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Evaluation of *Trichoderma* Species as Biocontrol Agents for Damping-Off and Wilt Diseases of *Phaseolus vulgaris* L. and Efficacy of Suggested Formula

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Rhizoctonia solani (RS) and *Fusarium oxysporum* f.sp. *phaseoli* (FOP) were isolate from a naturally occurring epidemic of damping-off and wilt in bean plants grown in Assiut governorate. *In vitro*, the ability of fifteen isolates of *Trichoderma* spp. isolated from the rhizosphere of bean plants to inhibit mycelial growth of RS and FOP, the causal agents of damping-off and wilt of bean were conducted. *Trichoderma harzianum* (Th1), *T. viride* (Tv1) and *T. spirale* (Ts3) isolates showed different inhibitory effect against growth of both tested pathogens. Th1 and Tv1 showed the greatest antagonistic effect to the pathogens followed by Ts3 isolate. In greenhouse and field experiments, soil treatment with a powder formulation of *Trichoderma* spp. two weeks before planting or at the time of planting reduced significantly the incidence of damping-off and wilt diseases on Giza 3 bean cultivar. The formulation of *Trichoderma* spp. treatments not only suppressed both damping-off and wilt diseases but also enhanced green yield of bean plants compared to infected control, especially, Th1 formulation gave equal green yield compared to healthy control.

Keywords: Bean, biocontrol, damping-off, powder formulation, wilt.

Bean (*Phaseolus vulgaris* L.) is one of the most important food legumes for direct human consumption in the world. Several soilborne fungi cause damping-off and wilt disease. The main pathogens responsible for damping-off and wilt incidence of bean are *Rhizoctonia solani* (Kühn) and *Fusarium oxysporum* f.sp. *phaseoli*, respectively (El-Mougy *et al.*, 2007). Under favourable conditions, the entire bean crop may be destroyed (Abawi, 1989). Also, wilt disease in dry bean is caused primarily by *F. oxysporum* f.sp. *phaseoli* in a complex with *Rhizoctonia solani* and *Fusarium solani*. Yield losses in severely infested areas may be as high as 50% (Estevez de Jensen *et al.*, 2001).

Various methods for controlling such diseases have been investigated including the use of resistant varieties (Brisa *et al.*, 2007), chemical control, cultural practices (Punja *et al.*, 1986), plant volatile compounds (El-Mougy *et al.*, 2007), plant extracts (Kumar and Tripathi, 1991) and biological control, particularly with species of *Trichoderma* and *Gliocladium* (Ristaino *et al.*, 1994). Many researchers have demonstrated the potential of *Trichoderma* spp. in controlling damping-off and wilt diseases of crop plants caused by *Rhizoctonia solani* and *Fusarium* spp. (Dubey *et al.*, 2007and Rojo *et al.*, 2007).

Isolates of the fungal genera *Trichoderma* are used most often in biocontrol studies and are generally applied as conidial or chlamydospore preparations (Coley-Smith *et al.*, 1991 and Papavizas, 1992). Fresh cells of potential antagonists have been mostly used for biocontrol *in vitro*. Such methods may not be suitable for routine use in the field (Pengnoo *et al.*, 2000).

So far, few biological control agents have achieved success under field conditions. Among the hundreds of organisms identified as potential biological disease control agent, only few have resulted in proving commercially acceptable control of these diseases (Warrior *et al.*, 2002). A fungal biocontrol preparation for control or prevention of plant fungal diseases comprises sporulated fungal biomass and a carrier preferably is vermiculite. Different formulations have been used in control soil borne pathogens, these are, fungal spores (Harman *et al.*, 1980), and powdery preparations of fungal mycelium (Latunde-Dada, 1993). A biocontrol formulation with agricultural potential should possess several desirable characteristics such as: easy preparation and application, stability, adequate shelf life, abundant viable propagules, and low cost (Churchill, 1982). The formulation should be amenable for application to both phylloplane and rhizosphere, depending on the pathogens and plants to be controlled.

The market for biological control products is not only determined by agricultural aspects such as the number of diseases controlled by one biocontrol product in different crops but also by economic aspects as cost-effective mass production, easy registration and the availability of competing means of control including fungicides.

Shelf life is a very important parameter to be considered in the development of a formulation, because most products will have to be stored for long periods of time before they can be marketed and later applied. More recently commercial formulations of biological controls have been developed which have consistently given good control of some plant diseases (Stewart *et al.*, 2001).

Formulation of the *Trichoderma* spp. to reduce incidence of the diseases caused by soilborne pathogens in the field is of great importance in biocontrol of such diseases. Therefore the present work was aimed to determine the efficacy of application of formulation contained the *Trichoderma* spp. as soil treatment against damping-off and wilt diseases of bean under greenhouse and field conditions.

Materials and Methods

Pathogens and inoculum preparation:

Pathogenic isolates of *Rhizoctonia solani* (RS) and *Fusarium oxysporum* f.sp. *phaseoli* (FOP) were isolated from naturally infected roots of diseased bean plants showing damping-off and wilt symptoms grown in Assiut Governorate, Egypt. Inocula were prepared by growing isolates of the tested fungi in 250 ml conical flasks each containing 100 ml of Czapek's broth medium. They were inoculated separately with 6 mm agar disc obtained from 7-days old cultures of FOP and RS. The flasks were incubated at 25°C for 15 days. Resulting mycelial growth of the tested fungi was decanted, washed with distilled water, suspended in 100 ml of distilled water and blended for 5 minutes using a Warring blender.

For soil infestation, 30 ml of fungal suspensions containing $5x10^6$ cfu/ml were added to 30 cm diameter pots, filled with steam sterilized sandy loamy soil 7 days before planting (Abdel-Kader, 1997). Seed infestation was carried out by dipping the seed in 1% sodium hypochlorite solution for 3 minutes, and then rinsing for several times with sterilized water. Pots containing non-infested soil were used as control. Five bean seeds were sown in each pot, and three pots were used as replicates for each isolate.

Isolation of Trichoderma spp.:

For isolation of *Trichoderma* spp., soil samples were collected from the rhizosphere of healthy bean plants using dilution plate techniques and purified by the single spore method. The isolated fungi were identified on the basis of their morphological characters (Rifai, 1969).The obtained fungal isolates were grown on PDA slants and kept at 4°C until being used.

In vitro screening test for antagonistic effect:

The tested isolates of *Trichoderma* spp. were grown on PDA medium at 20°C, for 6-days and used as inocula. Disks from each isolate of *Trichoderma* spp. (5 mm in diameter) were inoculated on PDA medium in one side in Petri plate and the opposite side was inoculated by FOP or RS inocula. Five replicates were used for each isolate of *Trichoderma* spp. and also for each pathogen. The inoculated plates with FOP and RS only were used as control. The experiment was repeated twice. After 4 and 7 days incubation periods at 20°C, linear growth of RS and FOP was recorded. Inhibition percent of growth was calculated using the following formula: Growth reduction (%) = (Growth in control - Growth in treatment / Growth in control) X 100.

Preparation of formulated antagonistic fungi:

For mass production, antagonists were grown in 1000 ml conical flasks, each containing 250g vermiculate soil (El-Halal company, El-Khatatpa, Egypt), 250g wheat bran and 250g CZ medium (CZ DIFCO) and autoclaved for 20 min. at 121°C, on two consecutive days. After 25 days incubation period, contents of flasks were transferred to plastic plates under sterile conditions, left to air dry then mixed in a blender to become powder and kept in polyethylene bags at room temperature until used. Colony forming units in all formulae of *Trichoderma* spp. mixtures was adjusted to 3×10^7 cfu/g.

Diseases severity assessments:

Percentages of damping-off incidence of bean at pre and post-emergence stages were calculated after 10 and 30 days, respectively. Pre-emergence damping-off (%) was based on the number of non-emerged seeds in relation to the number of sown seeds, while post-emergence (%) was based on the number of plants showing disease symptoms in relation to the number of emerged seedlings. Fifty days after sowing, the disease severity (DS) was recorded by using the CIAT scale, 1-9 (Van Schoonhoven and Pastor-Corrales, 1987), where 1= no visible symptoms and 9= plants are dead or severely infected, with 100% foliage showing wilting, chlorosis and/or premature defoliation. The experiment was repeated twice.

Greenhouse Experiments:

The trials were carried out in the greenhouse of Plant Pathology Dept. Faculty of Agriculture, Assiut Univ. The formulated antagonists (Th1, Tv1 and Ts3) were added to infested soil in pots (30 cm in diameter) at the same time or two weeks before planting. Fungal suspensions containing $5x10^6$ cfu/ml were added to 30 cm diameter pots, formulations of *Trichoderma* spp. were added at the rate of 1% w/w. The experiment was repeated twice in 2006 and 2007 growing seasons. Three replicates were used, each replicate consisted of three pots and each pot contains 5 plants. Disease rating for damping-off and wilt diseases were recorded as described before.

Field Experiments:

Field experiments were conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2006 and 2007 growing seasons. A (3.5x3.5 m) field plots each comprised of 7 rows and 15 holes/ row were used in split-plot design. Three plots were used as replicates for each treatment as well as for untreated control treatment. Soil treatments were done by applied 150 g of the prepared formulation/plot (Th1, Tv1 and Ts3) at two weeks before planting or at the same time of planting. Bean seeds, cv. Giza 3, were sown at the rate of 3 seeds/hole. Percent of pre, post-emergence damping-off and wilted plants were calculated as mentioned before. At harvest time, the average accumulated green yield was calculated for all applied treatments and control as well.

Effect of storage period on biological activity of formulated antagonists:

The effect of storage period on the biological activity of the formulated antagonists was tested. The powder formulations of *Trichoderma* spp. ($3X10^7$cfu/g) were stored in sealed polyethylene bags at room temperature (25-30°C). After 0, 1, 2, 3 and 4 months storage, one gram powder formulation was suspended in 100 ml of water. Serial dilutions of the homogenate were prepared and 1 ml aliquots from the dilutions were spread on PDA plates. The developed fungal colonies were counted on the agar plates after 5 days of incubation at 25°C and populations were reported as colony-forming-units (cfu) per g of powder formulation. Three replicates were used for each treatment.

Statistical analysis:

Split-plot design was used for the experiments in greenhouse and field condition. Data were subjected to statistical analysis using analysis of variance using the Statistical Analysis System, (SAS institute Inc., 1996), and means were compared using L.S.D. test according to Gomez and Gomez (1984).

Results

In vitro evaluation of antagonism of Trichoderma spp. against FOP and RS:

The highest inhibitory effect on growth of both pathogens FOP and RS was achieved by *T. harzianum* Th1 (51.4 and 42.5.4%, respectively) followed by Tv1 (41.4%) to FOP and Thm3 to RS (39.1%) then Ts3 (37.9 and 30.5, respectively). Th2 showed the lowest inhibitory effect to FOP (15.1%). On the other hand, the lowest inhibitory effect was obtained by Tv3 against RS (13.2%). According to these results, Tv1, Th1 and Ts3 were used in the further experiments (Table 1).

Table 1. Antagonistic effect of certain fungal isolates against growth of *Fusarium oxysporum* f.sp. *phaseoli* (FOP) and *Rhizoctonia solani* (RS) *in vitro*

Antagonists	Inhibition (%) of	
	FOP	RS
Trichoderma viride		
Tv1	41.4 b *	31.2 b
Tv 2	17.2 g	13.3 h
Tv 3	17.0 g	13.2 h
Tv 4	20.7 f	22.1 cd
T. harzianum		
Th1	51.7 a	42.5 a
Th 2	15.1 g	16.2 fgh
Th 3	31.0 d	25.5 c
T. polysoprium		
Tp1	31.0 d	25.0 c
T. hamatum		
Thm1	24.1 e	20.1 de
Thm 2	17.2 g	18.5 efg
Thm 3	31.0 d	39.1 a
T. pseudokoningii		
Tps1	24.1 e	15.6 gh
T. spirale		
Ts1	17.5 g	19.5 def
Ts2	20.7 ef	25.1 c
Ts3	37.9 c	30.5 b

* Values in the same column followed by same letter(s) are not significantly different according to ANOVA (L.S.D. p= 0.05).

Effect of soil treatment with powder formulation of Trichoderma species on incidence of damping-off diseases of bean under greenhouse conditions:

Under greenhouse conditions, soil treatments with the formulations two weeks before planting and at the time of planting have significantly reduced pre- and post-emergence damping-off disease caused by *R. solani* compared to the untreated control (Tables 2). Application of formulation of Th 1 to infested soil at the time of planting gave the highest reduction in disease incidence of damping-off on bean plants. The lowest percentage of disease incidence was obtained by application of Ts3 to infested soil. Application of *Trichoderma* spp. formulation at the time of planting gave more reduction in DS than after two weeks of planting in post emergence damping-off.

Effect of powder formulation of Trichoderma species on damping-off of bean under field conditions:

Under field conditions, all the applications of formulation reduced DS compared to untreated control. All applications varied in reduction of both pre- and post-emergence damping-off. Tv1 formulation caused the highest reduction of DS followed by the other treatments. There was no significant different were obtained in DS when the formulations were added two weeks before planting or at the time of planting in both pre and post emergence damping-off (Table 3).

Table 2. Effect of soil treatment with formulated *Trichoderma* species on incidence of damping-off disease of Giza 3 bean cultivar under greenhouse conditions

Pre-emergence damping off (%)					
Time of application	Treatment				
	Th1*	Tv1	Ts3	Infected control	Mean
Two weeks before planting	9	10	12	16	11.8
At time of planting	6	9	11	19	11.3
Mean	7.5	9.5	11.5	17.5	
L.S.D. at 0.05 Time of application (A): 1.1 Bioagents (B):1.08 Interaction (AxB): 1.5					
Time of application	Post-emergence damping off (%)				
	Th1	Tv1	Ts3	Infected control	Mean
Two weeks before planting	7	11	11.7	15	11.2
At time of planting	7	9	10	15	10.3
Mean	7	10	10.8	15	
L.S.D. at 0.05 Time of application (A): 0.3 Bioagents (B): 0.92 Interaction (AxB): 1.3					

* Th1, *Trichoderma harzianum*, Tv1, *T. viride* , Ts3, *T. spirale*.

Table 3. Effect of soil treatment with formulated *Trichoderma* species on incidence of damping-off disease of Giza 3 bean cultivar under field conditions

Pre-emergence damping off %					
Time of application	Treatment				
	Th1	Tv1	Ts3	Infected control	Mean
Two weeks before planting	16.3	15.2	18.5	22.3	18.1
At time of planting	13.9	12.2	15.4	23.4	16.2
Mean	15.1	13.7	17.0	22.8	
L.S.D. at 0.05 Time of application (A): 2.4 Bioagents (B): 1.75 Interaction (AxB): 2.5					
Time of application	Post-emergence damping off (%)				
	Th1	Tv1	Ts3	Infected control	Mean
Two weeks before planting	16.9	12.4	16.9	21.9	17.1
At time of planting	12.6	11.2	11.9	21.2	14.2
Mean	14.8	11.8	14.4	21.5	
L.S.D. at 0.05 Time of application (A): 2.1 Bioagents (B): 1.9 Interaction (AxB): 2.7					

* As described in footnote of Table (2).

Effect of powder formulations of Trichoderma species on Fusarium wilt disease of bean under greenhouse and field conditions:

Under greenhouse and field conditions, all tested treatments have significantly reduced wilt disease compared to control plants. Data also indicated that such treatments varied in their reduction of DS incidence in bean plants. The highest reduction was achieved by application of Th1 and the lowest disease reduction was achieved by application of Ts3.

To study the best time of application, formulation of *Trichoderma* spp. applied at the time of planting showed better effect in DS reduction than at two weeks before planting under greenhouse conditions but under field conditions applied formulations two weeks before planting or at time of planting show the same effect (Table 4).

Table 4. Effect of soil treatment with formulated *Trichoderma* species on incidence of Fusarium wilt disease of Giza 3 bean cultivar under greenhouse and field conditions

Wilt rating* under greenhouse conditions					
Time of application	Treatment				
	Th1**	Tv1	Ts3	Infected control	Mean
Two weeks before planting	3.7	4.1	5.2	8.0	5.3
At time of planting	2.5	3.1	4.0	7.0	4.2
Mean	3.1	3.6	4.6	7.5	
L.S.D. at 0.05 Time of application (A): 0.5 Bioagents (B): 0.43 Interaction (AxB): 0.61					
Time of application	Wilt rating* under field conditions				
	Th1	Tv1	Ts3	Infected control	Mean
Two weeks before planting	3.3	4.2	5.0	6.0	4.7
At time of planting	4.0	4.2	5.0	7.0	5.1
Mean	3.6	4.1	5.0	6.5	
L.S.D. at 0.05 Time of application (A): 0.2 Bioagents (B): 0.23 Interaction (AxB): 0.36					

* According to CIAT scale (Van Schoonhoven and Pastor-Corrales, 1987)

** As described in footnote of Table (2).

Effect of powder formulations of Trichoderma species on green yield of bean under field conditions:

Green yield (ton/fedden) of control plant infected by RS and FOP were significantly lower than that of uninfected control (Table 5). Results also, indicate that all tested treatments have significantly increased bean yield relative to untreated control. Application of Th1 resulted in highest bean yield compared to the control inoculated with both pathogens and equals the yield of uninfected control. Although application of Tv1 and Ts3 caused higher yield than the infected control, no significant differences were found between them.

Table 5. Effect of bioagents formulations on green yield (ton/fedden) of bean plants (cv. Giza 3) under field conditions

Damping-off (RS)						
Time of application	Treatment					
	Th1*	Tv1	Ts3	Control Healthy	Control infected	Mean
Two weeks before planting	3.0	2.2	2.0	3.0	1.2	2.9
At time of planting	2.7	2.1	2.1	3.0	1.5	2.9
Mean	2.9	2.2	2.1	3.0	1.4	
L.S.D.at 0.05 Time of application (A): 0.5 Bioagents (B): 0.43 Interaction (AxB): 0.61						
Time of application	FOP (Wilt disease)					
	Th1	Tv1	Ts3	Control Healthy	Control infected	Mean
Two weeks before planting	2.8	2.4	2.1	3.0	1.2	2.3
At time of planting	3.1	2.5	2.3	3.0	1.5	2.5
Mean	3.0	2.4	2.2	3.0	1.4	
L.S.D. at 0.05 Time of application (A): 0.2 Bioagents (B): 0.25 Interaction (AxB): 0.36						

* As described in footnote of Table (2).

Effect of storage period on viability of Trichoderma spp. in powder formulation:

Results in Table (6) show that the number of viable colonies of bioagents in the formulation was decreased gradually by prolonging storage time up to four months. In focus, the number of viable colonies in Th1 formulation, three and four month after storage were markedly higher than in Tv1 and Ts3 in the same time. There is no difference between the number of activity colonies of formulations Tv1 and Ts3 one, three and four month after storage. Although Th1 formulation was slightly stable than other formulation, the count of colonies in it was also decreased after storage.

Table 6. Effect of storage period on viability of *Trichoderma* spp. formulation

Treatment	CFU/g after storage time (months)				
	Zero	One	Two	Three	Four
Th1	3×10^7	2.1×10^6	1.2×10^6	2.0×10^5	1.3×10^4
Tv1	3×10^7	2.0×10^6	1.0×10^5	2.0×10^4	1.1×10^2
Ts3	3×10^7	2.9×10^6	1.1×10^6	2.0×10^4	1.1×10^2

* As described in footnote of Table (2).

Discussion

In vitro testing, antagonistic capability of *Trichoderma* spp. obtained from the rhizosphere of bean showed inhibitory effect against growth of both *Rhizoctonia solani* (RS) and *Fusarium oxysporum* f.sp. *phaseoli* (FOP) with differed degrees. Elad (1996) stated that the mechanisms of the antagonism of *Trichoderma* spp. against different pathogens may be due to mycoparasitism, competition and antibiosis.

The present study showed that tested formulations of *Trichoderma* spp. proved to be effective in controlling RS and FOP, the causal agents of bean damping-off and wilt diseases respectively, under greenhouse and artificially infested field conditions. *T. harzianum* (Th1) proved to be the most effective isolate in controlling the tested diseases than all the tested isolates. *Trichoderma viride* and *T. harzianum* were reported by several workers as the best antagonists against several soil and seed borne plant pathogens (Poddar *et al.*, 2004). The potentiality of *Trichoderma* spp. as biocontrol agents of phytopathogenic fungi in several crops is well known especially to *Fusarium* spp. and *Rhizoctonia* spp. (Poddar *et al.*, 2004 and Rojo *et al.*, 2007).

There are many mechanisms suggested to clarify the role of antagonistic organisms in suppression of growth pathogens and thus to control diseases. Their action could be through antibiosis (Ghisalberti and Rowland, 1993); mycoparasitism (Haran *et al.*, 1996). The competition for nutrients and/or space (Inbar *et al.*, 1994), this fact was already observed in the interaction among Trichoderma and other pathogens (Melo, 1991), the other mechanisms involved in Trichoderma are induction of resistance in plants (Yedidia *et al.*, 1999).

Results reported herein indicated that formulation of *Trichoderma* spp. treatments not only suppressed both damping-off and wilt diseases but also enhanced green yield of bean plants compared to infected control. In case of Th1 isolate the green yield equal, the healthy control. The reduction in disease severity and increasing the yield of different crops after treatment by formulations of *T. harzianum* has been reported by several workers (Singh and Singh, 2004 and Rojo *et al.*, 2007). The increase of bean yield obtained in this study, could be related to the effect of *Trichoderma* spp. as plant growth promoters. Several reports have shown that the addition of specific Trichoderma isolates to the rhizosphere can result in plant growth promotion (Naseby *et al.*, 2000). The plant growth promoting effects in some systems are prolonged even to the point of increasing yield (Lynch *et al.*, 1991). Also, may be the application of *Trichoderma* spp. as powder formulation into soil provides nutrient sources for other soil microorganisms such as growth promoting rizhobacteria.

Preparations of *Trichoderma* spp. led to control the disease in both pathogens whether applied 2 weeks before planting or at the time of planting. Such results agree with those reported by Lewis and Lumsden (2001), this may be due to apply biocontrol formulations at the time of planting avoid spread of the pathogen in soil. Coley-Smith *et al.* (1991) reported that formulations of biocontrol against were more effective when added at the time of planting more than two weeks before planting.

The effect of storage time at room temperature on the viability of *Trichoderma* spp. in the prepared formulations showed that more than 40% viability of the colonies was recorded at room temperature storage after 4 months. The lowest viability was observed in all fungal formulates after 4 months. Similar results were obtained by Walker and Connick (1983) who declared that formulations of *Trichoderma* spp. after stored at ambient conditions for 6 to 8 months. However.

Küçük and Kivanç (2005) mentioned that no viability was observed in different soils at 30°C after 9 weeks, whereas there was viability in all soils at 4°C even after 24 weeks.

In conclusion, application of powder formulation of *T. harzianum* (Th1) to the soil two weeks or at the time of planting as described herein could be recommended for controlling damping-off and wilt of bean plants.

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تقيم فاعلية مستحضر انواع من الـترايكوديرما كعوامل مقاومة حيوية ضد مرضى السقوط المفاجئ والذبول فى الفاصوليا

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تم عزل فطريات *Rhizoctonia solani* و *Fusarium oxysporum* f.sp. *phaseoli* من نباتات مصابة بمرض السقوط المفاجئ والذبول من محافظة أسيوط. وكذلك عزل الفطريات المضادة من حول النباتات السليمة من نفس الحقول والتى عرفت على انها انواع من الفطر ترايكوديرما.

فى المعمل تم دراسة تاثير ١٥ عزلة من أنواع الفطر ترايكوديرما المعزولة من حول نباتات الفاصوليا النامية فى الحقل ضد النمو الميسليومى للفطريين المسببين للمرض وقد كانت العزلات *Trichoderma harzianum* (Th1) ، *T. viride* (Tv) ، *T. spirale* (Ts3) اكثر العزلات تثبيطا لنمو الفطريات الممرضة.

وتحت ظروف الصوبة الزجاجية أظهرت مستحضرات العزلات Th1 وTv1 أكبر الأثر فى تثبيط الأمراض تليها Ts3. ايضا تحت ظروف الصوبة الزجاجية ادت اضافة مستحضرات الفطريات المضاده الى التربة قبل الزراعة باسبوعين إلى خفض كبير فى شدة المرض عن اضافتها اثناء الزراعة. وكذلك ادت اضافتها الى تربة الحقل المعدية الى خفض شدة المرض وكذلك زيادة الغلة الانتاجية للمحصول مقارنة بالنباتات غير المعامله.