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BIOCONTROL OF CERTAIN SOILBORNE DISEASES AND PROMOTION OF GROWTH OF *CAPSICUM ANNUUM* USING BIOFUNGICIDES

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Abstract

Colored pepper (*Capsicum annuum* L.) has great economic importance as a food vegetable crop in Egypt and all over the world. This crop is prone to infection with soilborne fungal pathogens such as *Rhizoctonia solani*, *Fusarium solani* and *Macrophomina phaseolina*. These mycopathogens were isolated from diseased pepper seedlings, identified; their virulence was confirmed in the greenhouse. Eight bacterial isolates mainly; (*Bacillus subtilis* and *Pseudomonas fluorescens)*, and many fungal isolates mainly, (*Trichoderma harzianum* and *T. viride)*, were isolated from the rhizosphere soil of pepper. They caused appreciable *In vitro* inhibition of the radial growth of the 3 pathogens in dual culture technique, in percentages ranging from (71-79%) and (80-87%), respectively. On infestation of pepper soil with these bioagents and the 3 pathogens separately in the greenhouse, they caused *In vivo* reduction of disease symptoms of pepper compared with the pathogens infested and non-infested control soils. In addition, they caused significant improvement of pepper growth compared with the control soil, however, promotion exerted by *B. subtilis* and *T. harzianum* was more than that of *P. fluorescens* and *T. viride*. These promoting activities could be attributed to the production of metabolites such as growth hormones; solubilization of phosphates and improvement of nutrient uptake. This is the first record of promoting the growth of pepper in greenhouse by *B. subtilis* and *T. harzianum* in Egypt. Thus these bioagents could be formulated then applied in the future in pepper fields of this country as safe, effective, ecofriendly biofungicides to control soilborne pathogens and also could be used as biofertilizers to promote the growth and productivity of this crop.

Key words: *Capsicum annuum*; Soilborne pathogens; Fungal diseases; Biocontrol; Biofungicides; Biofertilizers.

Introduction

Vegetable crops are used all over the world as sources of nutrients and fibers in the human diet. Pepper, *Capsicum* sp. belongs to the Solanaceous family and is divided into two main groups, pungent and non-pungent, which are also called hot and sweet pepper, respectively. Sweet pepper includes more than one cultivar used in greenhouse production, such as hybrids which have bell-shaped (*Capsicum annuum* L.) (Zayed *et al*., 2013).

In Egypt, sweet pepper (*Capsicum annuum* L.) is one of the most common and preferable vegetable crop used for local market and exportation. High cash crops such as sweet pepper is very important in Egyptian and world agriculture due to its high profit and nutritional values for human health (Fawzy *et al*., 2012).

Soilborne pathogens are considered to be one of the major problems in agricultural production throughout the world, causing reduction in yield and quality of crops. Damping-off; root rot; charcoal rot and wilt of vegetables caused by *R. solani*, *F. solani*, *M. phaseolina*, *F. oxysporum*, *Sclerotium rolfsii*, *Alternaria solani* and *Pythium* spp., are the most deleterious diseases (Steinkellner *et al*., 2008).

Generally, phytopathogenic fungi are chemically controlled using synthetic fungicides, however, the use of these is increasingly restricted due to their harmful effects on human health and the environment (Harris *et al*., 2001). The increasing demand of production, regulations on the use of synthetic fungicides and development of pathogens resistant to these chemicals employed, justifies the search for alternative control strategies such as biological control.

Antagonistic bacteria are mainly soil inhabitants and could be developed into biofungicides for the management of damping-off, rot and many soilborne diseases of different crops (Khabbaz & Abbasi, 2014). Many strains of Bacillus bacteria have been found to be potential biocontrol agents against fungal pathogens. Recently, results of Torres *et al*., (2016) study showed that *B. subtilis*, *B. amyloliquefaciens* significantly inhibited the growth of pathogenic *M. phaseolina* by two different mechanisms namely; lipopeptide synthesis and competition among microorganisms.

P. cepacia or *P. fluorescens* applied to pea seeds act as biological control agents against *Pythium* damping-off and *Aphanomyces* root rot, and were able to reduce diseases incidence (King & Parke, 1993). The mechanisms suggested to be involved in their biocontrol activities were antibiotics production; competition for nutrients; mycoparasitism and improvement of plant growth (Hjeljord *et al*., 2001).

Both of *T. viride* and *T. harzianum* were reported in many previous studies as good antagonists for inhibiting growth of several soil and seed borne plant pathogens (McLean *et al*., 2004; Poddar *et al*., 2004). In the study of Hussain *et al*., (2013), *T. harzianum* was highly antagonistic towards many soilborne pathogens such as; *R. solani*, *M. phaseolina*, *F. oxysporum*, *F. solani* and *Pythium* spp., as it showed a strong inhibitory effect on their growth and mycelial development. Sharon *et al*. (2001) demonstrated the possible role of chitinolytic and/or glucanases enzymes in the biocontrol exhibited by *Trichoderma* spp.

An alternative way to increasing the crop yield is by using biofertilizers besides chemical ones. These include

substances obtained from living organisms and microbial sources (Chen, 2006). Biofertilizers have many different benefits such as; increased access of nutrients to plants, providing growth-promoting factors for plants and effective recycling of solid wastes (Chen, 2006; Das *et al*., 2007). Addition of biofertilizers had a major effect on vegetative growth characters of sweet pepper (Berova & Karanatsidis, 2009; Berova *et al*., 2010); total yield (Berova & Karanatsidis, 2008; Bogevska *et al*., 2009) and quality of sweet pepper plants (Ghoname & Shafeek, 2005; Reyes *et al*., 2008). Accordingly, root infecting fungi can be controlled by pelleting seeds with biocontrol agents as a safe and effective method; in addition plant growth could be also promoted more by this method (Ramzan *et al*., 2016).

The aims of the current work are; to use the bacterial and fungal bioagents as safe, effective and eco-friendly biofungicides to control the major soilborne pathogens of colored pepper in Egypt. In addition to their use as biofertilizers to promote the growth and productivity of pepper for the first time in this country. Thus we could displace using the deleterious fungicides and chemical fertilizers in the fields of pepper.

Materials and Methods

Isolation and identification of the fungal pathogens of pepper plant: Naturally diseased pepper seedlings exhibiting typical symptoms of damping off, root and charcoal rot diseases were collected from pepper fields of El-Munofia governorate–Egypt at November 2014. The mycopathogens were isolated from root pieces of pepper on Potato dextrose agar medium (PDA) according to (Rashad *et al*., 2012). *R. solani* was identified as described by Sneh *et al*. (1991); *F. solani* (Nelson *et al*., 1983) and *M. phaseolina* (Barnett & Hunter, 1972).

Isolation and identification of the rhizosphere bacteria and mycoflora of pepper: Apparently healthy pepper plants with their intact roots and adhering soil were collected from pepper fields, and then bacterial and fungal antagonists were isolated from the rhizosphere soil of these pepper seedlings by serial dilution following the methods of Lodha & Webster (1990); Aneja & Sharma (2010).

Rose Bengal medium was used for isolation of fungi, whereas, soil extract agar medium (Gibbons & Rokas, 2013) and King's medium B (Schaad, 1980) were used for isolating rhizosphere bacteria. Developed bacterial isolates were identified according to Fahy & Hayward (1983); Holt & Krieg (1994); Cappuccino & Sherman (1996). However, fungal colonies were inspected microscopically and only *Trichoderma* spp., were selected and transferred to Gliotoxin fermentation agar medium (GFM) (Mukherjee *et al*., 2012). Colony morphology on petri dishes and microscopical studies on slide culture according to Leahy & Colwell (1990), were adopted for identification of *Trichoderma* spp. Isolates were compared to a taxonomic key for the genus *Trichoderma* (Rifai, 1969).

Pathogenicity assays of the mycopathogens against pepper plant in the greenhouse

Preparation of pathogens inocula: Inocula of *R. solani*, *F. solani* and *M. phaseolina* isolates were prepared by growing each isolate on PDA medium for 5 d. Flasks containing autoclaved corn sand meal medium supplemented with 0.2% peptone solution (Abd El-Moity, 1985) were inoculated separately with equal disks 0.5 cm of each isolate, and then inoculated flasks were incubated at 25°C. After 15 d incubation, inoculum concentration of each isolate was adjusted to contain 5 x 10^6 cfu/g by adding only sterilized corn sand meal medium and mixed through.

Soil infestation: Inocula of *R. solani*, *F. solani* and *M. phaseolina* isolates (5 x 10^6 cfu/g) were added separately to soil in pots at the rate of 10 g/ kg soil. Each pot was sowed with 5 seeds of pepper (var. Bunjii), separately. Pots containing non-infested soil but autoclaved corn sand meal served as control. Ten pots were used for each treatment.

***In-vitro* antagonistic potential of the rhizosphere bacteria and *Trichoderma* isolates against the pepper pathogens**

Rhizosphere bacteria: According to Sivanantham *et al*. (2013), bacterial isolates were streaked separately as thick bands on opposite edges of PDA plates. 4 mm diameter disc of each tested fungal pathogen was cut from of an actively growing culture by a sterile cork borer and then placed onto the center of these plates. The petri dishes were sealed using parafilm, incubated at 28-30°C in dark for 2-3 d. Mycelial disc of each pathogen only placed at center of PDA plates was maintained as control. The antagonistic potential of the bacterial isolates were recorded according to percentage of inhibition of radial growth of the fungal pathogens, compared with the control plates. This assay was conducted in five replicates for each isolate and repeated twice.

Rhizosphere *Trichoderma* spp.: Dual culture assay was conducted according to the methods of Sibounnavong *et al*. (2009a); Charoenporn *et al*. (2010). The selected mycoflora (*T. harzianum*, *T. viride*) and the 3 pathogens were cultured separately on PDA at 30-32°C for seven d. An agar plug of each pathogen was placed separately on one side of the PDA plate opposite to an agar plug of each tested *Trichoderma* isolate. Plates inoculated with a single plug of each pathogen only served as control, plates were then incubated at 30-32°C for 14 d. Five replicates were used for each fungal antagonist. Data were collected regarding pathogen colony diameter (cm). Percentage inhibition of pathogen radial growth was calculated using the following formula:

$$\% \text{ inhibition of pathogen radial growth} = A- B / A \times 100$$

where, A is the colony diameter of the pathogen on the control plate and B is the colony diameter of the pathogen when inoculated opposite to an antagonistic fungus. This experiment was repeated twice.

***In vivo* antifungal potency of the selected bacteria and *Trichoderma* spp. against the pepper pathogens in the greenhouse**

a. For bacterial antagonists

According to the modified method of Gopalakrishnan *et al*. (2010), 8 treatments of the 2 selected bacterial isolates (*B. subtilis* and *P. fluorescens* with the 3 mycopathogens separately, and each bacterial isolate alone) were evaluated in the greenhouse for their *In vivo* antagonistic potential against the 3 pathogens. Pathogens inocula were mass produced separately on corn grains according to Gupta *et al*. (2002). Pot mixture (800 g) was prepared by mixing soil; sand and farm yard manure at 3:2:2 and filled in 8″ plastic pots followed by inoculation with each pathogen inoculum (200 g pot $^{-1}$), inocula were mixed thoroughly with the pot mixture. 100 mL of water was added to each pot and then pots were covered with polythene sheets; the whole set-up was incubated at 32±2ºC. One week later, pepper seeds (var. Bunjii) were surface sterilized with sodium hypochlorite solution (2.5% for 5 min), rinsed with sterilized water (4 times) and then allowed to sprout overnight in a Petri plate.

Sprouted seeds were transferred into the 2 bacterial isolates separately grown in nutrient broth medium (10^8 cfu mL^{-1}) in presence of carboxymethyl cellulose for an hour, before being sown in pathogens infested soils (five treated seeds/ pot). Doses of each bacterial isolate (5 mL per seedling) were applied twice separately (at 7 and 14 d after sowing) by soil drench method. Soils treated with fungal pathogens inocula only served as positive control; whereas, non-infested soil served as negative control. Ten pots were used for each set of treatments.

Growth parameters recognized include; numbers of germinating seeds, root length, shoot length, root fresh weight, shoot fresh weight and visual disease symptoms on pepper seedlings, which were compared with the positive and negative control seedlings. Disease incidences were recorded at day 21 after sowing.

b. For *Trichoderma* isolates

Preparation of pathogens inocula: *R. solani, F. solani* and *M. phaseolina* were grown on PDA medium at 25ºC for 7 d in the dark. Inocula grown on crashed corn seeds were prepared according to Wong *et al*. (1984). Under aseptic conditions, the corn seeds were inoculated separately with four agar plugs (2 mm diameter each) cut from actively growing margins of each growing colony. Flasks were then incubated at 25ºC for one week in the dark, shaken occasionally to ensure uniformity of colonization. Corn seeds free of inoculum and autoclaved twice served as control.

Preparation of antagonistic *Trichoderma* inocula: Inocula of each fungal antagonist (*T. harzianum* and *T. viride*) were prepared on crashed corn seeds in the same way described before for pathogens inocula.

Soil infestation: Eight treatments of the fungal antagonists (*T. harzianum*, *T. viride* with the 3 pathogens separately, and each fungal isolate alone) were evaluated for *In vivo* antifungal potency in the greenhouse. In reference to Madbouly *et al*. (2014), ten free draining pots were used for each antagonistic treatment; each containing 3–4 Kg of non-sterile clay soil. One hundred g of crashed corn seeds infested with each pathogen inoculum were dispersed separately through the lower quarter of soil in each pot, and then left for 2 d. Two hundred g of corn seeds treated with inoculum of each antagonistic fungus (12.7×10^6 cfu/ g corn seed) were dispersed in the upper quarter of soil in each pot. After adding the antagonist's inocula, 5 pepper seeds (var. Bunjii) were sown in the upper quarter of soil in each pot and pots were watered daily. Pots containing soil treated with the pathogens only served as positive controls, whereas non treated soil served as negative controls. After 4–5 weeks, growth parameters of pepper seedlings were recorded as described before with the bacterial antagonists.

Statistical analysis: All treatments were replicated twice, data were recorded as mean ± SD (standard deviation) and subjected to analysis of variance (ANOVA) to analyze differences between applied treatments and disease incidence.

Results

Isolation of fungal pathogens, rhizosphere bacteria and mycoflora of pepper: Isolation of the fungal pathogens from roots of pepper plants showing typical symptoms of damping off; root and charcoal rot diseases respectively, led to the recovery of five fungal isolates in the isolation plates. These were identified mycologically according to the cultural; morphological and microscopical characteristics as; *R. solani, F. solani, F. oxysporum, F. sambucinum* and *M. phaseolina*.

Isolation of rhizosphere bacteria from healthy pepper plants led to the detection of 8 colony morphotypes in the soil extract agar and King's B isolation plates, these were coded as; Pep1-Pep8. They were identified biochemically as *B. subtilis, B. amyloliquefaciens, P. putida, P. fluorescens, P. aeruginosa, Enterobacter cowanii, Azospirillum* sp., and *Paenibacillus polymyxa.*

About 9 different fungal colonies isolated from the rhizosphere of healthy pepper plants were observed in the PDA plates. *Trichoderma* spp., colonies were specifically selected and then identified by examining their shape; size and development of conidiophores, they were identified as *T. harzianum* and *T. viride*.

Pathogenicity assay of the isolated fungal pathogens in the greenhouse: *R. solani, F. solani* and *M. phaseolina* isolates showed high virulent activities against the pepper seedlings in the greenhouse compared with the non-infested control soil, as they caused disease symptoms in about 85-90% of these seedlings. The other two isolates of *F. oxysporum* and *F. sambucinum* caused wilting of 25% of the pepper seedlings only, thus were regarded as aviurlent isolates and not-considered for further research.

***In vitro* antifungal activities of the rhizosphere bacteria and *Trichoderma* spp. against the pepper pathogens:** Five bacterial isolates (*Azospirillum* sp., *B. amyloliquefaciens*, *Paenibacillus polymyxa*; *P. aeruginosa* and *P. putida*) showed weak antifungal potential against the 3 pathogens (Table 1), consequently were neglected from further research. On the other hand, three promising bacterial isolates namely; *B. subtilis*, *Enterobacter cowanii* and *P. fluorescens* appeared to be effective in suppressing pepper pathogens under *In vitro* conditions.

Enterobacter cowanii showed moderate antifungal potency against the 3 pathogens, it was mostly effective against *F. solani* as it surrounded its colony and prevented its radial spread (60% inhibition). However, *B. subtilis* and *P. fluorescens* were highly antagonistic against all pathogens causing inhibitory activities ranging from 71-79%.

On the other hand, both of *T. harzianum* and *T. viride* expressed high *In vitro* antifungal efficacies against *R. solani*, *F. solani* and *M. phaseolina*, as they inhibited their radial growth by 80-87% as clear in (Table 1). Both isolates surrounded the pathogen colonies in dual culture plates and caused their restricted spread.

***In vivo* antifungal potency of the bioagents against the pepper soilborne pathogens in the greenhouse:** Results of *In vivo* effect of the soilborne pathogens on the growth parameters of pepper separately, and in combination with the bacterial, fungal bioagents in the greenhouse are shown in (Tables 2 and 3).

In addition to post-emergence damping-off symptoms of pepper in *R. solani* treated soil (germinated seeds become soft mushy and then disintegrated, slightly darkened water-soaked lesions become visible on stems of young seedlings), *R. solani* caused decrease in the number of germinating pepper seedlings (38), root and shoot length (4, 3.5 cm), fresh wt. of root and shoot (7, 4 g), compared with the non-treated control soil (50- 13, 20 cm- 21, 24.5 g, respectively). However, infestation of soil with *R. solani* and each of the bacterial bioagents separately (Table 2) caused significant reduction in disease symptoms and improvements in the same growth parameters of pepper (*B. subtilis*: 42- 10, 8.5 cm- 17.5, 12.5 g and *P. fluorescens*: 39- 8.5, 6.5 cm- 15.5, 10 g) compared with *R. solani* treated soil.

On treatment of pepper soil with *R. solani* and each of the fungal bioagents separately (Table 3), we observed complete absence of disease symptoms and major enhancements in the growth parameters of pepper compared with pathogen infested soil (*T. harzianum*: 50- 12.5, 11 cm- 21.5, 15.5 g and *T. viride*: 48- 11, 9.5 cm- 20, 12 g) and with the non-treated control soil.

In pepper soil infested with *F. solani* and *M. phaseolina* only, they caused typical disease symptoms. Similarly, the same antagonistic potential of the bacterial and fungal bioagents were observed against both of *F. solani* and *M. phaseolina* respectively, as clear in (Tables 2 and 3). Infestation of soil with *F. solani* and *M. phaseolina* in presence of both of the bacterial and fungal bioagents separately, caused reduction of the virulence of these pathogens in addition to improvements of growth parameters of pepper.

Inoculation of soil with each of the bacterial bioagents only (Table 2) caused promotion of the growth parameters of pepper even more than the non-treated soil (*B. subtilis*: 50- 14, 22 cm- 23.5, 26.5 g and *P. fluorescens*: 50- 13, 20.5 cm- 22, 24.5 g). The growth promoting effect of *B. subtilis* was more than that of *P. fluorescens*. On the other hand, treatment of soil with each fungal bioagent only (Table 3) enhanced the growth of pepper significantly (*T. harzianum*: 50- 15.5, 23 cm- 25.5, 27.5 g and *T. viride*: 50- 14, 21 cm- 24, 22 g) compared with the control soil. *T. harzianum* exerted stimulating effect on the growth parameters of pepper seedlings more than that of *T. viride*. The bioagents and the pathogens were re-isolated from the upper and lower soil layers respectively, thus verifying Koch's postulates.

Table 1. *In vitro* antifungal activities of the rhizosphere bacteria and *Trichoderma* isolates against *R. solani*, *F. solani* and *M. phaseolina* pathogens of pepper, using dual culture technique.

Bacterial\ fungal antagonists	% inhibition of mycelial radial growth		
	R. solani	***F. solani***	***M. phaseolina***
Azospirillum sp.	$29^{a} \pm 0.02$	$21^{a} \pm 1.07$	$28^{a} \pm 0.96$
B. amyloliquefaciens	$27^{a} \pm 0.10$	$32^{d} \pm 0.23$	$48^{c} \pm 0.98$
B. subtilis	$74^{b} \pm 0.02$	$79^{b} \pm 0.32$	$77^{b} \pm 0.76$
Enterobacter cowanii	$47^{c} \pm 0.09$	$60^{f} \pm 0.65$	$53^{f} \pm 1.20$
Paenibacillus polymyxa	$32^{d} \pm 0.07$	$26^{a} \pm 0.0$	$34^{d} \pm 0.17$
P. aeruginosa	$20^{a} \pm 0.16$	$26^{a} \pm 0.94$	$41^{c} \pm 0.41$
P. fluorescens	$71^{b} \pm 0.0$	$75^{b} \pm 1.03$	$77^{b} \pm 0.01$
P. putida	$31^{d} \pm 0.10$	$33^{d} \pm 0.14$	$56^{f} \pm 0.98$
T. harzianum	$85^{e} \pm 0.12$	$87^{e} \pm 0.75$	$82^{e} \pm 0.03$
T. viride	$81^{e} \pm 0.65$	$82^{e} \pm 1.0$	$80^{e} \pm 0.69$

- Results are averages of 5 replicates. ± mean SD (standard deviation); mean values followed by the same letter are not significantly different ($p \leq 0.05$)

Table 2. *In vivo* effects of *R. solani, F. solani, M. phaseolina* pathogens and the bacterial bioagents on the growth parameters of pepper in the greenhouse.

Pathogens and bacterial bioagents treatments	No. of emerging pepper seedlings (out of 50)	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Other visual symptoms of disease
a)-*R. solani*	38	$4^{a} \pm 0.15$	$3.5^{a} \pm 0.21$	$7^{a} \pm 1.01$	$4^{a} \pm 0.15$	seeds were soft and mushy
R. solani + B. subtilis	42	$10^{b} \pm 0.91$	$8.5^{b} \pm 0.00$	$17.5^{a} \pm 0.45$	$12.5^{c} \pm 0.21$	-
R. solani + P. fluorescens	39	$8.5^{b} \pm 0.21$	$6.5^{b} \pm 0.08$	$15.5^{d} \pm 1.04$	$10^{b} \pm 0.91$	-
b)-*F. solani*	46	$9.5^{b} \pm 0.00$	$12^{c} \pm 0.06$	$14^{d} \pm 0.87$	$12^{c} \pm 0.08$	roots were rotted; stems were soft and dark-brown, stunted seedlings
F. solani + B. subtilis	48	$12.5^{c} \pm 0.08$	$14.5^{d} \pm 0.06$	$17.5^{b} \pm 0.55$	$13.5^{d} \pm 1.00$	-
F. solani + P. fluorescens	48	$11^{c} \pm 0.47$	$13.5^{d} \pm 0.78$	$16.5^{a} \pm 0.73$	$12^{c} \pm 0.77$	-
c)-*M. phaseolina*	50	$12^{c} \pm 1.00$	$18^{e} \pm 0.12$	$20^{f} \pm 0.15$	$24^{f} \pm 1.01$	Dusty appearance of root and stem, yellowing of leaves and wilting of seedlings
M. phaseolina + B. subtilis	50	$13.5^{d} \pm 0.20$	$18.5^{e} \pm 0.91$	$21.5^{f} \pm 1.00$	$25.5^{g} \pm 0.54$	-
M. phaseolina + P. fluorescens	50	$12.5^{c} \pm 0.21$	$18^{e} \pm 0.77$	$20^{f} \pm 0.77$	$24.5^{f} \pm 0.47$	-
d)-*B. subtilis*	50	$14^{d} \pm 0.13$	$22^{f} \pm 0.13$	$23.5^{f} \pm 0.01$	$26.5^{g} \pm 0.89$	-
e)-*P. fluorescens*	50	$13^{d} \pm 0.68$	$20.5^{f} \pm 0.88$	$22^{f} \pm 0.00$	$24.5^{f} \pm 0.02$	-
h)-Non-treated control plants	50	$13^{d} \pm 0.24$	$20^{f} \pm 0.08$	$21^{f} \pm 0.21$	$24.5^{f} \pm 0.15$	-

-Results are averages of 50 replicates. ± mean SD (standard deviation); mean values followed by the same letter are not significantly different ($p \leq 0.05$)

Table 3. *In vivo* effects of *R. solani, F. solani, M. phaseolina* pathogens and *Trichoderma* spp. on the growth parameters of pepper in the greenhouse.

Pathogens and *Trichoderma* spp. treatments	No. of emerging pepper seedlings (out of 50)	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Other visual symptoms of disease
a)-*R. solani*	38	$4^{a} \pm 0.15$	$3.5^{a} \pm 0.21$	$7^{a} \pm 1.01$	$4^{a} \pm 0.15$	seeds were soft and mushy
R. solani + T. harzianum	50	$12.5^{b} \pm 0.06$	$11^{b} \pm 1.21$	$21.5^{ac} \pm 0.69$	$15.5^{a} \pm 0.00$	-
R. solani + T. viride	48	$11^{b} \pm 1.01$	$9.5^{c} \pm 0.15$	20 ± 0.77	$12^{b} \pm 0.12$	-
b)-*F. solani*	46	$9.5^{c} \pm 0.00$	$12^{b} \pm 0.06$	$14^{d} \pm 0.87$	$12^{b} \pm 0.08$	roots were rotted; stems were soft and dark-brown, stunted seedlings
F. solani + T. harzianum	50	$13.5^{b} \pm 0.77$	$22^{g} \pm 0.00$	$20^{a} \pm 0.54$	$15.5^{b} \pm 0.00$	-
F. solani + T. viride	50	$13^{b} \pm 0.12$	$20^{f} \pm 1.10$	$19.5^{f} \pm 0.06$	$14^{d} \pm 0.12$	-
c)-*M. phaseolina*	50	$12^{b} \pm 1.00$	$18^{f} \pm 0.12$	$20^{f} \pm 0.15$	$24^{g} \pm 1.01$	Dusty appearance of root and stem, yellowing of leaves and wilting of seedlings
M. phaseolina + T. harzianum	50	$13.5^{b} \pm 0.00$	$23.5^{g} \pm 0.54$	$24.5^{g} \pm 0.42$	$25.5^{g} \pm 0.21$	-
M. phaseolina + T. viride	50	$13^{b} \pm 0.15$	$18.5^{f} \pm 0.20$	$23.5^{g} \pm 0.08$	$26^{g} \pm 0.24$	-
f)-*T. harzianum*	50	$15.5^{d} \pm 0.12$	$23.5^{g} \pm 0.42$	$25.5^{g} \pm 0.91$	$27.5^{g} \pm 0.00$	-
g)-*T. viride*	50	$14^{d} \pm 1.01$	$21^{c} \pm 0.00$	$24^{g} \pm 0.31$	$22^{f} \pm 0.77$	-
h)-Non-treated control plants	50	$13^{b} \pm 0.24$	$20^{b} \pm 0.08$	$21^{f} \pm 0.21$	$24.5^{g} \pm 0.15$	-

-Results are averages of 50 replicates. ± mean SD (standard deviation); mean values followed by the same letter are not significantly different ($p \leq 0.05$)

Discussion

Isolation of fungal pathogens from infected pepper seedlings led to the recovery of 5 fungal isolates, three of them only were selected according to their virulence in the greenhouse, mainly: *R. solani* (causal agent of damping-off disease) (Yang *et al*., 1992), *F. solani* (root rot) (Agrios, 1988) and *M. phaseolina* (known of causing charcoal rot disease) (Jana *et al*., 2005).

Three rhizosphere bacterial isolates showed considerable *In vitro* antifungal activities against the three fungal pathogens. *Enterobacter cowanii* showed moderate antifungal potential against *F. solani* only, thus was excluded from further research. On the other hand, *B. subtilis* and *P. fluorescens* had good antifungal potency against the three mycopathogens in accordance with results of Tan *et al*. (2013); Rajeswari, (2015). This *In vitro* antifungal potency was attributed by Zarrin *et al*. (2009) to the production of inhibitory substances, antifungal antibiotics, cell wall degrading enzymes and siderophores released by the bacteria into the culture media.

Many species of rhizosphere fungi were observed in the PDA isolation plates but *Trichoderma* isolates (identified as *T. harzianum* and *T. viride*) only were selected, because of being potent biocontrol agents of many fungal pathogens (Mokhtar & Dehimat, 2014; Naglot *et al*., 2015). In the current study, *T. harzianum* and *T. viride* showed appreciable *In vitro* antifungal potential against the 3 pathogens, they overgrow and prevented them from radial spread. These observed activities might be attributed to more than one mechanism such as; mycoparasitism (as they overgrow the pathogens) and production of antifungal antibiotics (direct antagonism), in accordance with Zeilinger & Omann (2007); Vinale *et al*. (2008).

In the greenhouse, treatment of soil with the fungal pathogens and the bacterial bioagents (*B. subtilis* and *P. fluorescens*) reduced the virulence of these pathogens and improved the growth parameters of pepper

compared with soil infested with the pathogens only. These *In vivo* antifungal potential could be attributed to the production of antifungal antibiotics, cell wall degrading enzymes, and/ or Fe-chelating siderophores. In a previous study, Ahimou *et al*. (2000) attributed the antifungal potential of *Bacillus* sp. to its ability to synthesize a wide variety of antifungal lipopeptides such as classes of surfactin, iturin and fengicin which were able to modify hydrophobicity of bacterial surfaces, consequently their adhesion to fungal mycelia.

The growth promotion of pepper seedlings by *B. subtilis* and *P. fluorescens* was explained before by Weller, (2007) as rhizobacteria are aggressive root colonizers so may enhance plant growth by producing metabolites, incorporating root exudates and competing with other soil microbes. In later studies, they attributed these promoting activities to ACC deaminase and phosphate solubilization (Shaharoona *et al*., 2008) and production of siderophores (Braud *et al*., 2009).

On infestation of pepper soil with *T. harzianum* and *T. viride* together with the fungal pathogens separately, we observed considerable reduction of disease symptoms and pronounced improvement in the growth parameters of pepper. Howell, (2003) referred the strong antifungal potential of *T. harzianum* to the production of chitinolytic and glucanolytic enzymes, which break down chitin and β-glucan polysaccharides responsible for fungal cell wall rigidity.

The use of chemical fertilizers to enhance soil fertility and crop productivity have many disadvantages. *Trichoderma* have the ability to promote plant growth directly through solubilization of phosphates, minerals such as: Fe, Mn and Mg which have important role in plant growth, and indirectly through control of the rhizosphere root pathogens. Vinale *et al*. (2008) added that the enhanced plant growth by *T. harzianum* was due to the production of secondary metabolites as auxin like compounds, which cause development of the root system and exploration of a larger volume of the soil.

In accordance with our results, the protection exerted by *T. harzianum* against the fungal pathogens was more than *B. subtilis*, this difference might be due to several modes of actions exerted by *Trichoderma* spp., as they are well-known producers of cell wall-degrading enzymes and antibiotics. The antagonistic *T. viride* and *P. fluorescens* occupied significantly the second degree of reducing soilborne pathogens in accordance with Abdel-Kader *et al*. (2012).

This is the first time to record the *In vivo* growth promoting activities of *B. subtilis* and *T. hrazianum* on pepper plant in Egypt. Our future prospectus is to mass produce and then formulate these bioagents to be used as safe, ecofriendly biofungicides against pepper soilborne pathogens, and at the same time as effective biofertilizers to promote the productivity of this crop in the fields of Egypt.

Conclusion

B. subtilis and *T. harzianum* could be used as potent biofungicides to reduce the incidence of major soilborne fungal pathogens of colored pepper, moreover, they may be applied as biofertilizers to promote the growth and productivity of this crop. In the future, both bioagents could be applied on a wide scale in the pepper fields of Egypt, hence displace the use of deleterious, environmentally non-safe synthetic fungicides and chemical fertilizers.

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