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***In vitro* and *In vivo* Biocontrol of Soil-Borne Phytopathogenic Fungi by Certain Bioagents and Their Possible Mode of Action**

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The study aimed to investigate new wide-spectrum biological control agents against soil-borne fungal plant pathogens. Screening of 336 fungal strains and 256 bacterial strains was carried out in vitro to select the most promising isolates for controlling destructive pathogens of greenhouse-vegetables. In dual cultures, *Bacillus subtilis* JF419701 and *Trichoderma harzianum* JF419706 (teleomorph: *Hypocrea lixii*) inhibited the growth of *Alternaria alternata*, *Fusarium oxysporum*, *Exserohilum rostratum*, *Macrophomina phaseolina*, *Pythium ultimum* and *Rhizoctonia solani*. Microscopic examination showed the ability of *T. harzianum* JF419706 to parasitize the hyphae of all pathogens and kill them. *T. harzianum* produced the cell wall degrading enzymes; α -1-3-glucanase (0.83 U/ml), β -1-3-glucanase (0.89 U/ml) and chitinase (0.86 U/ml) in high concentrations. However, *B. subtilis* produced proteases in very high concentrations (9341.64 U/ml). The culture filtrate of *T. harzianum* did not show any antifungal effect. The cell free extract of *B. subtilis*, containing cyano-compounds, suppressed the growth of all phytopathogens, especially *M. phaseolina*. Results proved the efficacy of the two biological control agents to control the common soil pathogens either singly or in combination. We recommend further field experiments to study either the synergistic or antagonistic interactions between them under natural conditions.

Key words : Biocontrol / Soil-borne pathogens / *Bacillus subtilis* / *Trichoderma harzianum* / Chitinase.

INTRODUCTION

Vegetable crops are grown and used worldwide as a source of nutrients and fiber in the human diet. These vegetable crops as well as all crops are vulnerable to the possible attack by fungal pathogens (Punja and Utkhed, 2003). The challenges for producers in managing the damage caused by fungal pathogens are ever increasing, as consumer demand for the year-round production of fresh vegetables with reduced or no pesticide residues, continues to grow

(Robles-Yerena et al., 2010).

Biological control agents (BCAs) are frequently being tested, developed and used in an effort to control various soil-borne plant pathogens. Many reports indicated that a number of biological control agents could control plant pathogens with some efficiency in this field (Koch, 1999; Yobo et al., 2010). However, one of the most serious disadvantages of biological control is that in most cases the control rendered is not equivalent to chemical control (Guetsky et al., 2001). To overcome this effect, an effective combination of chemical agents (in low doses) and biological control agents has been sought. The combined use of BCAs and fungicides has attracted much attention in order to obtain synergistic or additive effects against

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target organisms (Raupach and Kloepper, 2000; Clarkson et al., 2006).

Effective use of BCAs is an important component of sustainable agriculture. The majority of strategies for biological control of soil-borne pathogens rely on a single microbial biocontrol agent for pathogen suppression (Larkin et al., 1998). However, application of BCAs, individually, is not likely to perform consistently against all crop pathogens or under diverse rhizosphere and soil environmental conditions. An approach to overcome this inconsistent performance is to include a combination of BCAs in a single preparation (Abo-Elyousr et al., 2009). Recently, there has been an increasing interest in using more than one BCA to control plant diseases in order to exploit potential synergistic effects among BCAs. There is need to assess the methodology for investigating such synergistic effects and to assess the available evidence so far for synergy (Xu et al., 2011). A combination of BCAs is more likely to have a greater variety of traits responsible for the suppression of one or more pathogens. It is also likely to have these traits expressed over a wide range of environmental conditions (Pierson and Weller, 1994; Crump, 1998). Combining different BCAs can contribute to the better control of pathogens (Spadaro and Gullino, 2005).

Trichoderma spp. and *Bacillus subtilis* are known as biological control microorganisms that have shown promising results in suppressing soil borne diseases in practice (Dawar et al., 2010; Mohiddin et al., 2010; Khan et al., 2011). The biological activity of the species of *Trichoderma* is based on different mechanisms such as the production of antifungal metabolites, mycoparasitism and competition for space and nutrients (Sivakumar, 2001). Their capability to synthesize different antagonistic compounds (proteins, enzymes and antibiotics) and micro-nutrients (vitamins, hormones and minerals) enhance their biological control activity (Al-Taweil et al., 2009). *B. subtilis* is able to synthesize more than 60 different types of antibiotics, many of which possess antifungal effects and belong to the iturin family (Zongzheng et al., 2009). Many studies have been devoted to applying both *Trichoderma* spp. and *Bacillus subtilis* to suppress plant disease; however, little information is available about how the two micro-organisms work together. In addition, the application of biological control in Saudi Arabia has not yet received considerable attention especially from the practical point of view.

Therefore, the objective of this study was to prove the efficiency of new strains of BCAs against a wide spectrum of root pathogens of common vegetables (tomato, cucumber and lettuce) *in vitro* and to study

their possible mode of action. In addition, the study aimed to explore the synergistic effect of the most effective strains of the biological control agents in combination to control the root pathogens of the vegetables under Saudi environmental conditions.

MATERIALS AND METHODS

Isolation of the fungal pathogens

The fungal pathogens: *Alternaria alternata* HQ905483, *Exserohilum rostratum* HQ905473, *Fusarium oxysporum* HQ905450, *Macrophomina phaseolina* JF419703, *Pythium ultimum* JF419705 and *Rhizoctonia solani* HQ905443 were isolated from the roots of naturally infected tomato, cucumber and lettuce plants with root rot disease grown in green-houses in the Asir region in Saudi Arabia. A pathogenicity test of these fungi was carried out to confirm their severity (data not included). They were maintained on potato dextrose agar (PDA) medium amended with streptomycin (60 µg/ml) for bacterial suppression.

Isolation of the antagonistic microorganisms

The antagonistic fungi: *Trichoderma harzianum* JF419706, *Penicillium raperi* HQ905444, *Emericella nidulans* HQ905459 and bacteria: *B. subtilis* JF419701 and *B. subtilis* JF419702 were isolated from the rhizosphere of healthy vegetables (tomato, cucumber and lettuce) and grown in the same farms where the pathogens were isolated using the dilution plate techniques on PDA for fungi and Nutrient Agar (NA) for bacteria.

Identification of microorganisms

The DNA of pathogenic and antagonistic fungi as well as the bacterium was extracted using the Qiagen extraction kit (Qiagen, Germany) following the method described by Ausubel et al. (1995). The fungal isolates were identified by amplification of the ITS gene using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The bacterial isolate was identified by amplification of the 16S rRNA gene using the primers: F DG74f (AGG AGG TGA TCC AAC CGC) and R RW01r (AAC TGG AGG AAG GTG GGGAT) according to Handschur et al. (2005). The PCR amplicones were sequenced in the Macrogen Company (Seoul, Korea). The DNA sequences were analyzed using DNA Blast and the obtained nucleotide sequences were deposited in the GenBank under specific accession numbers.

Preliminary test of antagonism

Screening for the antagonistic potentiality of 336 fungal strains and 256 bacterial strains was carried out against the fungal pathogens by the dual culture method (Hazarika and Das, 1998) at $28 \pm 1^\circ\text{C}$ on PDA medium (data not shown). The most promising strains, which suppressed the most fungal pathogens, were *Emericella nidulans* HQ905459, *Penicillium raperi* HQ905444, *Trichoderma harzianum* JF419706, *Bacillus subtilis* JF419701 and *B. subtilis* JF419702. These strains were selected to study their mode of action in the suppression of the pathogens.

Studying the mode of action of antagonistic microorganisms

Capability of the antagonists to utilize the cell wall of the phytopathogens

The antagonistic fungi and bacteria were inoculated onto Czapek's and nutrient agar containing the cell wall of the phytopathogens as a sole carbon source, respectively. The cell wall preparation of the phytopathogens was carried out as described by Saligkarias et al. (2002). The inoculated microorganisms were incubated at $28 \pm 1^\circ\text{C}$ for 4 days, and their ability to grow and utilize the cell wall of the phytopathogenic fungi was recorded as +++ (very strong), ++ (strong), + (weak) and -/+ (very weak).

Production of cell wall degrading enzymes

Cell wall preparations and preparation of colloidal chitin

Cell wall preparations of the six pathogens were obtained using the method described by Saligkarias et al. (2002). Colloidal chitin was prepared according to the method provided by Roberts and Selitrennikoff (1988) from shrimp shell chitin.

Enzyme production by the antagonists

The culture filtrates of *B. subtilis* JF419701 and *B. subtilis* JF419702 were obtained by growing them in modified Yeast Glucose Broth (YGB). *T. harzianum* JF419706, *E. nidulans* HQ905459 and *P. raperi* HQ905444 were grown in Czapek's broth. The cell wall preparation of fungal pathogens was added to the media as a sole carbon source. At the end of the incubation period, the cultures were filtered through filter membrane ($0.22 \mu\text{m}$) and the filtrate was used as the enzyme's source (Moita et al., 2005).

Enzyme assays

β -1,3-glucanase activity was determined by measuring the amount of reducing sugars released from laminarin, using glucose as a standard (Masih and Paul, 2002). α -1,3-glucanase activity was assayed

as described by Ait-Lahsen et al. (2001) using S-glucan (α -1,3-glucan) as a substrate. S-glucan was extracted from *A. niger* as described by Hasewaga et al. (1969) and Zonneveld (1971). Chitinase activity was determined by a dinitrosalicylic acid (DNS) utilizing the methods of Miller (1959) and Massimiliano et al. (1998). Protease activity was examined as described by Takami et al. (1989).

Antifungal activity of the culture filtrate of *T. harzianum* JF419706 and *B. subtilis* JF419701

The antagonists (fungus and bacterium) were grown under optimum conditions for growth and metabolite production. The cell-free supernatant was collected and filtered aseptically through a sterile membrane with $0.22 \mu\text{m}$ pore size and stored at 4°C . The fungal pathogens were grown on PDA plates, supplemented with 0, 10 or 20 % (v/v) of the antagonists' culture filtrates. Mycelial growth of fungi was monitored by measuring the colony diameter daily for 7 days.

Analysis of the extracellular metabolites in the culture filtrate of the effective antagonists

The cell free extract of *T. harzianum* JF419706 and *B. subtilis* JF419701 was obtained by cultivating them in modified Czapek's and Yeast Glucose Broth (YGB) respectively, containing the cell wall preparation of *M. phaseolina* as a sole carbon source. The expected extracellular metabolites were extracted using different solvents (ethanol, chloroform). Fractions in the extracts were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). An aliquot of $1 \mu\text{l}$ of the extract was injected into the GC-MS (6890 N/5975B). The HP-5MS column was 30 m in length, 0.25 mm i.d., and 0.25 mm in thickness. The carrier gas was helium with an average velocity of 36 cm/sec , and flow of 1 ml/min . The operating conditions of GC oven temperature were maintained as follows: initial temperature of 40°C for 9 min, 150°C for 8 min, at 15°C/min up to a final temperature of 310°C with the isotherm for 3 min at 25°C/min . The injector and detector temperatures were set at 250 and 280°C , respectively, according to the EPA standard method 8270 (Cakir et al., 2004). Identification of the components of the prepared extract was done by comparison of their retention indices, relative to a series of *n*-alkane indices on the capillary column and GC-MS spectra from the Wiley 6.0 MS data according to the method described by Simoes et al. (2007) with modification.

Effect of *T. harzianum* JF419706 and *B. subtilis* JF419701 on suppression of the root pathogens

of vegetables in pot culture

The inocula of both pathogens and biological control agents (BCA) were prepared by inoculation of sterilized 500ml bottles containing autoclaved wheat bran medium (100g/bottle) with 0.5cm agar blocks from the active growth margins from each organism, separately. The bottles were incubated for two weeks at $28 \pm 1^\circ\text{C}$. *B. subtilis* JF419701 was grown in 500-ml Erlenmeyer flasks containing 150 ml of nutrient broth medium supplemented with starch (5%) and sodium alginate (3%) on a rotary shaker at 200 rpm, $28 \pm 1^\circ\text{C}$ for six days.

Plastic pots (15 cm in diameter) containing three kg of soil (sandy loam texture) were inoculated separately with 5 g of an inoculum (one gram contained $\approx 10^7$ CFU) of each pathogen (*A. alternata* HQ905483, *E. rostratum* HQ905473, *F. oxysporum* HQ905450, *M. phaseolina* JF419703, *P. ultimum* JF419705 and *R. solani* HQ905443) to test their pathogenicity (infected control) in a plastic greenhouse. To evaluate the efficacy of the biological control agents (*T. harzianum* JF419706 and *B. subtilis* JF419701) against the pathogens, pots containing the same amount of the soil were inoculated separately with three g of an inoculum of a desired pathogen + 3 g of BCA (10^9 CFU per g) and/or 2 ml of *B. subtilis* (10^{10} cell per ml). Each pot was seeded with 10 seeds of a desired vegetable (tomato, lettuce or cucumber). Five replicates of each treatment were done. Pots inoculated with sterilized wheat bran without any fungal or bacterial inoculation served as the uninfected control (healthy). Pots were arranged in a complete randomized design and watered when necessary. After 10 days, the germination percentage was determined. After 25 days, the number of surviving plants was counted and 5 plants of each pot were uprooted and the degree of root rot infection was assessed and expressed as the mean disease rating (MDR). MDR was evaluated as described by Pal et al. (2001) with minor modifications. The root discoloration was classified into a 5-degree scale as follows:

0= healthy (no discoloration), 1=1-25% discoloration of roots, 2= 26-50% discoloration of roots, 3=51-75% discoloration of roots and 4=75-100% discoloration of roots.

$$\text{MDR} = \Sigma \frac{(A0+B1+C2+D3+E4)}{(A+B+C+D+E4)}$$

where A, B, C, D, and E, are the number of plants with the disease rating of 0, 1, 2, 3, and 4, respectively

STATISTICAL ANALYSIS

All experiments were repeated twice with each treatment done in triplicate. Analyses of data were done by analysis of variance (ANOVA) using the Mstatc program. The Least Significant Difference (LSD) at $P \leq 0.05$ was used to detect differences among treatments.

RESULTS

Capability of the antagonists to grow using the cell wall preparations of the pathogens

Table 1 shows the ability of antagonists to grow on synthetic medium containing the cell wall preparations of the phytopathogens as the sole carbon source. All antagonistic fungi were able to grow very well on the cell wall preparations of the six pathogens except *Emericella nidulans*, which showed relatively slow growth on preparations from *F. oxysporum* and *Pythium ultimum*. The two bacterial strains showed a poor growth with the cell wall preparations of the pathogens compared with antagonistic fungi. *B. subtilis* JF419702, showed very weak growth using the preparations of *F. oxysporum*, *E. rostratum* and *P. ultimum* and slight growth on the media containing the cell wall preparations of the other fungi. On the other hand, *B. subtilis* JF419702 showed good growth using the preparations of *A. alternata*, *M. phaseolina* and *R. solani* and showed slight growth on

TABLE 1. The growth potential of antagonists using the cell wall preparation of the pathogens as a sole carbon source

Antagonists	Pathogens	<i>Alternaria alternata</i>	<i>Fusarium oxysporum</i>	<i>Exserohilum rostratum</i>	<i>Macrophomina phaseolina</i>	<i>Pythium ultimum</i>	<i>Rhizoctonia solani</i>
<i>Bacillus subtilis</i> JF419701		++	+	+	++	+	++
<i>Bacillus subtilis</i> JF419702		+	+/-	+/-	+	+/-	+
<i>Trichoderma harzianum</i> JF419706		+++	+++	+++	+++	+++	+++
<i>Penicillium raperi</i> HQ905444		+++	+++	+++	+++	+++	+++
<i>Emericella nidulans</i> HQ905459		+++	++	+++	+++	++	+++

+/-, very weak growth, +, weak growth, ++, good growth, +++, very good growth

FIG. 1. Steps in the mycoparasitism of *T. harzianum* JF419706 on *Rhizoctonia solani*. (A) *Trichoderma* strains detect the target fungus and grow towards it. (B) *Trichoderma* attaches to the host and coil around it and subsequently form appressoria on the host surface.

media containing that of the other pathogens.

Mycoparasitic potential of *Trichoderma harzianum* JF419706 on hyphae of the pathogens

Microscopic examination of hyphal interaction between *T. harzianum* JF419706 and the fungal pathogens showed clear parasitism by the fungus *T. harzianum* on the six fungal pathogens. The process starts with simple coiling of the *T. harzianum* hyphae around the pathogen's hyphae (Fig. 1). Then, penetration into the pathogen's hyphae could be seen in some cases, supporting the hypothesis that *T. harzianum* hyphae obtain nutrients from the parasitized hyphae after excretion of cell wall degrading enzymes that facilitate their penetration. The results of this study showed a high parasitic affinity between *T. harzianum* and *R. solani*.

Production of cell wall degrading enzymes

Fig. 2 clearly demonstrates that the three fungal antagonists excreted considerable quantities of α -1-3-glucanase when they were grown on synthetic medium supplemented with the cell wall preparation of the six fungal pathogens. *T. harzianum* excreted the highest concentration of the enzyme especially when grown on the medium with the cell wall of *M. phaseolina* (0.83 U/ml). Growth with the cell wall preparation from 5 other pathogens produced this enzyme at a concentration of 0.59-0.76 U/ml. *E. nidulans* came after *T. harzianum*, where it produced 0.44-0.55 U/ml of the enzyme except when it was grown using the *R. solani* cell wall preparation, which

FIG. 2. Effect of the cell wall preparation of fungal pathogens on the production of α -1-3-glucanase enzymes by selected antagonists (B.s.= *B. subtilis*, T.h.= *T. harzianum*, E.n.= *E. nidulans*, P.r.= *P. raperi*). Bars indicate standard error (n=3).

stimulated the production of the enzyme up to 0.97 U/ml. *P. raperi* produced the enzyme in relatively low concentrations. It was noticed that the medium supplemented with the cell wall preparations of *A. alternata*, *M. phaseolina* and *R. solani* stimulated the production up to 0.69 U/ml. However, when it was grown using the cell wall preparations of *E. rostratum*, *F. oxysporum* and *P. ultimum*, the production did not exceed 0.25 U/ml. The two bacterial strains did not produce a considerable concentration of the enzyme, but when *B. subtilis* JF419702 was grown with the cell wall preparation of *P. ultimum*, it produced 0.345 U/ml.

Fig. 3 shows that all antagonistic fungi and bacteria that were able to excrete β -1,3-glucanase when grown using the cell wall preparations of the six

FIG. 3. Effect of the cell wall preparation of fungal pathogens on the production of β -1-3-glucanase enzymes by selected antagonists (B.s.= *B. subtilis*, T.h.= *T. harzianum*, E.n.= *E. nidulans*, P.r.= *P. raperi*). Bars indicate standard error (n=3).

FIG. 4. Effect of the cell wall preparation of fungal pathogens on the production of protease enzymes by selected antagonists (B.s.= *B. subtilis*, T.h.= *T. harzianum*, E.n.= *E. nidulans*, P.r. = *P. raperi*). Bars indicate standard error (n=3).

pathogens, but at different concentrations. It could be summarized that fungi are better producers than the bacteria, and *T. harzianum* was the best one except in some cases. When *P. raperi* was grown using the cell wall preparation of either *A. alternata* or *R. solani*, its production was markedly stimulated (0.66 and 0.89 U/ml, respectively). *B. subtilis* JF419701 produced 0.6 U/ml as a maximum value of production when it grew on medium containing the cell wall preparation of *M. phaseolina*.

The production of protease was completely different from that of both the α -1-3-glucanase and β -1-3-glucanase enzymes (Fig. 4), where the two bacterial strains produced protease in very high concentrations. Peak production was achieved by *B. subtilis* JF419701 when it was grown on medium with the cell wall preparation of *F. oxysporum* (9341.64 U/ml) and another pulse was noticed in the case of *P. ultimum*. Similar pulses were obtained when *B. subtilis* JF419702 was grown using the cell wall preparation of *F. oxysporum* and *R. solani* (6724.6 and 8125.7 U/ml, respectively). The two bacteria were not able to produce any detectable concentration of the enzyme when utilizing the cell wall of *A. alternata*. In contrast, *T. harzianum* and *P. raperi* did not show any activity of the enzyme when grown using the cell wall preparation of any pathogen. However, *E. nidulans* was able to produce protease in considerable amounts, but in lower quantities than bacteria. The maximum excretion was noticed when this fungus was grown on medium containing the *Rhizoctonia* cell wall (4794.03 U/ml).

Fig. 5 shows the productivity of chitinase by the antagonists grown using the cell wall of the pathogens as a sole carbon source. The results clearly showed

FIG. 5. Effect of the cell wall preparation of fungal pathogens on the production of chitinase enzymes by selected antagonists (B.s.= *B. subtilis*, T.h.= *T. harzianum*, E.n.= *E. nidulans*, P.r.= *P. raperi*). Bars indicate standard error (n=3).

the greater ability of fungal strains to produce chitinase than bacteria. *T. harzianum* JF419706 is the highest producer among the fungal strains except in the case of *E. rostratum*, where *E. nidulans* HQ905459 excreted the highest concentration of this enzyme. *E. nidulans* HQ905459 came after *T. harzianum* JF419706 regarding the production of chitinase using cell wall preparations from other pathogens. *P. raperi* HQ905444 produced a relatively low concentration of the enzyme when compared with the two fungi.

Effect of culture filtrate of the bioagents on the growth of the phytopathogens

The results demonstrated that the bacterial culture filtrate has a fungicidal effect on all phytopathogenic fungi in various degrees (Fig. 6). *P. ultimum*, *F. oxysporum* and *R. solani* showed some resistance to the fungicidal effect of the filtrate. They were inhibited by 27.4%, 14.0% and 15.3% of the control, respectively, when they were grown on media supplemented with 20% of the bacterial filtrate. The growth rate of *A. alternata* and *E. rostratum* was inhibited moderately by the application of 20% of the filtrate (58.4% and 50.0% from the control, respectively). However, *M. phaseolina* was the most sensitive organism, as its growth rate was inhibited by 79.7 and 87.8% compared to the control after 4 days of incubation when treated with 10 and 20% of the culture filtrate, respectively (Fig. 7).

The effect of the culture filtrate from *B. subtilis* JF419701 on the morphology of *M. phaseolina*, *A. alternata*, *E. rostratum* and *R. solani* as examined using a light microscope is shown in Fig. 8. It was observed that the hyphae of the fungi growing in the

FIG. 6. Effect the culture filtrate of *B. subtilis* JF419701 and *T. harzianum* JF419706 on the growth of phytopathogens on PDA at 27 °C. B.S.= culture filtrate of *B. subtilis*; T.H.= culture filtrate of *T. harzianum*. Bars indicate standard error (n=3).

absence of the filtrate (control) did not show any obvious growth aberrations. However, an abnormal hyphal swelling was observed in hyphae growing on media containing the filtrate. The abnormal hyphae distinguished itself from that of the control. In some instances, shrinking and vacuolated cytoplasm was observed (*E. rostratum*) as well as extensive lysis of

hyphae (*A. alternata*).

On the other hand, there was no considerable inhibitory effect on the six fungal pathogens produced when they were grown on medium supplemented with either two concentrations (10 and 20%) of the culture filtrate of *T. harzianum* JF419706.

FIG. 7. Effect of the culture filtrate of *B. subtilis* JF419701 on growth of *M. phaseolina* on PDA. Control (left), 10% of filtrate (middle) and 20% of the filtrate (right).

GC-MS analysis of culture filtrates of the bioagents (*T. harzianum* JF419706 and *B. subtilis* JF419701)

Analysis of the culture filtrate of *T. harzianum* JF419706 using the GC-MS technique showed the production of three dominant compounds. They were Phenol, 4,4'-(1-methylethylidene)bis, Diphenolic acid and Phenol, 2,4-isopropylidened (Table 2). The presence of phenol as a main constituent shows that the three compounds are phenolic derivatives and could have some toxic effect on microorganisms.

Nine compounds were identified in the culture filtrate of *B. subtilis* JF419701 (Table 3). They were; Oxetane, 2,3,4-trimethyl, Peroxide, bis (1-methylpropyl), Formic acid, ethyl ester, 3-Methyloxirane-2-carboxylic acid, Propanedioic acid, dihydroxy, Acetic

acid, cyano, Acetamide, 2-cyano, Oxirane, 2, 3-dimethyl and Crotonic anhydride. Among them cyanide was present in two compounds (cyano Acetic acid and 2-cyanoAcetamide).

Effect of *T. harzianum* JF419706 and *B. subtilis* JF419701 in suppression of the root pathogens of vegetables in pot culture

Table 4 shows the effect of application of two BCAs (*T. harzianum* JF419706 and *B. subtilis* JF419701) singly or in the mix on the percentage of seed germination and surviving plants as well as the progress of root rot disease of cucumber, tomato and lettuce, infected with six phytopathogens, in pot experiment. All pathogenic fungi were able to infect the three host plants significantly ($P \leq 0.5$), but in different degrees. *R. solani* and *P. ultimum* were the most aggressive pathogens on the three plants. These pathogens decreased the germination percentage of cucumber to 31.67% and 33.33%, respectively. They increased the MDR in all tested plants from 2.3 to 2.8. The dark fungi (*A. alternate*, *E. rostratum* and *M. phaseolina*) showed a lower degree of aggressiveness on the plants.

Application of the BCAs, either in single preparation or in combination, had a positive effect on the treated plants. They significantly lowered the negative effects that appeared as the result of infection with the fungal pathogens. The BCAs increased the percentage of seed germination and surviving plants and decreased the MDR in all treatments. Application of *T. harzianum* increased the seed germination percentage in the case of *F. oxysporum* to reach the level that was obtained in the healthy control (76.67%). In most cases, BCAs protected the seedlings after germination from the pathogen-related damages and the percentage of surviving plants reached 100%. The clearest observation was that in

TABLE 2. GC-MS analysis of the culture filtrate of *T. harzianum* JF419706.

Formula	Compound name	No.
C ₁₅ H ₁₆ O ₂	Phenol, 4,4'-(1-methylethylidene)bis	1
C ₁₇ H ₁₈ O ₄	Diphenolic acid	2
C ₁₅ H ₁₆ O ₂	Phenol, 2,4-isopropylidened	3

TABLE 3. GC-MS analysis of the culture filtrate of *B. subtilis* JF419701.

Formula	Compound name	No.
C ₆ H ₁₂ O	Oxetane, 2,3,4-trimethyl	1
C ₈ H ₁₈ O ₂	Peroxide, bis (1-methylpropyl)	2
C ₃ H ₆ O ₂	Formic acid, ethyl ester	3
C ₄ H ₆ O ₃	3-Methyloxirane-2-carboxylic acid	4
C ₃ H ₄ O ₆	Propanedioic acid, dihydroxy	5
C ₃ H ₃ NO ₂	Acetic acid, cyano	6
C ₃ H ₄ NO ₂	Acetamide, 2-cyano	7
C ₄ H ₈ O	Oxirane, 2, 3-dimethyl	8
C ₈ H ₁₀ O ₃	Crotonic anhydride	9

FIG. 8. Effect of 20% culture filtrate of *B. subtilis* JF419701 on the morphological structure of hyphae of different phytopathogens. Healthy hyphae in controls show smooth walls and cells filled with cytoplasm. Treated hyphae show swollen walls and shrinkage and vacuolated cytoplasm.

case of both *A. alternata* and *E. rostratum*, the application of the BCAs in combination was more effective than a single application. However, in case of the rest of the pathogens, the application of BCAs singly, especially that of *B. subtilis*, was better than a mixed application. However, the increase in most values was not significant as compared to each other ($P \leq 0.5$).

DISCUSSION

Our results showed that all antagonists grew on a synthetic medium containing the cell wall preparations of the phytopathogens as a sole carbon source. This indicates their potentiality to excrete enzymes that degrade the cell wall of the pathogens (Prapagdee et al., 2008). This finding was supported by detection of high concentration of glucanases, chitinase and proteases in the culture filtrate of the antagonists. Secretion of chitinases and glucanases is a common feature of many effective biological

TABLE 4. Effect of biological control agents' application on germination %, survival % and mean disease rating (MDR) of the tested vegetables in pot experiments

Treatments	Cucumber			Tomato			Lettuce		
	G%	S%	MDR	G%	S%	MDR	G%	S%	MDR
C	76.67	93.5	0.5	83.33	96.1	0.6	73.33	93.5	0.8
A	40.00	91.7	2.2	55.00	88.6	2.3	51.67	94.8	2.4
A+T	56.67	94.2	1.2	60.00	100.0	1.2	56.67	100.3	1.2
A+B	53.33	97.0	1.2	63.33	100.0	1.0	51.67	100.0	1.2
A+T+B	61.67	100.0	1.2	65.00	100.0	1.0	60.00	100.0	1.0
E	41.67	92.6	2.1	50.00	93.9	2.2	46.67	93.0	2.4
E+T	56.67	100.0	1.0	63.33	97.4	1.2	60.00	95.3	1.2
E+B	73.33	100.0	1.0	76.67	97.9	1.2	61.67	97.4	1.2
E+T+B	73.33	100.0	1.0	76.67	100.0	1.2	66.67	97.4	1.2
F	40.00	95.7	2.2	53.33	97.2	1.6	40.00	91.7	2.6
F+T	76.67	100.0	1.2	70.00	100.0	1.0	61.67	94.7	1.2
F+B	66.67	100.0	1.4	66.67	100.0	1.0	60.00	100.0	1.4
F+T+B	71.67	100.0	1.2	70.00	100.0	1.0	65.00	100.0	1.0
P	33.33	70.8	2.8	46.67	93.0	2.5	36.67	82.1	2.6
P+T	50.00	87.6	1.4	78.33	100.0	1.2	51.67	93.6	1.2
P+B	60.00	77.8	1.2	80.00	98.0	1.2	56.67	100.0	1.2
P+T+B	58.33	94.7	1.2	80.00	100.0	1.0	55.00	100.0	1.0
M	45.00	89.6	2.4	58.33	97.2	2.6	55.00	85.6	2.8
M+T	61.67	97.4	1.2	85.00	100.0	1.0	65.00	92.3	1.2
M+B	68.33	92.7	1.2	78.33	100.0	1.2	63.33	100.0	1.2
M+T+B	63.33	97.6	1.2	85.00	100.0	1.2	61.67	100.0	1.2
R	31.67	89.7	2.6	53.33	84.5	2.3	35.00	86.1	2.7
R+T	65.00	97.6	1.2	76.67	100.3	1.2	60.00	100.0	1.2
R+B	71.67	95.6	1.0	65.00	92.8	1.2	58.33	100.0	1.2
R+T+B	66.67	98.0	1.0	81.67	100.0	1.2	61.67	100.0	1.0
LSD ($P < 0.05$)	6.32	10.8	0.57	6.66	6.5	0.54	5.96	10.4	0.61

C, healthy control; **A**, *Alternaria alternata*; **E**, *Exserohilum rostratum*; **F**, *Fusarium oxysporum*; **P**, *Pythium ultimum*; **M**, *Macrophomina phaseolina*; **R**, *Rhizoctonia solani*; **T**, *Trichoderma harzianum*; **B**, *Bacillus subtilis*; G%, Germination %; S%, Survival %; MDR, Mean disease rating.

control agents (Peterson and Nevalainen, 2012; Saloheimo and Pakula, 2012). Studies by Rey et al. (2001) showed that a mutant strain of *T. harzianum* with an enhanced ability to hydrolyze pustulan (a polymer of β -1,6-glucans) showed an enhanced production of chitinase and β -1,3- and β -1,6-glucanases, and produced more extracellular proteins and other compounds.

Our results proved that the mechanism of *T. harzianum* JF419706 to suppress the pathogenic fungi is through competition for nutrients, mycoparasitizing and lysis of pathogens' cell walls. Harman et al. (2004; 2012) mentioned that *Trichoderma* spp. parasitize a range of phytopathogenic fungi. The mycoparasitic process starts when *Trichoderma* detects the target fungus and grows toward it (Chet et al., 1981). Remote sensing is at least partially due to the sequential

expression of cell-wall-degrading enzymes. Production of lytic enzymes and antibiotics might be induced through the mycoparasitism itself, which could be controlled by some factors (Harman et al., 2004).

The culture filtrate of *T. harzianum* JF419706 did not show any significant inhibitory effect on the growth of the pathogenic fungi. Chemical analysis of the filtrate using the GC-MS proved the presence of three phenolic compounds. In agreement with our results, Samapundo and his co-workers (2007) reported that phenolic compounds (vanillic and caffeic acid) did not affect the mycelial growth of the *Aspergillus* species. Similarly, Miller et al. (1996) reported that 4-acetyl-benzoxazolin-2-one (4-ABOA) had no significant effect on the mycelial weight of *Fusarium culmorum*. In contrast, some phenolic compounds have previously been found to have a

potential inhibitory effect on the growth of several fungi (Siranidou et al., 2002). In the liquid culture media of *Trichoderma* spp., some secondary metabolites such as nonanoic acid, alkanes, alcohols, ketones, pyrones, furanes, monoterpenes, and sesquiterpenes have been detected (Aneja et al., 2005; Stoppacher et al., 2010). However, the type and constituents of culture filtrates of this fungus depend on various environmental factors such as relative humidity, substrate and temperature (Polizzi et al., 2011).

The culture filtrate of *B. subtilis* JF419701 showed an inhibitory effect on the growth of all tested pathogens; however, *M. phaseolina* was the most sensitive fungus. The filtrate affects the normal structure of the fungal hyphae through the swelling, shrinking and vacuolation of the cytoplasm leading to complete lysis. It was reported that *Bacillus subtilis* inhibited mycelium growth of numerous plant pathogenic fungi *in vitro* (Liu et al., 2009). Our results were supported by many other studies that established the fungal toxicity of the culture filtrate against many fungal pathogens (Liu et al., 2007; Chakraborty et al., 2006). San-Lang and his co-workers (2002) observed a clear deformation and lysis in hyphae grown on media supplemented with 20% of the crude extract of *B. subtilis* W113.

GC-MS analysis showed the presence of nine aliphatic compounds in the culture filtrate of *B. subtilis* JF419701. Among them, two compounds belong to the cyano-group (cyano acetic acid and 2-cyano acetamide) and two other compounds are oxirane derivatives (3-methyl oxirane-2-carboxylic acid and oxirane, 2, 3-dimethyl). Cyanide is an inhibitor of cytochrome c oxidase in the fourth complex of the electron transport chain (found in the membrane of the mitochondria of eukaryotic cells). It makes complexes with the ferric iron atom in this enzyme. The binding of cyanide to this cytochrome prevents transport of electrons from cytochrome c oxidase to oxygen. As a result, the electron transport chain is disrupted and the cell can no longer aerobically produce ATP for energy (<http://www.t3db.org/toxins>). Oxiranes are important intermediates and starting materials which have found much use in synthetic organic chemistry (Alliouche et al. 2011). In addition, naturally occurring oxiranes are associated with various biological activities (Marco-Contelles et al., 2004). Cyanoacetamides are highly reactive compounds (El-Bayouki et al., 2011) and have shown antifungal or antibacterial effects (Bartoli et al., 1998). We could attribute the inhibitory effect of the culture filtrate of *B. subtilis* JF419701 to the presence of such compounds; however, further studies are needed to confirm this hypothesis.

The results of the pot culture proved that both BCAs suppressed the phytopathogens significantly. Except in some cases, mixing of the BCAs enhanced their performance. This finding indicates that mixing of the BCAs leads to a weak synergistic effect, but does not result in any antagonistic effect between them. We could assume that each of the bioagents could stand alone to control the root diseases of the vegetables; however, mixing them could expand their biocontrol spectrum against multiple pathogens. The weak synergistic effect, which we noticed between the bioagents might be because each of them has more than one mechanism of action. *T. harzianum* showed three different mechanisms of action (mycoparasitism, competition and production of cell wall degrading enzymes) and *B. subtilis* showed two different mechanisms (production of antifungal metabolites and cell wall degrading enzymes). In agreement with our suggestion, Xu et al., (2011) after examining 36 publications involving the combined use of BCAs, concluded that studies suggested that a single BCA with two biocontrol mechanisms usually results in better control than the use of individual or combinations of two BCAs with single mechanisms of action. Recently, there has been an increasing interest among researchers in using combinations of BCAs to exploit potential synergistic effects among them (Xu et al., 2011). Mixing of BCAs showed different results depending on the ecological and application processes (Freeman et al, 2004; Steinberg et al., 1997). The outcome of the combined use of two BCAs, each with a single but different mechanism, does not necessarily result in an appreciable improvement in the biocontrol efficacy over an application of a single BCA with a single mechanism. In most cases, the combined use of BCAs did not lead to an appreciable increase in biocontrol over the use of the more efficacious component and, therefore, was even less likely to show improved results over the expected efficacy under the assumption of Bliss independence. (Xu et al., 2011).

We can conclude that our isolates *T. harzianum* and *B. subtilis* are good candidates for application as safe biological control agents against most soil-borne phytopathogens *in vitro* and *in vivo*. They are characterized by different modes of action like mycoparasitism, and the production of cell wall degrading enzymes and that of fungal toxic metabolites. The wide variety of action expands their biological control spectrum. *In vivo* results proved the presence of a weak synergistic effect between them. We recommend further field experiments dealing with the application of the BCAs singly and in combination, in different modes such as seed treatment and the use

of soil drench, to study the synergistic or antagonistic effects of the BCAs under natural conditions.

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