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In vitro screening of soil bacteria for inhibiting phytopathogenic fungi

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At present, the greatest interest resides with the development and application of specific biocontrol agent for the control of diseases on plant and this form the focus of this work. Several soil bacteria were evaluated *in vitro* for their effectiveness on the basis of their ability to suppress fungi in plate inhibition assays. 51 strains of 12 bacterial species were performed against 12 strains of 10 phytopathogenic mould species. Almost all soil bacteria species; but about 50% of the bacteria strains, showed an antagonistic activity against at least one phytopathogenic fungus. *Sphingomonans* spp was the only specie that did not show any antagonistic effect to all fungi. *Bradyrhizobium japonicum* could highly inhibit the mycelial growth of five moulds (*Botrytis cinerea*, *Phoma medicaginis*, *Fusarium verticilloides*, *Rhizoctonia solani* and *Phytophtora infestans)* with a growth inhibition varying between 12.38 and 37.61%. 12 *Bacillus* strains and five *Pseudomonas* strains were antagonistic to the major phytopathogenic moulds used in this trial. *Bacillus subtilis* exhibited strong antagonism against fungi both from cultural medium and from sterile filtrate. Results show that bacterial suspension and bacterial supernatant did not operate in the same way. Supernatant from bacterial strains seemed to be efficient against phytopathogenic moulds. The mycelial growth of *R. solani*, *P. medicaginis* and *F. verticilloides* was inhibited by 12-fold dilution of the supernatant from *B. japonicum*. The latter draws a conclusion that bacteria isolated from soil are promising natural biocontrol agents and should be further studied and tested for the control of numerous plant diseases. Additional studies are required to definitively determine their mode of antifungal action, safety and biocompatibility.

Key words: Bacteria, phytopathogenic fungi, antagonism.

INTRODUCTION

In Tunisia, farmers have to use chemicals to control the diseases of crop plants. Chemicals create environmental pollution and health hazards. In this regard, certain studies have shown that some fungicides widely used in agriculture may potentially alter the normal development of human reproductive organs and can also reduce the populations of beneficial mycorrhiza (Brimner and Boland, 2003; Blystone et al., 2007). On the other hand, poor farmers do not purchase costly chemicals, that is why the development and application of environmentally safe biopreparations and most of all, biological agents, are of particular importance in the contemporary methods for plant diseases control (Ahamad and Srivastava, 2000; Deacon and Berry, 1993; Grosch et al., 1999; Montesinos, 2007; Nunes et al., 2001; Phae et al., 1992).

Soil is a very complex system that comprises a variety of microhabitats with different physicochemical gradients and discontinuous environmental conditions where micro-organisms adapt to microhabitats and live together in communities interacting with each other and with other components of the soil biota (Trabelsi et al., 2009). Bacterial

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Table 1. Bacterial and fungal species, and strains used in the antagonistic activity screening. Each bacteria strain possesses a code number.

Bacteria species	Strain number	Code	Fungus species	Strain number
Bacillus subtilis	15	1;3;7;10;14;15;16;18;23;25;28;34;40;41;45	*Botrytis cinerea*	1
Bacillus simplex	8	4; 6; 8;13; 19; 24; 43; 49	*Fusarium culmorum*	2
Bacillus cereus	1	9	*Fusarium graminearum*	1
Pseudomonas fluorescens	10	2; 5; 11; 12; 20; 27; 33; 42; 46 ;47	*Fusarium oxysporum*	1
Pseudomonas aeruginosa	4	21; 26; 36; 44	*Fusarium verticillioides*	1
Stenotrophomonas spp	1	17	*Phoma medicaginis*	1
Sinorhizobium meliloti	1	22	*Phytophtora infestans*	2
Sinorhizobium etli	1	39	*Phytoptora nicotianae*	1
Bradyrhizobium japonicum	2	29; 30	*Rhizoctonia solani*	1
Microbacterium spp	3	31; 32; 48	*Sclerotinia sclerotiorum*	1
Bordetella spp	2	35; 38		
Sphingomonas spp	3	37; 50; 51		

biocontrol agent originally obtained from soil has been demonstrated previously to inhibit a broad range of soil borne fungi (Utkhede and Sholberg, 1986; Utkhede and Smith, 1997). Substantial interest of microbiologists in rela-tion to rhizosphere bacteria, reporting variable anti-fungal properties and soil phytosanitary effect, resulted in the occurrence of different methods aimed at studying their fungistatic and growth-stimulating activities (Minaeva et al., 2008).

Considering the potential environmental and health haz-ards and drawbacks in the chemical measures of plant disease control, this study was aimed at screening, the *in vitro* antagonism of soil bacterial against fungal plant pathogens. Thus, the development of a new bio-control agent based on using new rhizobacterial strains not only possessing pronounced antagonistic activity against phyto-pathogens but also as good nitrogen fixers, is recommended and their use is not associated to ecological risks on the indigenous microbial community.

MATERIALS AND METHODS

Bacterial strains

51 bacterial strains (Table 1) were isolated from Tunisian soil and bacterium taxonomy was identified with 16S partial sequencing (Trabelsi et al., 2009). Media used were Lysogeny Broth (LB) [1.0 Bacto Tryptone, 0.5 yeast extract (Difco), and 0.5% NaCl (Mallinckrodt)], and Tryptic Soy Broth (TSB-pH 7) [1.7% Bacto Tryptone, 0.3% Bacto soytone, 0.25% D-glucose (pH 7.0), 5 mM sodium chloride and 5 mM potassium phosphate]. Solid media con-tained 1.5% Bacto agar.

Fungal strains

12 fungal strains (Table 1) were obtained from the cryptogamy laboratory collection (National Institute of Agricultural Research of Tunisia). The media used was potato dextrose broth (PDB) (Difco). The solid media contained 1.5% Bacto agar, and PDA.

In vitro antagonistic activity of bacterial strains against fungal strains

In vitro screening of bacterial strains for inhibiting effect against phytopathogenic moulds was conducted by the methods described by Gupta et al. (2006) with some modifications. The screening was tested with a bacterial suspension and a bacterial extract.

Agar plugs (5 mm diameter) from 5-old day fungal culture was placed in the center of the PDA agar plate. Four wells were made 2 cm apart from the pathogen. The single colony of bacterial strain was mixed with 10 ml of LB fresh medium. After two days of incubation at 150 rpm and 28°C, the bacteria were grown in an incubator shaker at 28°C with 150 rpm. One-old day fungal culture plate was filled with three replicates of 30 µl of bacterial suspension in each and the 4th with 30 µl of sterile medium. Three different media (LB, TSB and PDB) were used for each bacterium. Growth inhibition was calculated by measuring the distance between the edges of the bacterial and fungal colonies by using the formula:

$$\frac{100 \times (C - T)}{C}$$

C is the radial growth of fungus in control and T is the radial growth in dual culture.

Bacteria strains showing a high antagonistic activity were tested with their bacterial supernatant. The bacteria were grown in medium in which they have shown the best inhibitory activity. Bacterial sus-pensions were centrifuged at 10000 rpm for 20 min. The supernatants were collected and filtered through 0.2 µm Nalgen sterilized filter. Various concentrations (8.3, 12.5, 16, 25, 50 and 100%) of culture supernatants were tested to determine the minimum inhibitory con-centration (MIC) which was defined as the minimal concentration that inhibited fungal growth (Islam et al., 2008). Here the disk-diffusion method was used. 5 mm diameter of the filter-paper disk (Whatman filter-paper), impregnated with 100 µl of the bacterial supernatant (triplicate for each samples), was placed on the agar surface.

The supernatant diffused out from the filter paper into the agar. The concentration is higher next to the disk, and decreases gradually as distance from the disk increases. If the compound is effective against fungi at a certain concentration, no mycelium will grow wherever the concentration in the agar is greater than or equal to that effective concentration. This region is called the "zone of inhibition." Thus, the size of the zone of inhibition is a measure of the bacterial supernatant effectiveness: the larger the clear area
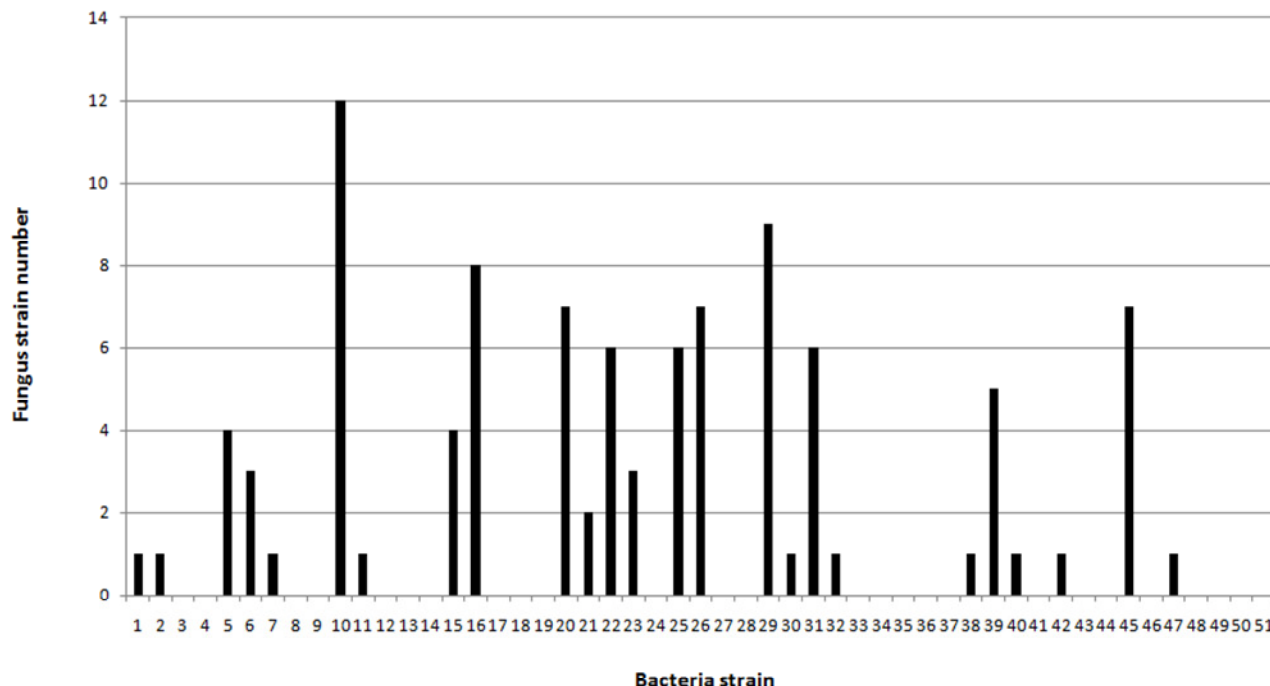
Figure 1. *In vitro* screening of antagonistic activity of bacterial suspension against fungal strains.

around the filter disk. The control test was impregnated with 100 μl of sterile medium.

Statistical analysis

Data were analyzed by the basic module of STATISTICA 6.0 (Stat-soft, 2001). Means, standard errors, and variance were calculated for all data, and per replication. Analyses of variance (ANOVA) were computed for the continuous variables to indicate the significance between strains, media and bacterial supernatant concentration (Dowdy and Wearden, 1991).

RESULTS

In vitro screening of the antagonistic activity of bacterial suspension against fungal strains

About 50% of the bacterial strains were antagonistic against, at least, one phytopathogenic mould. *B. subtilis* (10) showed an antagonistic activity against all the fungi (Figure 1). It was shown that both *B. japonicum* (29) and *B. subtilis* (10, 16 and 45) strains exhibited very broad spectra of action (Figure 1) with an efficient antagonistic activity (Figure 2). *B. subtilis* strain (10 and 16) showed, respec-tively: (a) maximum of 47.5 and 32.02% of *F. culmurum* and (b) mycelial growth inhibition. *B. subtilis* strain (45) and *B. japonicum* strain (29) exhibited, respectively, a maxi-mum of 40.79 and 37.61% of *B. cinerea* mycelial growth inhibition. It is worth noting that the three strains of *Sphingomonas spp* and *Stenotrophomonas spp* did not show any antagonistic

activity against the fungi used (Figure 2).

Media effect on the antagonistic activity of the most efficient bacteria strains

In this trial, only the most antagonistic bacteria [*B. subtilis* (10, 16 and 45) and *B. japonicum* (29)] was tested, as suspension culture in different media against the phyto-pathogenic moulds (Figure 2). Three media were used: LB, TSB and PDB.

B. subtilis strain 10, cultivated in LB medium, produced and secreted an antifungal compound able to inhibit more than 30% the growth of five different phytopathogenic fungal strains. This strain, cultivated in TSB medium, could inhibit more than 30% the growth of just one phyto-pathogenic mould, *F. verticilloides* with growth inhibition of 45.83%. No fungal strain growth was inhibited at more than 30% when *B. subtilis* strain 10 was cultivated on PDB medium (Figure 2).

B. subtilis strain 16, cultivated in LB medium, produced and secreted an antifungal compound that was able to inhibit 32.02% the growth of *F. culmorum* (b). This bacte-rium strain, grown in TSB medium, was able to inhibit 22.81% the growth of *R. solani*. For the other phyto-pathogenic moulds, the growth inhibition did not exceed 12% with any medium culture (Figure 2).

B. subtilis strain 45, cultivated in TSB medium, could produce and secret antifungal compound able to inhibit more than 30% the growth of four different phytopathogenic moulds. Cultivated in LB medium, this
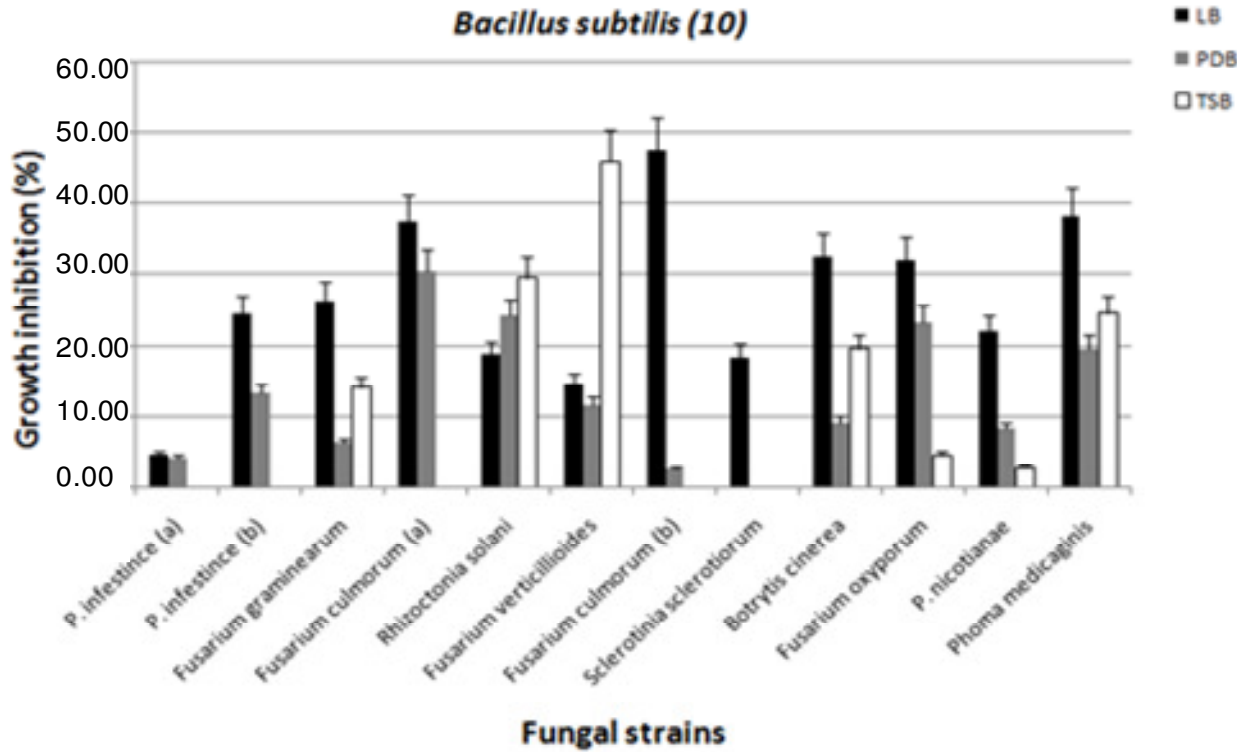
Figure 2. Comparison of the antagonistic activity of *B. subtilis* strain code 10, 45 and 16, and *bradyrhizobium japonicum* strain code 29 cultivated in three different media (LB, TSB and PDB).
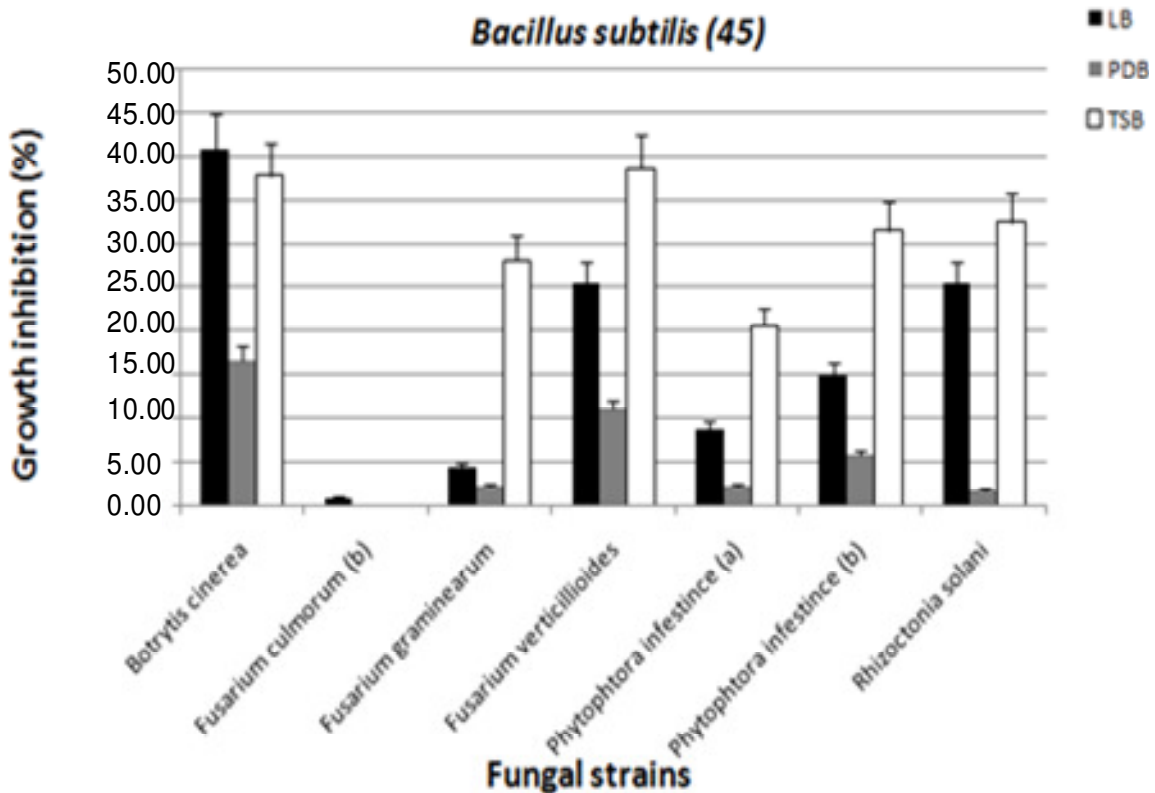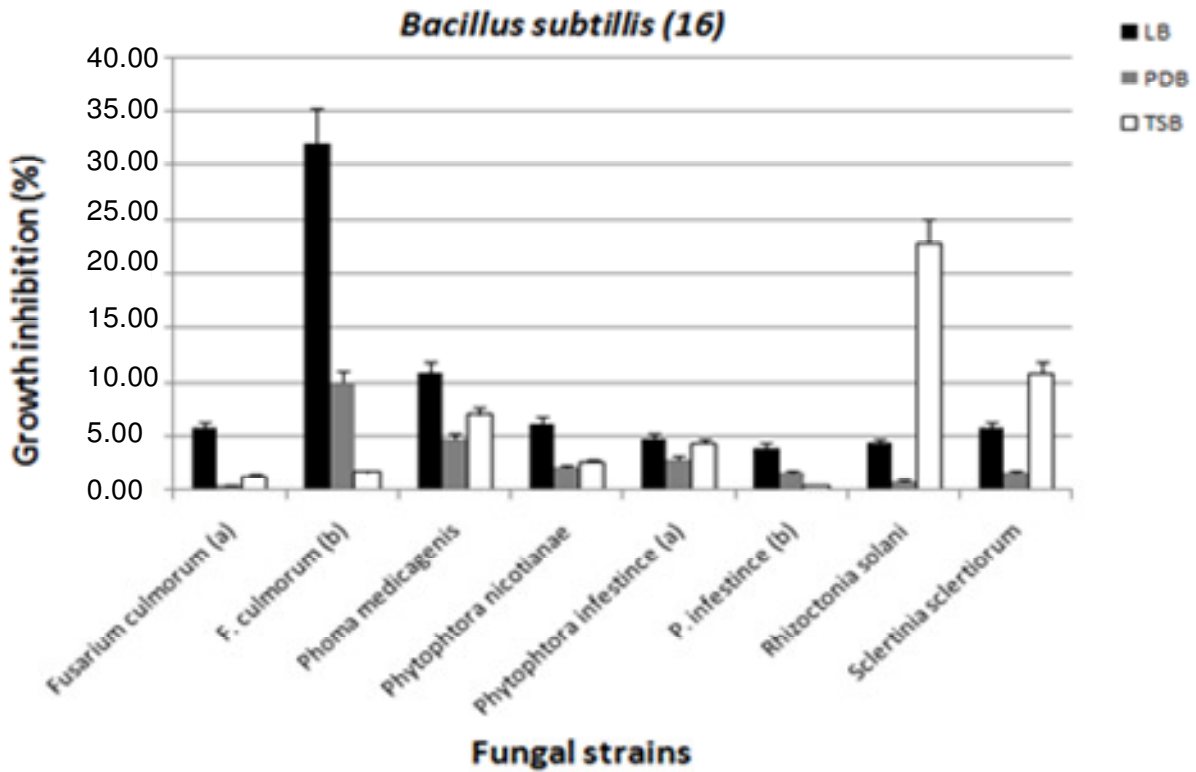

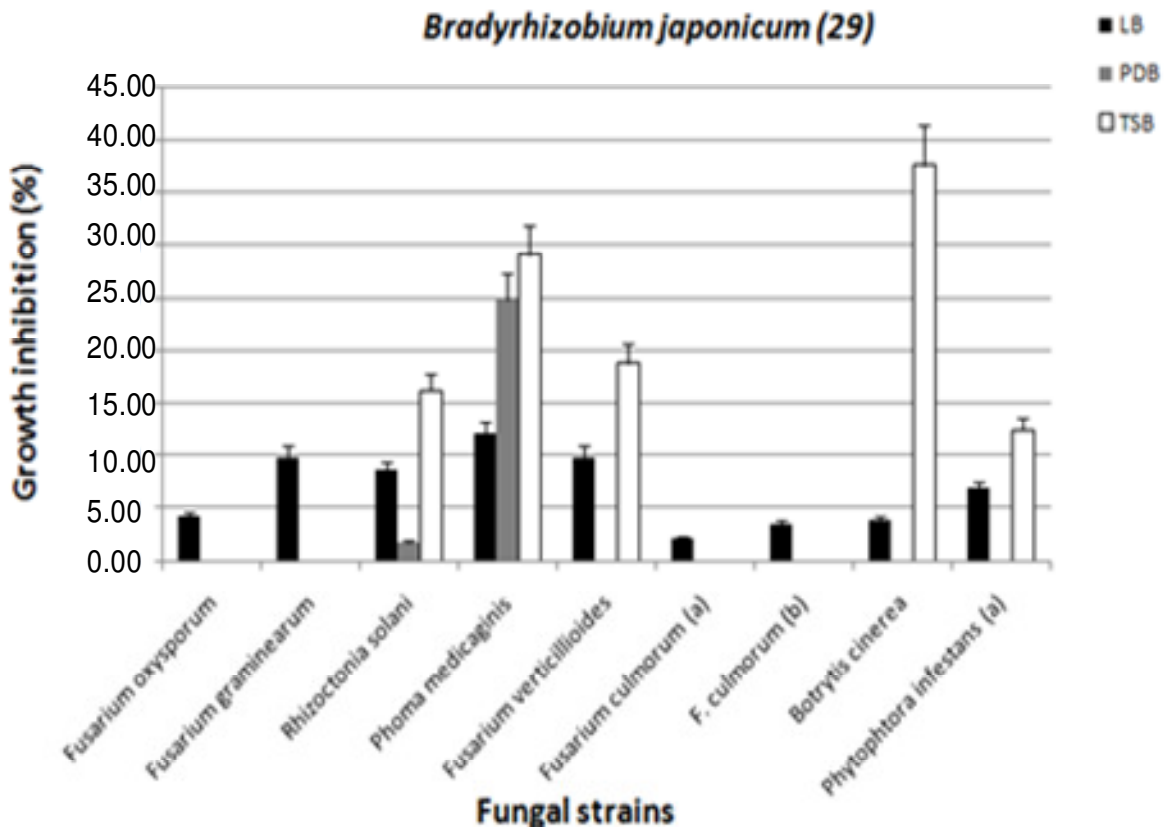
Figure 2. Contd.

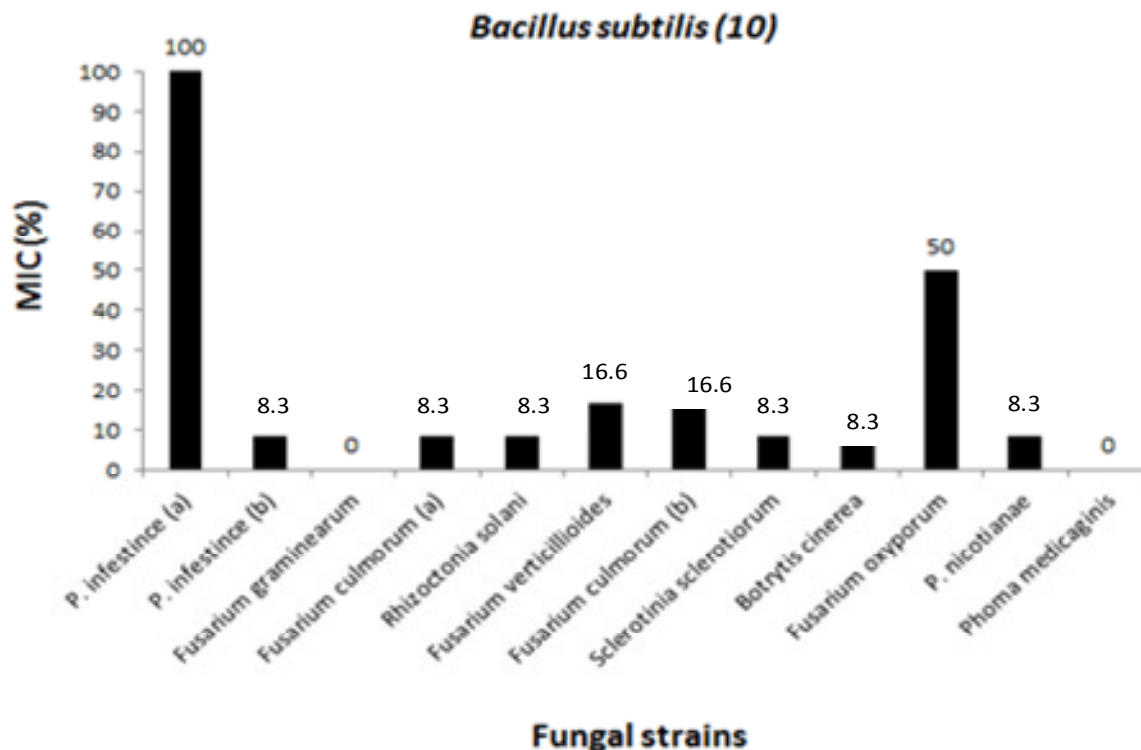**Figure 2.** Contd.

Figure 2. Contd.

Figure 3. *In vitro* extract antagonistic activity of the most efficient bacteria: *B. subtilis* strain 10 cultivated in LB medium, *B. subtilis* strain 45 cultivated in LB medium, *B. subtilis* strain 16 cultivated in TSB medium and *Bradyrhizobium japonicum* strain 29 cultivated in TSB medium.

strain inhibited 40.79% of *B. cinerea* mycelia growth; for the other fungi, the growth inhibition did not exceed 25%. The bacteria suspension in PDB medium was unable to inhibit the growth of the phytopathogenic fungus more than 17% (Figure 2).

B. japonicum strain 29, cultivated in TSB medium, could inhibit the mycelia growth of *B. cinerea*, *P. medicaginis*, *F. verticilloides*, *R. solani* and *P. infestans* (a) with a growth inhibition of 37.61, 29.06, 18.80, 16.24 and 12.38%, res-pectively. This strain, cultivated in LB medium, was able to inhibit the mycelia growth of nine phytopathogenic moulds, but the growth inhibition did not exceed 12%. The sus-pension of *B. japonicum* strain 29 in PDB medium could inhibit the growth of 2 fungi; *P. medicaginis* and *R. solani* with a growth inhibition of 24.79 and 17.1%, respectively (Figure 2).

***In vitro* antagonistic activity of bacterial extract against fungal strains**

Supernatant from *B. subtilis* strain 10 did not inhibit the growth of *F. graminearum* and *P. medicaginis*. Three fungi were not inhibited by the extract from *B. subtilis* strain 16 and strain 45 suspentions; they were *F. culmorum (b)*, *P. nocotianae*, *S. sclerotiorum*, *P. infestans* (b), *F. graminearum*, *F. culmorum* (b),

respectively. Extract from *B. japonicum* suspension did not inhibit the growth of *P. infestans* (a) (Figure 3).

Low concentration (8.3 and 16%) of the supernatant from *B. subtilis* strain (10) could inhibit the growth of eight phytopathogenic moulds and the growth of two fungi was inhibited by a high concentration of the extract (full con-centration, and ½ dilutions). The growth of *R. solani*, *P. infestans* (a) and *P. medicaginis* was inhibited by a low concentration of the extract from *B. subtilis* strain (16), 12.5, 12.5 and 8.3% respectively; in contrast, the growth of *P. infestans* (a) and *F. culmorum* (b) was inhibited by a high concentration, 50% and full concentration, respectively. The growth of *F. verticillioides*, *R. solani* and *B. cinerea* was inhibited by a low concentration (8.3 and 12.5%) of the supernatant from *B. subtilis* strain (45) and *B. japonicum* strain (29) suspensions. A 12-fold dilution of the extract from *B. subtilis* strain (45) and from *B. japonicum* strain (29) could inhibit the growth of *P. infestans* (a) and *P. medicaginis*, respectively (Figure 3).

DISCUSSION

The need to produce innocuous food crops and reduce the pollution generated by synthetic pesticides has led to a search for biocontrol agents against pathogens which are safe for both the crops and for human consumption.
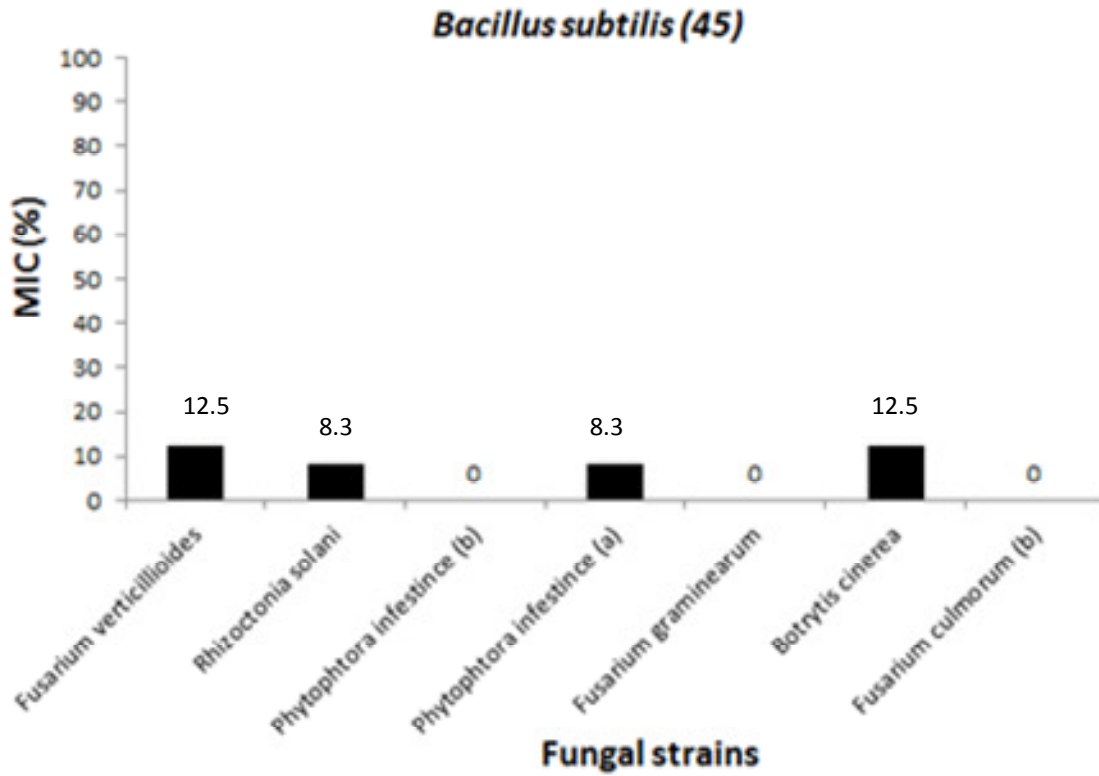
Bacillus subtilis (45)

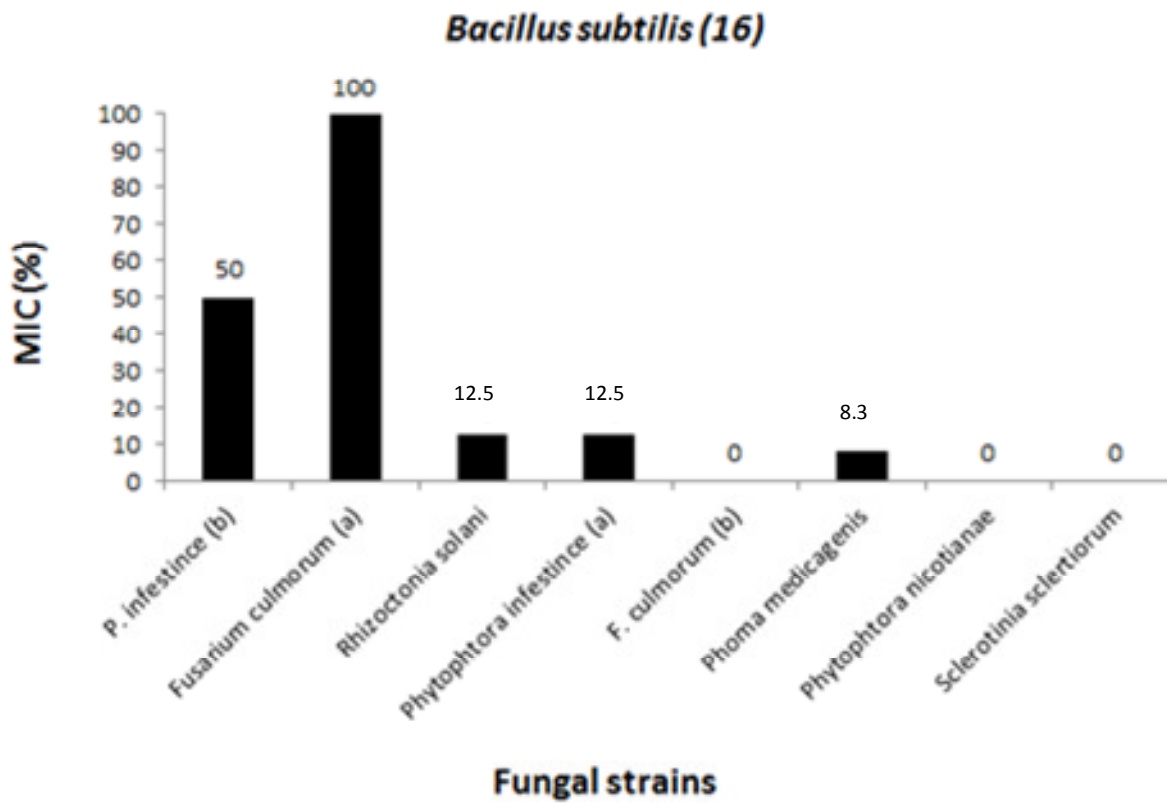**Figure 3.** Contd.

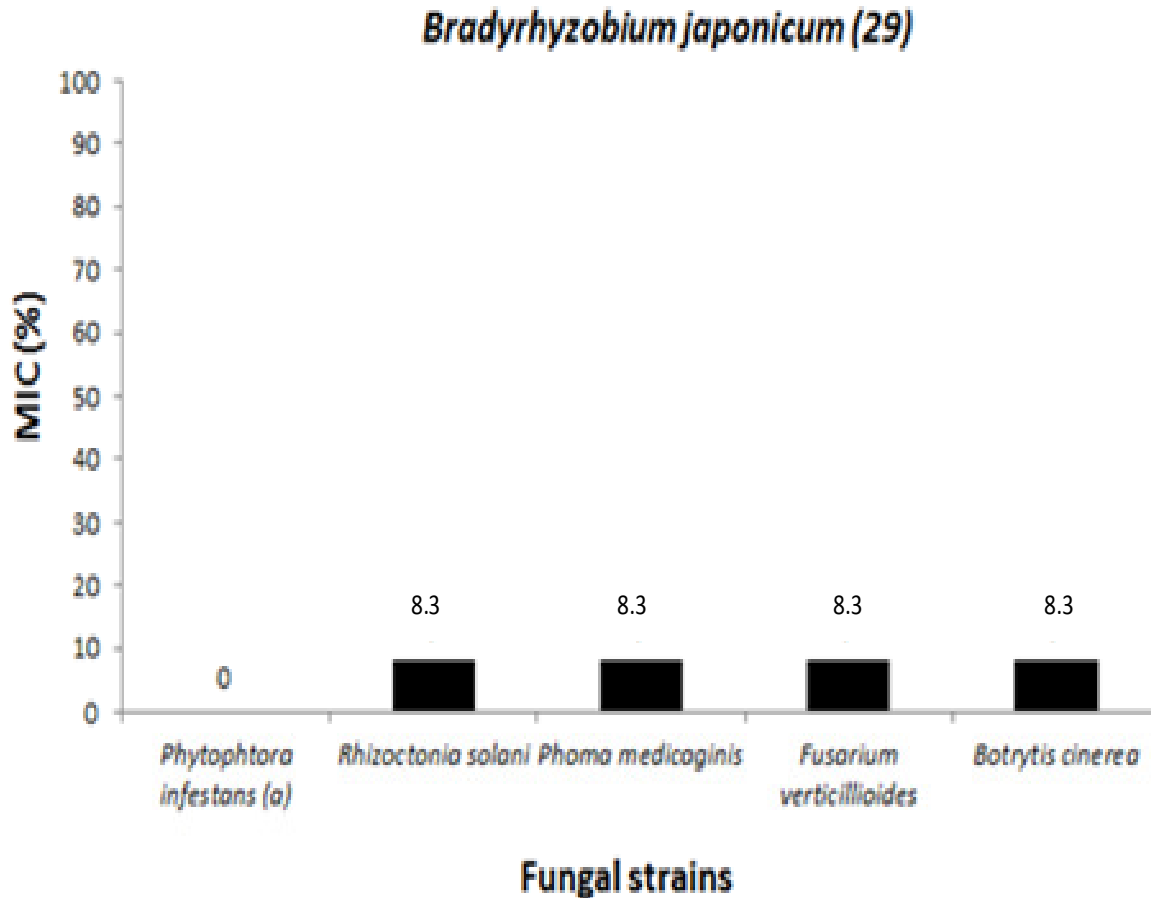**Bacillus subtilis (16)**

Figure 3. Contd.

Figure 3. Contd.

A variety of microorganisms with different modes of action has demonstrated substantial biocontrol activity against phytopathogens. These biocontrol agents have different mechanisms of action which reduce the effects of the pathogens such as: induction of defense responses in the host plant (both local and systemic resistance) (Herr, 1995; Cordier et al., 1998), parasitism (Howell, 2003), and production of metabolites which inhibit pathogen growth (Shen et al., 2002; Robles-Yerenaa et al., 2010).

From these results obtained from the conducted studies, we can summarize that approximately all soil bacteria species, but about 50% of bacteria strains, showed an antagonistic activity against at least one phytopathogenic fungus. *Sphingomonans* spp was the only specie that did not show any antagonistic effect to all fungi (Table 1 and Figure 1). Bacterial isolates consisted mainly of genera *Bacillus*, (by far the most frequent), *Pseudomonas*, *Micro-bacterium*, *Sinorhizobium*, *Bordetella*, *Sphingomonas* and *Stenothrophomonas* (Table 1). 12 *Bacillus* strains and five *Pseudomonas* strains were antagonistic to the major phytopathogenic moulds used in this trial (Table 1). Several publications also reported the broad-spectrum antagonistic activity of

bacteria (*Bacillus* and *Pseudomonas*), isolated from the rhizosphere, against wide range of fungal pathogens (Anjaiah et al., 1998). Jonathan and Stefan (2001) found that several strains *P. fluorescens* known to produce antifungal metabolites were able to induce substantial laccase production by *Rhizoctonia solani*. *P. aeruginosa* has been reported as strong antagonist of *Fusarium oxysporum* (Gupta et al., 1999) and *Sclerotinia sclerotiorum* (Gupta et al., 2006). Loper and Gross (2007) showed that the genome of *P. fluorescens* Pf-5 was characterized by the presence of at least nine gene clusters for secondary metabolite production. Four of these gene clusters specify the biosynthesis of antibiotics with a well-established role in biological control, and the organization and sequences of the biosynthetic, transport, and regulatory genes therein are very similar to those described in other strains of *Pseudomonas spp*. Loper and Gross (2007) mentioned that *Pseudomonas spp*. are prolific producers of antibiotics, and the availability of genomic sequences for several *Pseudomonas spp*. now opens the door for discovery of novel natural products with potential roles in the ecology and plant growth-promoting properties of these bacteria. In this survey, *P. aeruginosa* strain 22 was antagonistic against six fungi

which were among them *F. oxyporum* and *S. sclerotiorum* (data not shown).

In this study, three species and 24 strains of the *Bacillus* genus were used to test their antagonism activity against 12 strains of ten different phytopathogenic mould species. 13 *Bacillus strains* including 12 *B. subtilis* inhibited the growth of a large number of fungi. This corroborate with the finding of Tabbene et al. (2009), who noted that many species and strains of the *Bacillus* genus produce more than 70 different antibiotics and *B. subtilis* is one of the most important antifungal meta-bolites against a number of phytopathogenic micro-organisms. *B. subtilis* is an antagonist for many mould fungi, suppressing their growth in both *in vitro* and *in vivo* (Sevdalina and Lubka, 2009). It is effective against *B. cinerea* (Touré et al., 2004), *Fusarium* sp (Chan et al., 2003), *F. oxysporum* (Ortega et al., 2009) and *R. solani* (Asaka and Shoda, 1996). Besides, the isolated *Bacillus* strains exhibited inhibitory action not only against single species but against all the investigated species during the screening moulds. Most pronounced antagonism was observed with three of *Bacillus* isolates which were identified as *B. subtilis* and designed as strain 10, 16 and 45. These strains exhibited strong antagonism against fungi both from cultural medium and from sterile filtrate. This is probably due to the production of antibiotic substance with antifungal action. The spectra of the antifungal action of the three strains were close but not identical. This clearly indicates that the antibiotic sub-stances produced by them are, probably, close by nature but not the same.

Our result shows that *B. japonicum* could highly inhibit the mycelial growth of five moulds from 12 tested which are *B. cinerea*, *P. medicaginis*, *F. verticilloides*, *R. solani* and *P. infestans* (a) with a growth inhibition varying between 12.38 and 37.61% (Figure 2). A little is known about the antifungal activity and the bioactive compound that could be excreted by this bacterium. In fact, *B. japonicum* are known to be important in symbiosis (Dunlap et al., 1996). One hypothesis is that they increase resistance against low osmotic conditions (Spaink, 2000); while the other is that they serve as supramolecular transporters for other signaling compounds (Morris et al., 1991). They have been hypothesized to act as suppressing agonists that inhibit defense reactions and could be important factors in the successful establishment of symbiosis (Jorn, 2004) and synergistic effects of arbuscular mycorrhiza fungus, and *B. japonicum* have a high potential to improve the nutrient supply of soybean including phosphorus and soil quality (Mukesh et al., 2008). Fabio et al. (2002) showed that *B. japonicum* bacteroids could excrete alanine which is known to occur in bacterial cell walls and in some peptide antibiotics. This compound also has a significant antimycotic effect against phytopathogenic fungi such as *Phythium ultimum*, *B. cinerea* and *R. solani* (Werner et al., 1994). Flávia et al. (2009) mentioned that 35% of *Pythium aphani-dermatum* mycelial growth was inhibited by

B. japonicum suspension. In this study, it is clear that bacterial suspension and bacterial extract did not operate in the same way. Indeed, *B. subltilis* strain 10 suspensions could inhibit the growth of *F. graminearum* and *P. medicaginis* but it was not done with the extract fraction. It was the same with the other strains. The development of three fungi was impeding with strain 16 and 45 suspension but it was not with the extract fraction. Likewise, *B. japonicum* strain 29 suspension impeded the expansion of *P. infestans* (a) but it was not done with the extract fraction (Figure 3). In this case, the fungi inhibition was not due to the bioactive substance that could be released by the strains but it may be a com-petition for nutrients mainly the carbon in the solid PDB media. Competition for nutrients occurs when one organism attempts to obtain a limited food resource for itself at the expense of other organisms competing for the same resou-rce. Competitive success may result from a faster growth or a greater capacity to metabolize organic molecules. Microbes generally compete with each other for carbohydrates, growth factors, nitrogen, iron and other micronutrients (Salvatore and Alessandro, 2008). Minaeva et al. (2008) showed that growth inhibition of fungi *Fusarium* and *Bipolaris* by rhizosphere bacteria was caused mainly by competition for the nutrient substances necessary for the growth and development in the restricted habitat.

Supernatant from bacterial strains seems to be efficient against phytopathogenic moulds. Low concentration (between 8.3 and 16.6 %) of the supernatant from *B. subtilis* strain 10, 16 and 45 could inhibit the growth of 8, 3 and 4 fungi, respectively. The mycelial growth of *R. solani*, *P. medi-caginis* and *F. verticilloides* was inhibited by 12-fold dilution of the extract from *B. japonicum*. Antibiosis phenomenon consists in the production of antibiotics (example, toxins) or other compounds that are toxic for the pathogens or which cause fungistasis (Salvatore and Alessandro, 2008). Cell wall lysis is well established in the biocontrol of fungal pathogens by rhizosphere bacteria (Gabriele and Hallmann, 2006). Pleban et al. (1997) analyzed the importance of lytic enzymes in antagonism of *Bacillus cereus* strain 65 towards the soil borne fungal pathogen *R. solani*. Addi-tionally, chitinolytic *B. subtilis* strains were able to reduce symptoms of *Verticillium dahliae* in several host plants (Tjamos et al., 2004). Douglas et al. (1990) indicated that extracts have the advantage of greater stability in storage than whole-cell preparations; they lacked detectable pro-tease activity and exhibited minimal loss in potency over four to six weeks when kept at 4°C.

Antagonists are naturally occurring organisms with the potential to interfere with pathogen infection, growth and survival (Chernin and Chet, 2002). A better under-standing of the spectrum of indigenous antagonistic bacteria will (1) increase our knowledge of plant/ endophyte interactions, (2) facilitate screening efforts for effective biocontrol orga-nisms, (3) allow breeding of cultivars supporting a high level of antagonistic bacteria,

and (4) may even lead to management strategies for increasing the antagonistic potential of rhizosphere bacteria.

REFERENCES

Ahamad S, Srivastava M (2000). Biological control of dry root rots of chickpea with plant products and antagonistic microorganisms. Ann. Agric. Res. 21:450-451.

Anjaiah V, Koedam N, Nowak-Thompson B, Loper JE, Hofte M, Tambong JT, Cornelis P (1998). Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. Mol. Plant-Microbe Interact. 11:847-854.

Asaka O, Shoda M (1996). Biocontrol of *Rhizoctonia solani* dampingoff of tomato with *Bacillus subtilis* RB14. Appl. Environ. Microbiol. 62: 4081-4085.

Blystone CR, Furr J, Lambright CS, Howdeshell KL, Ryan BC, Wilson VS, LeBlanc GA, Gray LE (2007). Prochloraz inhibits testosterone production at dosages below those that affect androgen-dependent organ weights or the onset of puberty in the male Sprague Dawley rat. Toxicol. Sci. 97:65-74.

Brimner TA, Boland GI (2003). A review of the non-target effects of fungi used to biologically control plant diseases. Agric. Ecosyst. Environ. 100: 3-6.

Chan YK, McCormick WA, Seifert KA (2003). Characterization of an antifungal soil bacterium and its antagonistic activities against *Fusarium* species. doi:10.1139/ Can. J. Microbiol. 49:253-262.

Chernin L, Chet I (2002). Microbial enzymes in biocontrol of plant pathogens and pests. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment: Activity, Ecology, and Applications, Marcel Dekker, New York, pp. 171-225.

Cordier C, Pozo MJ, Barea JM, Gianinazzi S, Gianinazzi-Pearson V (1998). Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular-mycorrhizal fungus. Mol. Plant-Microbe Interact. 11: 1017-1028.

Deacon JW, Berry L.A (1993). Biocontrol of soil-borne pathogens: concepts and their application. Pestic. Sci. 7:417-426.

Douglas AS, Russell D, Alan TM (1990). Seasonal, regional and secular variations of cardiovascular and cerebrovascular mortality in New Zealand. Australian and New Zealand J. Med. 20:669-76.

Dowdy S, Wearden S (1991). Statistics for research. John Wiley and Sons, New York.

Dunlap J, Minami E, Bhagwat AA, Keister DL, Stacey G (1996). Nodulin gene expression by nodule development (ndv) mutants of *Bradyrhizobium japonicum*. Mol Plant Microbe Interact. 9: 546-555.

Fabio OP, Mariangela H, Geoffrey Y, William EN (2002). Alanine excretion from *Bradyrhizobium japonicum* Bacteroids in Nitrogen Fixation: From Molecules to Crop Productivity. Kluwer Academic Publishers New York, Boston, Dordrecht, London, Moscow. ISBN: 978-0-7923-6233-3 (Print) 978-0-306-47615-0 (Online). pp 355-358. DOI: 10.1007/0-306-47615-0. Vol. 38

Flávia MPM, Marli FF, Luiz ABM, Maria ES, Shirlei Sc, Manoel AT, Itamar SM (2009). Antifungal compound produced by the cassava endophyte *Bacillus pumilus* MAIIIM4A. Sci. Agric. (Piracicaba, Braz.). 66:583-592.

Gabriele B, Johannes H (2006). Control of Plant Pathogenic Fungi with Bacterial Endophytes. In: Microbial Root Endophytes B. Schulz, C. Boyle, T. N. Sieber. (Eds) Springer-Verlag Berlin Heidelberg. DOI: 10.1007/3-540-33526-9_4. Soil Biol. 9:53-69.

Grosch R, Junge H, Krebs B, Bochow H (1999). Use of *Bacillus subtilis* as a biocontrol agent. III. Influence of Bacillus subtilis on fungal root diseases and on yield in soilless culture. J. Pl. Dis. Prot. 106:568-580.

Gupta CP, Bhavesh K, Dubey RC, Maheshwari DK (2006). Chitinase-mediated destructive antagonistic potential of *Pseudomonas aeruginosa* GRC₁ against *Sclerotinia scleotiorum* causing stem rot peanut. BioControl 51:821-835. doi:10.1111/j.1365-2672.2004. 02252.x. J. Appl. Microbiol. 96:1151-1160.

Gupta CP, Sharma A, Dubey RC, Maheshwari DK (1999). *Pseudomonas aeuriginosa* as a strong a strong anatagonis of *Macrophomina phaseolina* and *Fusarium oxyporum*. Cytobios. 99:183-189.

Herr LJ (1995). Biological control of Rhizoctonia solani by binucleate Rhizoctonia spp. and hypovirulent R. solani agents. Crop Protection. 14:179-186.

Howell CR (2003). Mechanisms employed by Trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. 87:4-10.

Islam MA, Alam MM, Choudhury ME, Kobayashi N, Ahmed MU (2008). Determination Of Minimum Inhibitory Concentration (Mic) Of Cloxacillin For Selected Isolates Of Methicillin- Resistant *Staphylococcus Aureus* (Mrsa) With Their Antibiogram. Bangl. J. Vet. Med. 6(1):121-126.

Jonathan DC, Stefan O (2001). Induction of laccase activityin Rhizoctonia solani by antagonistic *Pseudomonos fluorescens* strains and a range of chemical treatements. Appl. Environ. Microbiol. 67(5): 2088-2094.

Jorn P (2004). Metabolites from symbiotic bacteria. Nat. Prod. Rep. 21:519-538.

Loper JE, Gross H (2007). Genomic analysis of antifungal metabolite production by Pseudomonas fluorescens Pf-5. Eur. J. Plant Pathol. 119:265-278.

Montesinos E (2007). Antimicrobial peptide and plant disease control. doi:10.1111/j.1574-6968. 2007.00683.x. FEMS Microbiol. Lett. 270(1): 1-11.

Minaeva OM, Akimova EL, Evdokimov EV (2008). Kinetic Aspects of Inhibition of the Phytopathogenic Fungi Growth by Rhizosphere Bacteria. Appl. Biochem. Microbiol. 44(5):512-517.

Morris VJ, Brownsey GJ, Chilvers GR, Harris JE, Gunning AP, Stevens BJH (1991). Possible biological roles for *Rhizobium leguminosarum* extracellular polysaccharide and cyclic glucans in bacteria-plant interactions for nitrogenfixing bacteria. Food Hydrocoll. 5:185-88.

Mukesh KM, Prasad K, Harwani D, Mahna SK (2008). Response of soybean cultivars toward inoculation with three arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* in the alluvial soil. Euro. J. Soil Biol. 44:316-323.

Nunes C, Usall J, Teixido N, Vinas I (2001). Biological control of postharvest pear diseases using a bacterium Pantoea agglomerans CPA-2. doi:10.1016/ S0168-1605(01)00523-2. Int. J. Food Microbiol. 70:53-61.

Ortega-Morales BO, Ortega-Morales FN, Lara-Reyna J, De la Rosa-García SC, Martínez-Hernández A, Jorge Montero M (2009). Antagonism of *Bacillus* spp. Isolated from Marine Biofilms Against Terrestrial Phytopathogenic Fungi. DOI 10.1007/s10126-008-9152-3. Mar. Biotechnol. 11:375-383

Phae C, Shoda M, Kita N, Nakano M, Ushiyama K (1992). Biological control of crown and root rot and bacterial wilt of tomato by *Bacillus subtilis* NB22. Ann. Phytopath. Soc. Jpn. 58:329-339.

Pleban S, Chernin L, Chet I (1997). Chitinolytic activity of an endophytic strain of Bacillus cereus. Lett. Appl. Microbiol. 25:284-288.

Robles-Yerenaa L, Rodríguez-Villarreala RA, Ortega-Amarod MA, Fraire-Velázquezb S, Simpsonc J, Rodríguez-Guerraa R, Jiménez-Bremontd JF (2010). Characterization of a new fungal antagonist of *Phytophthora capsici*. Sci. Hortic. 125:248-255.

Salvatore M, Alessandro R (2008). Biological and Integrated Means to Control Rust Diseases. In Integrated Management of Diseases Caused by Fungi, Phytoplasma and Bacteria. (Eds) Springer Science+Business Media B.V. DOI: 10.1007/978-1-4020-8571-0_15. pp 303-329.

Sevdalina T, Lubka K (2009). Charaxteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. DOI 10.1007/s11274-009-0290-1. World J. Microbiol. Biotechnol. 26:1207-1216.

Spaink HP (2000). Root nodulation and infection factors produced by rhizobial bacteria. Annu. Rev. Microbiol. 54:257-288.

Shen SS, Choi OH, Lee SM, Park CS, (2002). In vitro and in vivo activities of a biocontrol agent, Serratia plymuthica A21-4, against Phytophthora capsici. Plant Pathol. J. 18(4):221-224.

Stat-Soft (2001). Statistica for Windows (Computer Program Manual). Tulsa. Oklahoma. USA. p. 623.

Tabbene O, Ben SI, Bouabdallah F, Mangoni ML, Urdaci MC, Limam F

(2009)/ Production of anti-methicillin-resistant Staphylococcus activity from *Bacillus subtilis* sp. strain B38 newly isolated from soil. doi:10.1007/s12010-008-8277-1. Appl. Biochem. Biotechnol. 157(3): 407-419.

Tjamos EC, Tsitsigiannis DI, Tjamos SE, Antoniou P, Katinakis P (2004). Selection and screening of endorhizosphere bacteria from solarised soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. Eur. J. Plant Pathol. 110:35-44.

Toure´ Y, Ongena M, Jacques P, Guiro A, Thonart P (2004). Role of lipopeptides produced by Bacillus subtilis GA1 in the reduction of grey mould disease caused by Botrytis cinerea on apple. doi:10.1111/j.1365-2672.2004. 02252.x. J. Appl. Microbiol. 96:1151-1160.

Trabelsi D, Mengoni A, Mohammed EA, Mhamdi R, Bazzicalupo M (2009). Genetic diversity and salt tolerance of bacterial communities from two Tunisian soils. Ann. Microbiol. 59(1):25-32.

Utkhede RS, Smith EM (1997). Impact of chemical, biological and cultural treatments on the growth and yield of apple in replant-disease soil. DOI: 10.1071/AP00021, Aust. Plant Pathol. 29(2):129-136.

Utkhede RS, Sholberg PL (1986). In vitro inhibition of plant pathogen by Bacillus subtilis and Enterobacter aerogenes and in vivo control of two post-harvest cherry diseases. Can. J. Microbiol. 32:963-967.

Werner D, Bernard S, Gorge E, Jacobi A, Kape R, Kosch K, Miiller P, Parniske M, Schenk S, Schmidt P, Streit W (1994). Competitiveness and communication for effective inoculation by *Rhizobium*, *Bradyrhizobium* and vesieular-arbuscular mycorrhiza fungi. Experientia, 50:884-889.