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Biological control of Pythium root rot of broccoli plants under greenhouse conditions

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Pythium ultimum, *Rhizoctonia solani* and *Fusarium solani* were the most isolated fungi from roots of broccoli (*Brassica oleracea* L. var. *italica*) plants showing root rot disease symptoms collected from different governorates in Egypt . Pathogenicity test proved that *Pythium ultimum* is the main causal agent of root rot disease on broccoli plants .No significant difference between broccoli varieties (Atlantic F1 and southern star) in their susceptibility to *Pythium ultimum* infection. *Trichoderma harzianum ,T. virdi* , *Bacillus subtilis* and *Pseudomans fluorescens* isolated from the rhizospheric soil of healthy broccoli plants could completely inhibit the growth of *Pythium ultimum* on PDA medium *in vitro* .Under greenhouse trials, A combination of soil mixing plus root dipping method was generally more effective in reducing disease incidence than each method applied individually. Dipping roots of broccoli seedlings in water suspensions of each bio control agent i.e., *Trichoderma harzianum* , *T. virdi* ($5x10^6$ spore/ml) , *Bacillus subtilis* and *Pseudomans fluorescens* ($8x10^7$ spore/ml) and mixing soil with the same suspensions of bio control agents during transplanting ,gave the highest effect in reducing Pythium rot disease if compared with applying the two methods individually.The use of bio control agents as soil mixing or root dipping treatments as applicable safe and fungicides alternative might be used for controlling Pythium root rot on many crops especially under organic farming system.

Key words: Broocoli, Pythium rot, *T. harzainum*, *B. subtilis*, and *P. fluorescens*, biological control.

Introduction

Several diseases caused by many biotic agents are known to attach broccoli Broccoli (*Brassica oleracea* L.var. *italica*) plants all over the world causing serious losses in the yield. *Pythium* root rot is the most serious disease (Abdelzaher, 2003; Tanina *et al.*2004).

Pythium spp are ubiquitous soil borne pathogens they cause damping–off and root rot diseases on many plant species such as cabbage, Chinese cabbage, broccoli, carrot, cucumber, melon, turf grass, cotton, wheat and others

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(Abdelzaher, 2001). *Pythium* spp. could be controlled by steaming the soil which is applied in a small scale. Although, fungicides could supply a good suppression of Pythium diseases, they have a hazardous effect to the environment. Currently, increasing attention has been paid to biological control through the use of antagonistic microorganisms such as fungi and bacteria as an alternative to fungicides (Gravel *et al*. 2004). Many biological control agents such as *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. suppress diseases caused by *Pythium* spp and *Rhizoctonia solani* (Moller *et al.* 2003; Carisse *et al.* 2003). *Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soilborne fungi (Abdel-Kader (1997). Georgakkopoulos *et al*. (2002) found that better biocontrol in cucumber was achieved when *B. subtilis* and *P. fluorescens* were applied by drenching or by coating seed in peat carrier. Pseudomonas antagonists were superior to Bacillus antagonists for controlling Pythium root rot on cucumber and sugar beets.

The purpose of the present work was designed to survey the incidence of Pythium root rot of broccoli in different governorates of Egypt, isolation and identification of the causal fungi and evaluate its pathogenic ability. The effectiveness of rhizospheric bio agents against *Pythium* root rot disease incidence on broccoli under green house conditions.

Materials and methods

Survey of broccoli root rot diseases

Survey study on the incidence of root rot diseases of broccoli was carried out during 2008/2009 season. Four Governorates, *i.e*. Giza, Kalubeia, Ismaelia and Beheira (Nobaria province) were selected for survey where broccoli are widely spread especially in the new reclaimed lands. Plants of broccoli showing Pythium root rot symptoms were recorded. Hundred plants were examined in five farms at each surveyed governorate and the percentage of root rot infection were recorded.

Isolation and identification of the causal organism (s)

Samples of broccoli plants showing typical root rots symptoms were collected from surveyed fields. The root and basal parts of plant samples were rinsed in ethanol (5% v:v) for 30 sec, washed with tap water to remove the adhering soil particles, followed by sterile tap water, dried between two sheets of sterilized filter paper, and then cut to small pieces (2 sq cm each) and placed into Petri-dishes containing sterilized media. The media used were Martin's

medium (Allen 1961); VP3 selective medium (Ali-Shtayeh *et al*. 1986) and Pythium selective agar medium (Davison and McKay 1998).Identification of isolated fungi. was made using cultural and morphological characteristics and the aid of a light microscope according to Gilman (1957) and Barnett and Hunter (1972).

Pathogenicity test

Five isolates of *P. ultimum* (isolates G1, G2, B1,I1 and K1) were evaluated for their pathogenic ability against two cultivars of broccoli plants (Atlntic F1 and Souther star).The experiment was carried out in autoclaved (121^{o}C for two hours) clay loamy soil (50% sand, 40% clay and 10% silt) artificially infested with the tested fungal isolates. Soils were infested individually at a ratio of 5% (w:w) with tested pathogenic fungal cultures and mixed thoroughly to ensure equal distribution of fungal inoculum, then filled in plastic pots (25-cm-diameter) and irrigated every second day for 1 week before sowing. A set of pots were also amended with the same rate of sand barley medium free of fungal inoculum and reserved as control treatment. Healthy seedlings of broccoli of either cultivar were transplanted in both infested and non-infested soils, five seedlings per pot and ten replicates (pots). At the end of the experiment, plant roots showing rot lesions in addition to the visual root rot symptoms on the shoot system were considered diseased plants. The average percentages of root rot incidence (infected seedlings) were recorded.

Plates antagonistis test

The antagonistic ability of different microorganisms isolated from rhizospheric soil of healthy broccoli plants against *P. ultimum* was evaluated on PDA plates. Two isolates of each *T. harzianum*, *T. viride* , *B. subtilis* , and two isolates of *P. fluorescent*, were tested.. Interactions between *P. ultimum* and bio control agents were assayed using dull culture technique after Ferreira *et al.* (1991) on potato dextrose agar medium. This test was repeated three times and the inhibition was calculated as the percentage reduction in colony diameter growth compared with the control.

Greenhouse experiment

The evaluation of the bio agents against root rot incidence caused by *P. ultimum* was carried out under greenhouse conditions at NRC. One isolate of each *T. harzianum*, *T. viride*, *B. subtilis* and *P. fluorescens* were tested. The used microorganisms inocula prepared as follows:

Fungal mass production of either pathogenic or antagonistic fungi used for soil infestation was obtained by growing the tested isolates on sand-barley medium as mentioned above. As for spore suspension of antagonistic fungi, the tested bio agents individually grew on PDA medium at 25±2^{o}C until the abundant heavy growth of conidia was evident. Conidia were harvested by scraping the surface of the colonies with a spatula and then transferred to sterilized distilled water and filtered through nylon mesh. All spore suspensions were adjusted with sterile water to give a spore concentration of 10^{6}-10^{7}/ml. Meanwhile, bacterial bio agents were grown individually for 48 h in nutrient broth medium, and then cells were harvested by centrifugation. Bacterial isolates were re-suspended in sterile distilled water and the concentration adjusted to give 10^{9}-10^{10} cells/ml.

The pot experiment was carried out using a clay loamy soil artificially infested with the pathogen. Healthy transplants, 21 days old, of broccoli grown in autoclaved peat moss soil, obtained from Vegetable Research Department, National Research Center, Egypt were used.

Different applied treatments for bio control evaluation were performed as follows:- root dipping (RD) method, soil infestation (SI) method (SI) and root dipping (RD) plus soil infestation (SI) method.

Root dipping (RD) method

Roots of broccoli transplants were immersed for 30 min in either fungal or bacterial suspension supplemented with 1% (w/v) carboxymethyl cellulose (CMC) as adhesive polymers, then planted in infested soil with the pathogenic fungi.

Soil infestation (SI) method

Untreated broccoli transplants were planted in pots containing soil previously artificially infested with the pathogenic fungi followed by infestation with either fungal or bacterial bio agents. The fungal inocula of *T. harzianum* or *T. viride* were added to the soil at the rate of 5% w:w, meanwhile the bacterial inocula were added to the soil as cell suspension at the rate of 50 ml/pot. Infested soils were carefully mixed thoroughly to ensure equal distribution of the added inocula one week before transplanting.

Root dipping (RD) plus soil infestation (SI)

Immersed broccoli transplants roots for 30 min in either fungal or bacterial suspension supplemented with 1% (w/v) carboxymethyl cellulose (CMC) were planted in pots containing soil infested with both pathogenic fungus and fungal bio agents inocula as stated earlier, while bacterial inocula were added to the soil as a cell suspension (10^9-10^{10} cells/ml) at the rate of 50 ml/pot. Ten replicates (pots) with five transplants were used for each particular treatment. Control treatment was untreated transplants planted in pots containing soil artificially infested with the pathogenic fungus only. The average percentage of *Pythium* rot incidence were recorded up to 45 days from transplanting (the experimental period).

Statistical analysis

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyze differences between treatments . A general linear model option of the analysis system SAS (SAS Institute Inc. 1996) was used to perform the ANOVA. Duncan's multiple range test at $p \leq 0.05$ level was used for means separation (Winer 1971).

Results and discussions

Survey of root rots diseases of broccoli

Survey study on broccoli root rot diseases was preformed during 2008/2009 season at four Governorates, i.e. Giza, Kalubeia, Ismaelia and Beheira (Nobaria province) where broccoli are widely spread.. Data in Table (1) show that the high Pythium rot incidence were 38.0 and 35.2% respectively, at Beheira governorate followed by Ismaelia, Kalubeia and Giza governorate in respective order. On the other hand, the mean value of recorded root rot incidence throughout the surveyed governorates were 33.25%.

Table 1. Survey of the average root rots infection of broccoli plants at different governorates in Egypt

Location (governorate)	Root rot infection %
Giza	31.4*
Beheira	38.0
Ismaelia	35.2
Kalubeia	28.0
Mean	33.25

*Each figure represents the average recorded percentage of diseased broccoli plants at different surveyed locations belonging to the cited governorate

In this regards, *Pythium* rot on broccoli, chinese cabbage ,cauliflower has been reported by many investigators (Moller *et al*. 2003; Tanina *et al*. 2004; Tojo *et al*. 2001; 2005 ,El-Mohamedy and El-Mougy, Nehal, 2008, 2009). Agrios (1997) stated that the disease threshold in the field caused by soil borne plant pathogens is highly variable (depending on, e.g., the soil, climate conditions, crop species and variety, to the infesting phytopathogen. In this regard, high soil moisture levels are a key factor for the soil-borne pathogen *Pythium* to become a problem.

Isolation and identification of the causal organism (s)

Pythium, *Rhizoctonia*, *Fusarium* and *Phytophthora* were isolated from broccli plants showing typical root rots symptoms, collected from different surveyed fields (Table 2). Tabulated data revealed that *P. ultimum* is a dominant fungus and its frequency is between 48.6-57.3 %, followed by a less frequent of *R. solani, F. solani* and *Phytophthora* spp. in addition to other unidentified fungi, respectively.

Table 2. Frequency occurrence of fungi isolated from broccli plants showing root rots infection under field conditions in different governorates in Egypt

Location (governorate)	% Frequency of isolated fungi				
	Pythium ultimum	*Rhizoctonia solani*	*Fusarium solani*	*Phytophthora* pp.	Others (unidentified)
Giza	43.4	24.0	19.2	11.0	2.4
Kalubeia	44.0	25.6	15.8	12.6	2.0
Ismaelia	48.8	25.0	13.2	10.4	2.6
Beheira	46.0	22.8	18.0	10.2	3.0

*Each figure represents the percentage of isolates in relative to the whole isolated fungi

Several reports confirmed the isolation of *P. ultimum* from Chinese cabbage, cauliflower and broccoli in at high frequency (Kageyama *et al*. 1997; Tojo *et al*. 2001; Tanina *et al*. 2004, El-Mohamedy and El-Mougy, 2008 and 2009). The high frequency of isolated *P. ultimum* in this study may be attributed to environmental conditions during cultivation season These environmental conditions could be most favourable for phycomycetes fungi. This explanation is similarly reported by Tojo *et al*. (2001) who stated that abundant soil moisture and low temperature are the two most important environmental factors that regulate the distribution of *P. ultimum.* Previous studies have shown that among several *Pythium* and *Rhizoctonia* species that cause damping-off and root rot, *Pythium ultimum* is the most consistently virulent and the most frequently isolated (Georgakkopoulos *et al*. 2002 , El-Mohamedy and El-Mougy ,2008 and 2009).

Pathogenicity test

The pathogenic ability of Five isolates of *P. ultimum* to induce root rot of broccoli cv. AtlanticF1 and Souther star was tested. All the tested fungal isolates were able to cause root rot at different degrees on the two tested cultivars of broccoli seedlings is presented in Table 3. *Pythium ultimum* isolate B1 caused a significantly high disease incidence recorded as 64.0% and 58.0% of broccoli, respectively. Meanwhile, *P. ultimum* isolate G1 caused less disease of incidence recorded as 37.4% and 30.8% of broccoli cvs., respectively.

Table 3. Pathogenic ability of different *Pythium ultimum* to induce *Pythium* rot disease on broccoli under greenhouse conditions

Pythium isolates	**Root rot incidence %**	
	Broccoli cultivars	
	Atlantic F1	Souther star
P. ultimum (G)[A]	37.4[B] dc	30.8[B] c
P. ultimum (B)	64.0 a	58.0 a
P. ultimum (I)	55.4 b	46.2 b
P. ultimum (K)	52.2 b	49.0 b
P. ultimum (G)	45.0 b	42.4 b
P. ultimum (B)	50.8 b	41.2 b
Control	0.0 e	0.0 e

Mean values within columns followed by the same letter are not significantly Different ($p \leq 0.05$), [A] the letters G, B, I and K are codes of location sites:, Giza, Beheria, Ismaelia and Kalubeia Governorates in Egypt .

It is clear to note that the Atlantic F1 cv. of broccoli showed higher susceptibility to *Pythium* root rot infection than the souther star cv. The presented results indicate that *P. ultimum* is the causal agents of root rot of broccoli in Egypt. This was also reported by El-Mohamedy and El-Mougy (2008). They stated the first record of *Pythium* rot of Chinese cabbage in Egypt caused by *P. ultimum*. Also, the present findings are in agreement with those reported by Kikumoto (1987) and Tojo *et al*. (2005). *Pythium* spp. were reported to cause rot infection to cabbage and other brassicaceous plants in many countries. In Japan stem and crown root rot of Chinese cabbage caused by *P. ultimum* has been named as *Pythium* rot (Kikumoto, 1987). In Moller and Hockenhul (1997) noted that *P. tracheiphilium* causes leaf and head rot of maturing Chinese cabbage, with losses of 40 to 50 %. Furthermore, Pythium rot of Chinese cabbage was reported to be caused by *P. aphanidermatum* (Saha and Singh 1988). Similar rot diseases of other brassicaceouss plants caused by *Pythium* species are also known as Pythium rot (Tanina *et al*. 2004).

In vitro antagonism

The inhibitory effect of antagonistic fungi and bacteria *in vitro* was tested in this study with antagonistic agents applied as growth culture disks. Percentages of the reduction in growth of *P. ultimum* (isolate B1) in response to antagonistic agents are presented in Table (4). The presented data show that the growth of pathogenic fungi was significantly reduced by the inhibitory action produced by all antagonistic agents tested. The antagonistic fungi had a greater effect on the retardation of growth compared with the bacterial agents. The inhibitory effects of *T. harzianum* (isolates G1 and B1) and *T. viride* (isolates G1and G3) were higher than other isolates of *T. harzainum* and *T. viride*, as they completely reduce the growth of the pathogen (100% reduction).The antagonistic bacteria also showed significantly reduction in the growth of *P. ultiumum* isolates .The higher inhibitory effect was recorded with *B. subitilis* isolate B3 and *P. fluorescencs* isolate B3 .

Similar results were reported by many investigators (Andersen *et al.* 2003; Carisse *et al.* 2003; Leclère *et al.* 2005). They reported the inhibitory effect of antagonistic fungal and bacterial such as *Trichoderma* spp., *B. subtilis* and *P. fluorescen*s against growth reduction of *P. ultimum* under *in vitro* conditions. The inhibition in the growth of the pathogen could be attributed to antibiosis, hyperparasitism (We *et al*. 1986) or production of chitinase and *ß*-1, 3 glucanase enzymes which degrade the cell wall leading to lyses of mycelium of the pathogen (Ahmed and Baker 1987).

Table 4. Growth reduction of *P. ultumum* (isolate B1) in response to the inhibitory effect of antagonistic agents *in vitro*

Antagonistic agent [A]		***P. ultumum* (isolate B1)**	
		Linear growth	Reduction [B]
T. harzianum	(G1)	0.0d	100
T. harzianum	(G2)	18.0bc	80
T. harzianum	(G3)	0.0 d	100
T. harzianum	(B1)	0.0d	100
T. harzianum	(B2)	0.0 d	100
T. harzianum	(B3)	0.0 d	100
T. viride	(G1)	0.0d	100
T. viride	(G2)	20.0 b	77.7
T. viride	(G3)	0.0 d	100
T. viride	(B1)	18.0bc	80.0
T. viride	(B2)	18.0 bc	80.0
T. viride	(B3)	20.0b	77.7
B. subtillis	(G1)	18.0bc	80.0
B. subtillis	(G2)	25.0 b	72.2
B. subtillis	(G3)	30.0 b	66.6
B. subtillis	(B1)	16.0bc	82.2
B. subtillis	(B2)	16.0 bc	82.2
B. subtillis	(B3)	12.0 b	86.6
P. fluorescens	(G1)	18.0b	80.0
P. fluorescens	(G2)	20.0 bc	77.7
P. fluorescens	(G3)	18.0 bc	80.0
P. fluorescens	(B1)	21.0b	76.6
P. fluorescens	(B2)	18.0 bc	80.0
P. fluorescens	(B3)	15.0	83.3
Control		90.0a	

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$), [A]selected bioagents isolated from the rhizosphere of healthy broccoli plants, [B] values are percentage of reduction in growth of *P. ultumum* in the presence of bioagents calculated in relation to its growth in medium free of an antagonistic agent

Greenhouse experiment

The efficacy of highly antagonistic *in vitro* fungal and bacterial rhizispheric isolates was evaluated against Pythium root rot diseases in a pot experiment using soil artificially infested under greenhouse conditions. Two isolates of each *T. harzainum* (isolate G1 and B1), *T. viride* (isolate G1 and G3), as well as one isolate of *B. subtilis* isolate B1 and *P. fluorescens* isolate B3 were tested. The percentages of reduction of Pythium root rot incidence of broccoli plants was presented in Table 5. All tested bio control agents obviously significantly suppressed Pythium root rot incidence on broccoli compared to control treatment.

Applying bio control agents as a combination of soil mixing plus root dipping method was generally most effectively than each individually for suppressing Pythium root rot incidence followed by soil mixing and root dipping methods. The high reduction in Pythium root rot incidence with *T. harzainum* isolate G1 and B1(88.0 and 84.4%), *T. viride isolate G1and G3* (80.2 and 77.2%), *P. fluorescens* isolate B3 and *B. subtilis isolate B1* (80.0and 75.0 %) when thess bio agents applied as soil mixing plus root dipping methods. Moderate effect in the same trend was observed concerning root dipping and soil mixing methods. This observation could be attributed to the high introduced inoculm density throughout soil mixing and root coating with the tested bioagents. It is expected that when the antagonists introduced and established in the rhizosphere of the root court of broccoli transplants, then it could compete with the target pathogen and suppress its ability to infect the host plant. The use of *Trichoderma* spp., *B. subtilis* and *P. fluorescens* for controlling Pythium and Rhizoctonia root rot diseases is reported by many workers (Harman, 2001; Whipps and Lumsden, 2001; Georgakkopoulos *et al*., 2002). Moreover, the reduction in *Pythium* rot incidence in soil mixed with bacteria may probably due to competition as bacteria may compete with germinated oospores of the pathogen for soluble carbon and nitrogen sources supplied by root exudates that stimulate oospore germination and by eliminating these sources (Weller 1988). Presented data revealed that the applied bioagents could be arranged according to their activity for suppressing disease incidence as follows: *T. harzainum*, *B. subtilis*, *T. viride* and *P. fluorescens*, respectively.

Table 5. *Pythium* root rot reduction (%) of Broccoli plants as affected by various antagonistic agents under greenhouse conditions

Antagonistic agent [A]	**Pythium root rot reduction %**		
	Soil mixing [B]	Root dipping [B]	soil mixing + root dipping [B]
T. harzianum (B1)	60.5a	52.8a	88.0a
T. harzianum (G1)	66.0 a	58.8a	84.4a
T. viride (G3)	55.0 c	40.0b	77.2b
T. viride (B1)	57.0c	42.6b	80.2a
B. subtillis (B1)	62.4a	58.0a	80.0a
P. fluorescens(B3)	58.0c	44.0b	75.0 b

Mean values within columns followed by the same letter are not significantly different ($p \leq 0.05$) [A] microorganisms isolated from the rhizosphere of healthy broccoli and proved to have high antagonistic effect against the pathogenic fungus *in vitro,* [B] different approaches of bio agents treatments

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