filtrate, strain B2 showed higher effect with MB medium (46 % reduction in *R. solani* growth) compared with only 33 % reduction with NG medium, or compared with

only 18 % when strain B5 culture filtrate was used at the same concentration. Culture filtrates from MB medium added at a concentration of 30 or 40 % were the most desirable for stimulating antifungal metabolite production by *Bacillus* spp.

Table 4: Effect of culture filtrate concentrations of *Bacillus* spp. on percentatge of reduction in *R. solani* mycelial growth.

Treatment	Mannitol broth			Nutrient glucose broth		
	% of reduction in mycelial growth of *R. solani* with culture filtrate at concentrations					
	20%	30%	40%	20%	30%	40%
B. subtilis 1	36.4 p	50.6 e	53.0 dc	36.4 p	36.4 p	47.7 hg
B. subtilis 2	46.0 ji	52.0 d	79.4 a	33.1 r	34.1 qr	48.7 fg
B. subtilis 3	44.5 k	47.0 hi	52.0 d	42.3 L	40.0 nm	45.0 jk
B. subtilis 4	38.1 o	54.1 c	67.1 b	35.2 qp	47.0 hi	49.4 fe
B. subtilis 5	18.0 t	18.0 t	36.4 p	28.2 s	29.4 s	35.0 qp
B. cereus 6	36.3 p	36.4 p	40.3 nm	29.4 s	39.0 no	41.1 m
Control	0.0 u	0.0 u	0.0 u	0.0 u	0.0 u	0.0 u

Means with same letters are not significantly (*P*=0.05) different.

The highest percentages of reduction (79.4 % and 67.1 %) were obtained when MB culture filtrates of strains B2 and B4, respectively, were used at a concentration of 40 % whereas the lowest effect (18 %) was obtained when strain B5 was used at a concentration of 20 or 30 %. In general, alleviation of the mycelial growth of *R. solani* was positively correlated with increasing the culture filtrate concentration (Figure, 6) which might be led to increase in the amount of inhibitory substances.

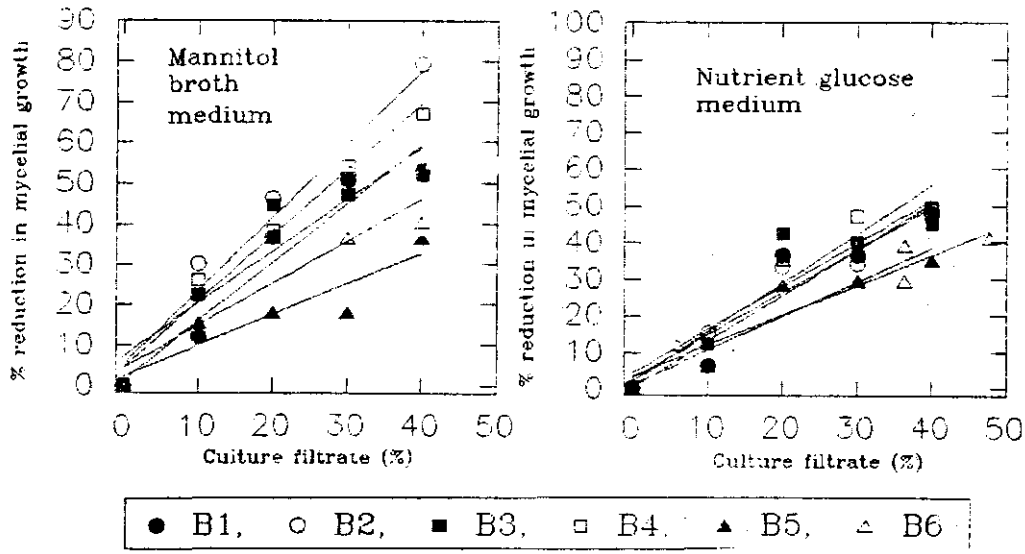

Fig.6: Linear regressions among *Bacillus* spp. culture filtrate concentrations and percentage of reduction in *R. solani* mycelial growth.

Correlations differed from strain to another and *r* values for respective bacilli strains were 0.97, 0.98, 0.94, 0.99, 0.92 and 0.93 (MB medium) as well as 0.95, 0.98, 0.91, 0.97, 0.95 and 0.98 (NG medium) respectively. These findings are in agreement with those of Kaparr and Kar (1989), who reported that the cell-free culture filtrate of *B. subtilis* inhibited the growth of *Fusarim oxysporum f.sp. lycopersici.* Different investigators used the culture filtrate of different bacterial isolates such as *B. cereus* (Fravel and Spurr, 1977) to inhibit the conidial or spore germination of the pathogenic fungi.

Greenhouse studies

Evaluation of two *Bacillus* spp. formulations in controlling *R. solani* root rot disease of bean

Treatment of bean seeds with either powder or suspension formulation caused significant reduction in pre- and post-emergence damping-off and increased surviving plants (Table, 5). Percentages of survived plants were almost higher in seeds treated with suspension. Strain B2 showed survived plants of 87.5 % and 73.3 %, respectively, compared with 12.5 % in control. On the contrary, strains B1 and B6 showed the lowest effect. The main differences in survival percentages are due to the differences in percentages of pre-emergence, while very slight differences in percentages of post-emergence were noticed. All bacilli members caused increases in fresh and dry weights of roots and shoots compared with control treatment (Table, 5). The type of formula had significant effect. Strain B4 was the most effective as powder. However, strain B2 showed the highest effect when comparison was carried out among *Bacillus* spp. prepared as suspension. In general, the effect of suspension formula was almost higher than the other formulation. These results are in conformity with those of Edwards and Seddon (1992) that antifungal antibiotics were known to be produced *in vitro* by *B. subtilis* strains which have *in vivo* activity against plant diseases. The present study has shown that all *Bacillus* spp tested. demonstrated in vitro antibiosis against *R. solani* (Fig., 2a) and also increased the growth and yield of bean plants treated with *R. solani in vivo* (Table, 5), suggesting that the antifungal substances produced *in vitro* were responsible for biocontrol *in vivo*. Correlation between percentages of antifungal production *in vitro* and those of biocontrol activity *in vivo* ($r = 0.92$) was significantly positive (Fig., 7). This finding was in accordance with Edwards and Seddon, (1992). In other studies, *B. subtilis* did not demonstrate *in vitro* antibiosis but increased the growth and yield of plants inoculated with pathogenic fungus (Amer and Utkhede, 2000). The value of *in vitro* studies into the mode of action was questioned because antibiotic activity produced by different strains *in vitro* plate assays correlated very little with *in vivo* biocontrol activity (Leifert *et al.*, 1993). The high antagonistic effect of powder formula in the present study might be due to the increase in stability and establishment of the antagonist in the infection court. This establishment caused reduction in the pathogenic propagules and consequently reduction in disease incidence (Kay and Stewart, 1994).

Granular formulations of *B. subtilis* used in the studies of Estevez de Jensen *et al.* (2002) were both convenient and likely to provide longer lasting protection and extra protection beyond the seed surface. However, the higher effect of suspension form in the present study is might be due to the faster spreading compared with the powder preparation. The variation among *Bacillus* spp. may be due to the production of different secondary metabolites which affect different pathogens. Also some *Bacillus* spp. were reported to produce growth regulators which improve plant growth and increased yield in peanut (Kokalis-Burelle *et al.*, 2003).

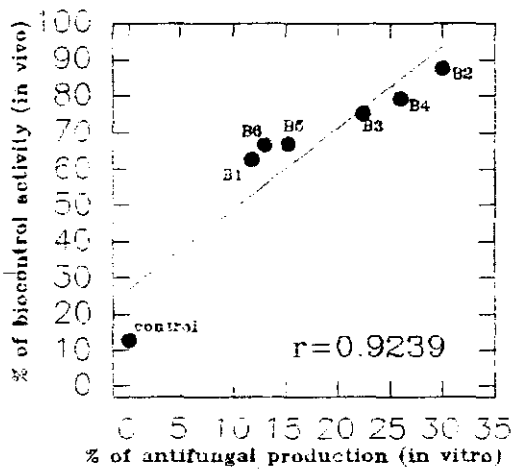

Fig. 7: Linear regression among *in vitro* plate antifungal activities of culture filtrate (MB medium) of *Bacillus* spp. (B1-B6) and *in vivo* biocontrol activities of the same formulated bacteria as suspension expressed as percentage of survived plants.

Effect of *Bacillus* spp. inocula sizes

Treatment with the majority of bacilli strains in any form or doses led to significant reduction in disease incidence and significantly increased survived plants compared with control treatment (Table, 6). In most cases, there was negative correlation between increasing the dose and percentage of pre-emergence damping-off. On the other hand, positive correlation was noticed between increasing the dose of bacteria and the number of survived plants (Fig., 8). The correlation coefficients (r) for respective bacilli were 0.84, 0.81, 0.82, 0.83, 0.84 and 0.89 (powder) as well as 0.76, 0.73, 0.72, 0.74, 0.67 and 0.82 (suspension). The greatest effect was obtained when *Bacillus* spp. were applied at a rate of 10 ml or 10 g. This could be explained by the fact that increasing the dose of *Bacillus* increased the number of bacterial cells and consequently the establishment, production of antifungal substances and zone of protection surrounding the treated roots (Abd-El-Momeim, 2005).

Table 5: Effect of *Bacillus* spp. formulations on controlling pre- and post-emergence damping-off disease as well as fresh and dry weights of bean seedlings

Assessment	Pre-emergence damping-off (%)		Post-emergence damping-off (%)		Survival (%)		Fresh weight (g/plant)				Dry weight (g/plant)			
							Shoot		Root		Shoot		Root	
Treatment	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension
B. subtilis B1	37.5 cb	37.5 cb	4.2 c	0.0 d	58.3 ef	62.5 ed	13.0 i	20.4 f	1.2 dc	1.7 bac	2.2 i	3.6 hc	0.35 ba	0.50 ba
B. subtilis B2	26.7 d	12.5 f	0.0 d	0.0 d	73.3 c	87.5 a	18.7 g	28.7 a	1.3 dc	1.9 ba	2.5 hi	4.1 a	0.30 ba	0.60 a
B. subtilis B3	37.5 cb	25.0 ed	0.0 d	0.0 d	62.5 ed	75.0 cb	21.0 ed	21.3 d	1.6 bac	1.3 dc	3.1 efd	3.3 ecd	0.40 ba	0.40 ba
B. subtilis B4	33.3 c	20.8 e	0.0 d	0.0 d	66.7 d	79.2 b	24.5 b	22.9 c	2.0 a	1.5 bdac	3.5 bcd	2.8 hfg	0.43 ba	0.37 ba
B. subtilis B5	33.3 c	33.0 c	4.2 c	0.0 d	62.5 ed	67.0 d	20.1 f	20.2 f	1.3dc	1.4 bdc	2.9 efg	3.8 ba	0.35 ba	0.55 a
B. cereus B6	41.6 b	25.0 ed	4.2 c	8.3 b	54.2 f	66.8 d	13.8 h	20.6 ef	1.2 dc	1.6 bac	2.9 efg	2.6 hig	0.30 ba	0.47 ba
Untreated control	58.3 a		29.2 a		12.5 g		12.1 j		1.0 d		1.2 j		0.20 b	

Means with same litters of the same assessment are not significantly (P=0.05) different.

Table 6: Effect of inocula sizes of *Bacillus* spp. formulations on pre- and post-emergence damping-off as well as survival of bean plants

Assess-ment	Pre-emergence damping-off %						Post-emergence damping-off %						Survival %					
Formula conc	Powder			Suspension			Powder			Suspension			Powder			Suspension		
Treatment	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
Bacillus 1	58.3 a	37.5 e	41.6 d	33.3 f	37.5 e	25.0 i	4.2 d	4.2 d	0.0 e	4.2 d	0.0 e	0.0 e	37.5 n	58.3 j	58.4 j	62.5 i	62.5 i	75.0 e
Bacillus 2	41.6 d	26.7 ih	23.3 ij	20.8 j	12.5 L	8.3 m	0.0 e	0.0 e	0.0 e	0.0 e	0.0 e	0.0 e	58.4 j	73.3 ef	76.7 ed	79.2 d	87.5 b	91.6 a
Bacillus 3	46.7c	37.5 e	33.3 f	29.2 gh	25.0 i	20.8 j	4.2 d	0.0 e	0.0 e	0.0 e	0.0 e	0.0 e	49.1 Lk	62.5 i	66.7 h	70.8 gf	75.0 e	79.2 d
Bacillus 4	50.0 b	33.3 f	29.2 gh	20.8 j	20.8 j	16.7 k	0.0 e	0.0 e	0.0 e	8.3 c	0.0 e	0.0 e	50 Lk	66.7 h	70.8 gf	70.9 gf	79.2 d	83.3 c
Bacillus 5	45.8 c	33.3 f	29.2 gh	29.2 gh	33.0 f	31.7 gf	8.3 c	4.2 d	4.2 d	4.2 d	0.0 e	0.0 e	45.9 m	62.5 i	66.6 h	76.7 h	67.0 h	66.3 gh
Bacillus 6	45.8 c	41.6 d	41.7 d	33.3 f	25.0 i	20.8 j	25.0 b	4.2 d	0.0 e	8.3 c	8.3 c	0.0 e	29.2 o	54.2 k	58.3 j	58.3 j	66.8 h	79.2 d
Control			58.3 a						29.2 a				12.5 p		12.5 p			

***Bacillus* 1-5 (*B. subtilis*) and no. 6 (*B. cereus*); means with same letters of the same assessment are not significantly (P=0.05) different.**

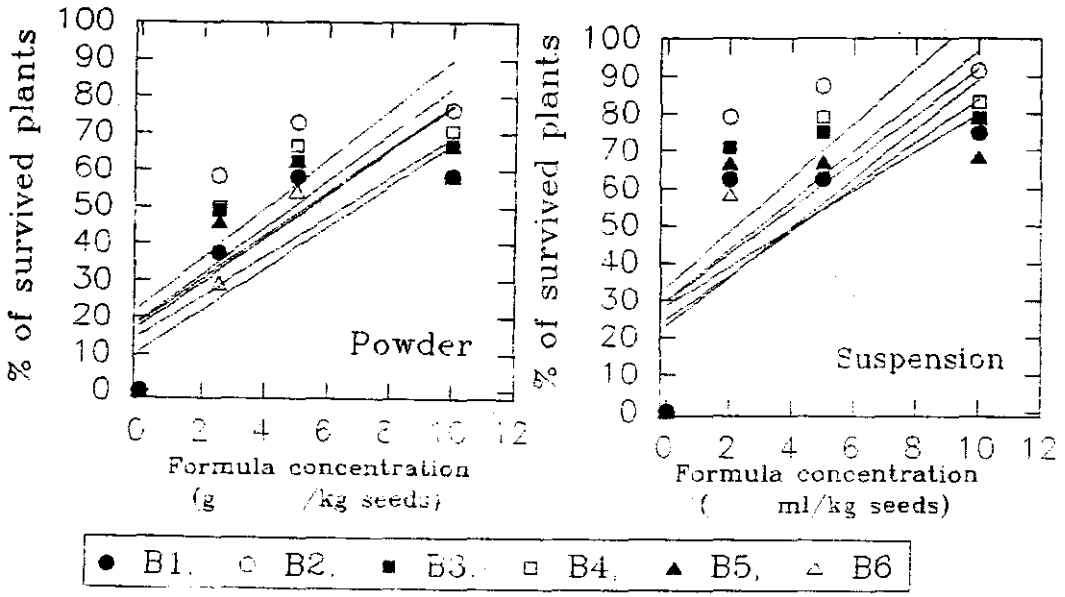

Fig. 8: Linear regressions among different inocula sizes of *Bacillus* spp. and percentage of survived bean plants.

Effect of various *Bacillus* spp. composite inocula on controlling *R. solani*

The dual bacilli inocula; B1+B2, B2+B4, B3+B4, B4+B6 and B5+B6 were tested to control *R. solani*. All these combinations significantly reduced disease incidence, increased the numbers of survived plants and increased fresh and dry weights of roots and shoots compared with the control (Table, 7). The majority of combinations were more effective when applied as suspension than those applied as powder. Of mixtures tested, B3+B4 showed the most effective protection (91.7 % survival). This mixture seemed the most effective in powder form and gave the highest fresh weight of root and shoot of 3.3 and 41 g/plant, respectively. When the mixture B2+B4 was used as powder, 29.2 % pre-emergence damping-off was recorded compared with 15.2 % as suspension. No differences were noticed with respect to post-emergence damping-off. The highest fresh shoot and root weights of 47.6 and 4.1 g/plant were obtained when this mixture was applied as suspension.

Up to 20 % of bacilli interactions were superior in the control of *R. solani* disease incidence in comparison with the mono bacterial cultures (Fig., 9). A finding contradicting those of other investigators that composite inocula of bioagents are strongly recommended in the pest biocontrol programs. The incidence of the majority of single strains authenticate the concept "environment selects", a phenomenon should be considered for on-going research planes. Raupach and Kloepper (1998) reported that different mechanisms of action for different antagonistic strains may explain why combinations of strains provided more consistency in disease suppression.

Table 7: Effect of combinations of *Bacillus* spp. formulated as powder or suspension on controlling pre-and post-emergence damping-off disease as well as fresh and dry weights of bean

Assess-ment	Pre-emergence damping-off (%)		Post-emergence damping-off (%)		Survival (%)		Fresh weight (g/plant)				Dry weight (g/plant)			
							Shoot		Root		Shoot		Root	
Treat-ment	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension	Powder	Suspension
B1+B2	33.3 c	25.0 d	3.3 d	0.0 e	63.5 g	75.0 d	34.2 e	38.8 d	2.8 e	3.3 c	6.5 d	7.4 b	0.9 bc	1.0 b
B2+B4	29.2 dc	15.2 f	0.0 e	0.0 e	70.8 e	87.5 b	38.1 d	47.6 a	2.5 f	4.1 a	6.0 e	7.9 a	1.0 b	1.4 a
B3+B4	25.0 d	8.3 g	0.0 e	0.0 e	75.0 d	91.7 a	41.0 c	42.0 cb	3.3 c	3.1 dc	6.8 cd	7.9 a	1.2 ba	0.9 bc
B4+B6	33.3 c	20.8 e	0.0 e	0.0 e	66.7 f	79.3 c	37.4 d	43.5 b	2.9 de	3.8 b	7.1 cb	6.9 cd	0.6 d	1.2 ba
B5+B6	37.5 b	29.1 dc	8.3 b	4.2 c	54.3 h	66.7 f	29.1 f	26.3 g	2.3 f	2.5 f	3.5 g	4.1 f	0.5 d	0.7 dc
Control	54.3 a		37.5 a		8.3 i		13.5 h		1.0 g		1.5 h		0.2 e	

B1, B2, B3, B4, and B5 (*B. subtillus*); B6 (*B. cereus*); means with same litters of the same assessment are not significantly (P=0.05) different.

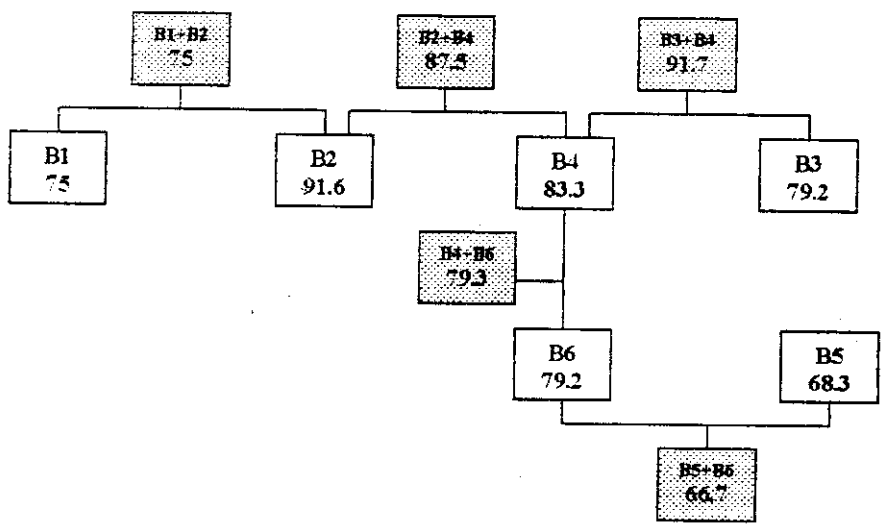

Figure 9: Flow diagram showing the effectiveness of combination of *Bacillus* spp. with each other in control of disease incidence of *R. solani* using the most effective formula (10 ml suspension /kg seeds) compared with each single *Bacillus*. Numbers in the chart are percentages of survived bean plants.

The combined use of *B. amyloliquifaciens* and *B. subtilis* had shown beneficial effects on growth (Reddy *et al.*, 1999). As mentioned by Peypoux *et al.* (1999), *Bacillus* spp. produce different numbers, amounts and kinds of antibiotics. By using more than one isolate the number of secondary metabolites and the slope of antifungal substances are increased which eventually lead to increase the efficacy of the treatment and also increase the plant protection. Kokalis-Burelle *et al.* (2003) suggested that different *Bacillus* spp. isolates in the combination act with different mechanisms *i.e.* competition for space and nutrients, the production of antifungal metabolites, induction of systemic resistance in the plants and also it successfully colonized roots of seedlings and provided protection against soil borne pathogens.

Survival of *Bacillus* spp. strains in the prepared formulations

All *Bacillus* strains survived in both formulas up to 60 days (Fig., 10). Calculating the difference between the percentage of decrease in numbers in powder and those in suspension after 60 days for each strain, the calculated value was either 0 (B1), 3.3 (B2), 3.4 (B3), 6.6 (B4, B6) or 13.3 % (B5), indicating that bacilli members varied in their reaction to the two formulas applied. Here, it could be concluded that suspension form was more favorable for *Bacillus* spp. and reduction percentages in viable cell numbers after 60 days were always higher in powder form. This might be due to that in suspension form the cells can obtain their requirements from water and different salts. On the contrary, in dry form (powder) only spores can survive, whereas all vegetative cells can not. Although a number of some bacterial strains used as a seed treatment with cell suspension have been found to be effective in controlling several soil borne diseases (Levy *et al.*, 1992), this methodology seems impractical because of difficulty in handling, transport, and storage. Amer and Utkhede (2000) showed that *B. subtilis* can survive in powder formulations up to 45 days at room temperature (22 °C). They added that the populations at the end of storage period were significantly higher in bacterial broth carrier (bacterial broth alone) compared with talc powder carrier. However, the population of this bacterium decreased by *ca.* 2 logs after 45 days in both formulations.

Survival of *Bacillus* spp. strains in soil

The cells of all tested *Bacillus* strains were recovered from soil as well (Figure, 10). Cells of strain B2 as suspension showed the best survival pattern and reached 100 % increase in number of propagules. Similar observation was noticed with the same strain as powder form where 93.3 % increase was observed. In general, suspension form did support better bacterial survival compared with powder. The ability of *Bacillus* spp. strains to biocontrol pathogens may relies on its long lasting stable activity. This stable survival of *B. subtilis* was the result of spores formed during incubation in soil (Tokuda *et al.*, 1995). Besides, the good survival of a *Bacillus* spp. is a reflection for its survival in different temperatures (Chet *et al.*, 1990), production of different antibiotics (Fiddaman and Rossall, 1993), ability to compete with other organisms in soil (Raupach and Kloepper, 1998) and to colonize plant roots efficiently (Bais *et al.*, 2004).

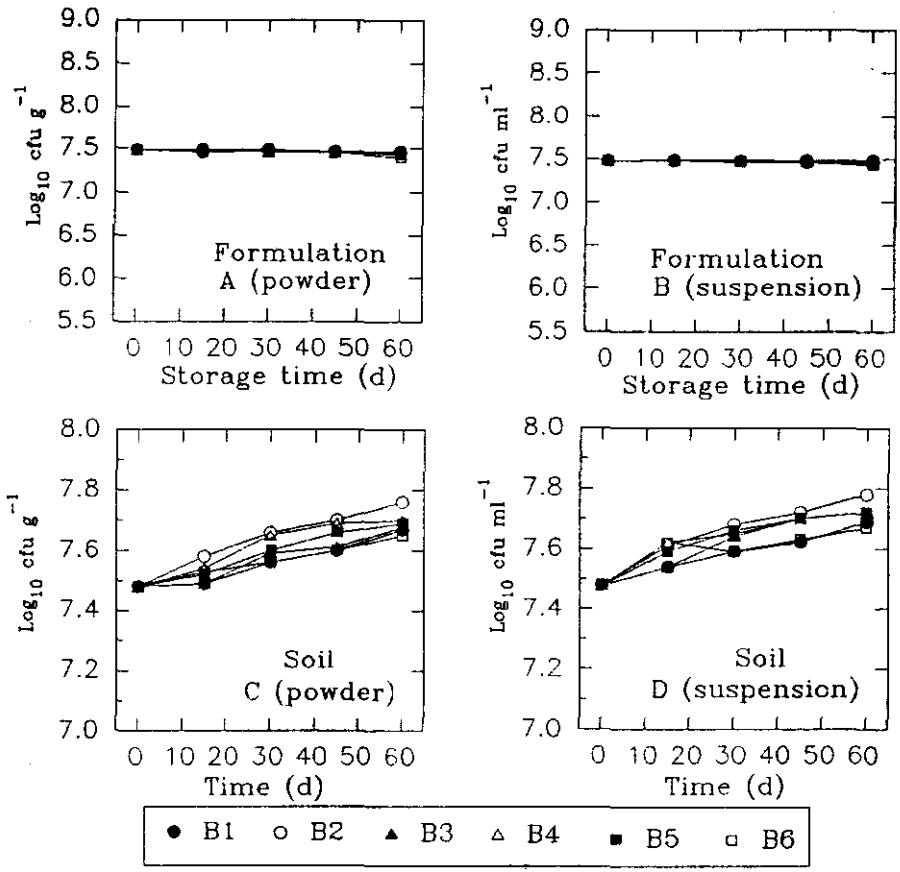

Fig. 10: Survival of the antagonistic bacilli either in prepared formulations (A & B) or in soil (C & D).

Conclusion

The use of emergence-promoting biocontrol agents inoculants, such as *B. subtilis* and *B. cereus*, may go some way towards promoting "healthier" crops in the future. The overall strategy of biological control fits in well with the current concerns of sustainable agriculture, whereby renewable resources are used for crop production, whilst incurring less of an impact on the environment as a whole. The present study has shown that root rot of bean caused by *R. solani* can be managed by bacterial suspension- and talc-based formulations of *B. subtilis* and *B.cereus*, and that these formulations may be exploited commercially. Since suspension formulation was the most effective, treatment of seeds with bacterial suspension is recommended. The isolated bacilli proved to be good biocontrol agents. The value of these isolates for root rot control was evident in greenhouse studies. Biological control using introduced antagonistic bacilli could play a promising alternative/integral role in overall disease control strategies.

REFERENCES

Abd-El-Moity, T.H. (1976). Studies on the biological control of white rot of onion. M.Sc. Thesis, Fac. of Agric., Monoufia Univ.

Abd-El-Moity, T.H. (1985). Effect of single and mixture of *Trichoderma harzianum* isolates on controlling three different soil-borne pathogens. Egypt J. Microbiology, Special Issue, 111-120.

Abd-El-Moneim, M.L. (1996). Studies on the biological control of Sclerotium rolfsii on bean.M.Sc.Thesis, Fac. of Agric., Univ. of Zagazig. pp. 110.

Abd-El-Moneim, M.L. (2005). Effect of some non-chemical, seed and foliage applications on cotton damping-off disease incidence and yield characteristics. Zagazig J. Agric. Res., 32, 6: 1829-1853.

Abou-Zeid, N.M.; El-Garhy, A.M. and Sohir A. Mokhtar (2002). Biological and chemical control of root-rot/wilt diseases in some legume crops under greenhouse conditions in Egypt. Egyp.J.Agric.Res.80(4): 2002.

Akhtar, C.M. (1982). Investigation of the biological control of *Rhizoctonia solani* and *Fusarium oxysporum f. lini* causing root-rot and wilt diseases in cotton and linseed crops, resoectively. Final report of linseed project No. PKARS-83, AARI / UA. Faislabad.

Amer, G.A. and Utkheda, R.S. (2000). Development of formulations of biological agents for management of root rot of lettuce and cucumber. Canad. J. Microbiol., 46, 9: 809-816.

Anker, H.S.; Johnson, B.A.; Goldberg, J. and Meleny, F.L. (1949). Bacitracin, methods of production, concentration, and partial purification, with a summary of the chemical properties of crude bacitracin. J. Bacteriol., 55: 249-255.

Asaka, O. and Shoda, M. (1996). Biological Control of *Rhizoctonia solani* Damping-off of Tomato with *Bacillus subtilis* RB14. Appl. and Environ. Microbiol., 62, 11: 4081-4085.

Bais, H.P.; Fall, R. and Vivanco, J.M. (2004). Biocontrol of *Bacillus subtilis* against Infection of Arabidopsis Roots by *Pseudomonas syringae* Is Facilitated by Biofilm Formation and Surfactin Production. Plant Physiology, 134: 307-319.

Bergey's Manual of Determinative Bacteriology (1994). Williams and Wilkins Co., Baltimore.

Brian, P.W. and Hemming, H.G. (1945). Gliotoxin a fungistatic metabolic product of *Trichoderma viride*. Ann. Appl. Biol., 32: 214-220.

Chappll, J.and Nable, R. (1987). Induction of sesquiterpenoid biosynthesis in tobacco cell suspension culture by fungal elicitor, Plant Physiol, 85: 469.

Chet, I.; Ordentlich, A.; Shapira, R. and Oppenheim, A. (1990). Mechanisms of biocontrol of soil-borne plant pathogens by *Rhizoctonia*. Plant and soil, 129, 1: 85-92.

Dowson, W.J. (1957). Plant Diseases Due To Bacteria. Second ed., Cambridge, the University Press, London, pp. 231.

Edwards, S.G. and Seddon, B. (1992). *Bacillus brevis* as a biocontrol agent against Botrytis cinerea on protected Chinese cabbage. In: Recent Advances in *Botrytis* Research, Verhoeff, K., Malathrakis, N.E. and Williamson, B. (eds.) pp. 267-271. Wageningen: Pudoc Scientific Publishers.

Estevez-de-Jensen, C. ; Kurle, J.E. and Percich, J.A. (2004). Integrated management of edaphic and biotic factors limiting yield of irrigated soybean and dry bean in Minnesota. Fields Crops Research, 86: 211-224.

Estevez-de-Jensen, C.; Percich, J.A. and Graham, P.H. (2002). Integratd management strategies of bean root rot with *Bacillus subtilis* and *Rhizobium* in Minnesota. Fields Crops Research, 74, 2-3: 107-115.

Ezzat, S.M.; Sarhan, M.M.; Tohamy, M.R.A. and El-Essawy, A.A. (2001). Isolation, Optimization and Characterization of an Antifungal Substance from *Bacillus subtilis* Against *Fusarium oxysporum* f. sp. *Lycopersici*. Egypt J.Microbiol., 36, 2: 191-209.

Fahy, P.C. and Persley, G.J. (1983). Plant Bacterial Diseases: A Diagnostic Guide. Academic Press, New York, 393 pp.

Ferreira, J.H.S.; Matthee, F.N. and Thomas, A.C. (1991). Biological Control of *Eutypa lata* on Grapevine by an Antagonistic Strain of *Bacillus subtilis*. Pytopathology, 8, 3: 283-287.

Fiddaman, P.J. and Rossall, S. (1993). The production of antifungal volatiles by *Bacillus subtilis*. J. of Applied Bacteriology, 74: 119-126.

Fravel, D.R. and Spurr, H.W.Jr. (1977). Biocontrol of tobacco brown-spot disease by *Bacillus cereus* subsp. *mycoides* in a controlled environment. Phytopathology, 67: 930-932.

Hammer, P.E.; Evensent, K.B. and Janisiewicz, W.J. (1993). Post harvest control of *Botrytis cinerea* on cut rose flowers with pyrrolnitrin. Plant Diseases, 77: 283.

Hassanein, A.M.; El-Barougy, E.; Elgarhy, A.m.; Parikka, P. and El-Sharkawy, T.A. (2000). Biological control of damping-off, root-rot/ wilt diseases of alfalfa in Egypt. Egyptian J. of Agric. Research, 78: 1, 63-71.

Hawker, L.E. (1960). Physiology of Fungi. Univ. of London Press, LTD War-Wich Square, London.

Iqbal, S.M. and Akhtar, C.M. (1987). Biological control of sugarcane red rot (*Colletotricum falcatum* Went.). J. Agric. Res., 25, 2: 195-202.

Johnson, L.E., Curt, E.A., Bond, J.H. and Fribourg, H.A. (1960). Methods for studying soil microflora-plant disease relationships. Second printing. Burgess Publishing Company, pp. 178.

Kaparr, I.J. and Kar, B. (1989). Antagonism of *Azotobacter* and *Bacillus* to *Fusarium oxysporum* f.sp. *lycopersici*. Indian Phytopathology, 42, 3: 400.

Kay, S.J. and Stewart, A. (1994). Evaluation of fungal antagonists for control of onion white rot in soil box trails. Plant Pathology, 43: 2: 371-377.

Kilian, M.; Steiner, U.; Krebs, B.; Junge, H.; Schmiedeknecht, G. and hain, R. (2000). FZB24 *Bacillus subtilis* – mode of action of a microbial agent enhancing plant vitality. Pflanzenschutz-Nachrichten Bayer, 1: 72-93.

Kloepper, J.w., Hume, D.L., Scher, F.M., Singleton, C., Tipping, B. Labiberte, M., Frauley, K., Kutchaw, T., Simonson, C., Lifshitz, R. Zaleska, I. and Lee, L. (1988). Plant growth-promoting rhizobacteria on canola (rape seed). Plant Disease, 72: 42.

Kokalis-Burelle, N.; Vavrina, C.S.; Reddy, M.S. and Kloepper, J.W. (2003). Amendment of Muskmelon and Watermelon Transplant Media With Plant Growth-Promoting Rhizobacteria: Effects On seedling Quality, Disease, and Nematode Resistance. Hort Technology,13, 3: 476-482.

Kondoh, M.; Hirai, M. and Shoda, M. (2001). Integrated biological and chemical control of damping-off caused of *Rhizoctonia solani* using *Bacillus subtilis* RB14-C and Flutolanil. J. of Bioscience and Bioengineering, 91, 2: 173-177.

Krebs, B.; Hoding, B.; Kubart, S.; Alemayehuworkie, M.; Junge, H.; Schmiedknecht, G.; Grosch, R.; Bochow, H. and Hevesi, M. (1998). Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. Z. Pflanzenkrankh. Pflanzenschutz, 105: 181-197.

Leifert, C.; Sigee, D.C.; Stanley, R.; Knight, C. and Epton, H.A.S. (1993). Biocontrol of *Botrytis cinerea* and *Alternaria brassicicola* on Dutch white cabbage by bacterial antagonists at cold-store temperatures. Plant Pathology, 42: 270-279.

Levy, E.; Gough, F.J.; Berlin, K.D.; Guiana, P.M. and Smith, J.T. (1992). Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescence* and its antibiotics. Plant Pathology, 41: 335-341.

Loeffler, W.; Kratzer, W.; Kremer, S.; Kugler, M.; Petersen, F.; Jung, G.; Rapp, C. and Tscen, J.S.M.(1990).Gegen Pilze Wirksame Antibiotika der *Bacillus subtilis*-Gruppe. Forum Mikrobiologie, 3: 156-163.

Montealegre, J.R.; Reyes, R.; Perez, L.M.; Herrera, R.; Silva, P. and Besoain, X. (2003). Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato. Electronic J. of Biotechnology, 6, 2: 115-126.

Peypoux, F.; Bonmatin, J.M. and Wallach, J. (1999). Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotech., 51: 553-563.

Raupach, G.S. and Kloepper, J.W. (1998). Mixtures of plants growth-promoting rhizobacteria enhance biological control of multible cucumber pathogens. Phytopathology, 88, 11: 1158-1164.

Reddy, M.S.; Rodríguez-Kábana, R.; Kenney, D.S.; Ryu, C.M.; Zhang, S.; Yan, Z.; Martinez-Ochoa, N. and Kloepper, J.W. (1999). Growth promotion and induced systemic resistance (ISR) mediated by a biological preparation. Phytopathology, 89: S65.

SAS, (1996). SAS/ STAT user's Guide, version 6, 12th ED. Vol.2 SAS Institute Inc. Cary, North Carolina, 846pp.

Siddiqui, I.A.; Ehteshamul-Haque, S. and Shaukat, S.S. (2001). Use of rhizobacteria in the control of root-rot knot disease complex of mungbean. J. Phytopathology, 149: 337-346.

Silosah, L.A.; Lethbrige, B.J.; Raffel, S.J.; He, H.; Clardy, J. and Handelsman, J. (1994). Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol., 60: 2023-2030.

Sneh, B.; Burpee, I. and Ogoshi, A. (1992). Identification of *Rhizoctonia* species. APS. Press. USA 133pp.

Tokuda, Y.; Ano, T. and Shoda, M. (1995). Survival of *Bacillus subtilis* NB22 and its transformant in soil. Appl. Soil Ecology, 2: 85-94.

Toyoda, H.; Hashimoto, H.; Utsumi, R.; Kobayashi, H. and Ouchi, S. (1988). Detoxification of fusaric acid by a fusaric acid resistant mutant of *Pseudonionans solanacearum* and its application to biological control of *Fusarium* wilt of tomato. Phytopathology, 78: 1307.

Yu, G.Y.; Sinclair, G.L.; Hartman, G.L.; Bertagnolli, B.L. (2002). Production of iturin-A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani.* Soil Biology & Biochemistry, 34: 955-963.

المقاومه الحيويه لفطر رايزوكتونيا سولانى المسبب لمرض سقوط البادرات فى الفاصوليا بواسطة أنواع من بكتريا الباسيلس

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تم اختبار ٦ سلالات من بكتريا الباسيلس (٥ باسيلس ساتلس) و (١ باسيلس سريس) معزوله من ريزوسفير نباتات فاصوليا، بسله، فراوله، نعناع سليمه ناميه فى حقول مصابه بكثافه بمرض سقوط البادرات وذلك لاستخدامها فى المكافحه الحيويه لفطر رايزوكتونيا سولانى. الدراسات المعمليه أوضحت أن كل سلالات البكتريا ثبطت معنويا (٣٥-٤٨ % - على حسب نوع السلاله) نمو هيفات الفطر فظهرت منطقة تحلل واضحه فى هيفات الفطر التى تعرضت لنواتج بكتريا الباسيلس وبلغ تأثير انتاج المواد المثبطه لنمو هيفات الفطر بواسطة أنواع الباسيلس أقصاه (٥٨-٧١ %) فى نهاية مرحلة الثبوت لمنحنى النمو بعد١٨-٣٩ ساعه لسلالات باسيلس المختبره. السلالات البكتيريه التى تميزت بالنمو الذائد (٣,١ - ٤,٣ × ١٠[٧] مستعمره/ مل) كانت أكثر كفاءه فى الحد من نمو هيفات الفطر الممرض. اتضح أن بيئة المانيتول السائله كانت الأفضل فى انتاج المواد المثبطه للفطر وتناسب تثبيط نمو هيفات الفطر طرديا مع زيادة تركيز راشح سلالات بكتريا الباسيلس. اختبر تأثير البكتريا تحت ظروف الصوبه حيث أدت معاملة بذور نبات الفاصوليا بأى من سلالات الباسيلس الى خفض نسبة موت البادرات وعفن الجذور وبالتالى زيادة نسبة النباتات السليمه. تم تحضير لقاحات من سلالات الباسيلس فى صورة مسحوق أو محلول وكلاهما كان فعالا فى المقاومه وأن نسبة بقاء النباتات السليمه كانت أعلى بالمعامله بالمحلول مقارنة بالمسحوق. لوحظت علاقه ارتباط طرديه بين زيادة حجم لقاح البكتيريا وعدد النباتات السليمه وكان أفضل تأثير (باستخدام المسحوق ٥٨-٧١ % وباستخدام المحلول ٦٨-٩٢ %) باستخدام تركيز ١٠ مل أو ١٠ جرام لقاح / كج بذور. كانت ٢٠ % من مخاليط البكتريا ذو فعاليه عاليه فى مقاومة درجة الاصابه بفطر رايزوكتونيا سولانى مقارنة بالسلالات الفرديه. استطاعت سلالات الباسيلس البقاء سواء فى اللقاح المحضر أو فى التربه حتى ٦٠ يوم و كان اللقاح المجهز على صورة محلول أكثر كفاءة فى بقاء الخلايا البكتيريه وبالتالى ينصح باستخدامه فى برامج المقاومه المستقبليه.