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Induction of Defence Responses Against Fusarium Wilt of Faba Bean (*Vicia faba* L.) by the Biocontrol Agents *Trichoderma* spp.

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Fusarium oxysporum f.sp. *fabae* (Schlecht) (*FO*) was isolated from diseased faba bean roots collected from different localities at Assiut governorate. The antagonistic capability of *Trichoderma* spp. isolates against linear growth of *FO* isolate was tested and all *Trichoderma* spp. isolates gave positive reaction of antagonism to the pathogen isolate in *vitro*. In greenhouse and field experiments, all applied treatments protected faba bean seedlings against *FO*. The most effective treatments were *T. harzianum* (Tz1) followed by *T. viride* (T1) and *T. harzianum* (T15). Soil treatments by *Trichoderma* spp. isolates reduced the disease index. All treatments significantly increased cellulose, pectin and lignin contents in treated plants compared with untreated plants. The highest increase of pectin was obtained with *T. harzianum* (Tz1).

Keywords: Biocontrol, cellulose, faba bean, lignin, pectin, *Trichoderma* spp. and wilt.

Faba bean (*Vicia faba* L.) is considered one of the most important leguminous crops cultivated in Egypt for green pods and dry seeds. A major problem of faba bean cultivation is controlling wilt disease caused by *Fusarium* spp. (Khalifa, 1997). This soil borne fungus is responsible for serious losses in the production.

Complete control of soil borne pathogens is often difficult to achieve. In recent years the goal of biological control as viable and reliable practice in modern agriculture has increased dramatically. The application of biological control using microorganisms proved to be successful for controlling various plant diseases in many countries (Sivan, 1987). The use of bioagents for controlling root rot disease of faba bean is considered important especially in view of its wide prevalence in Egypt (Abdel-Kader *et al.*, 2002).

Several bioagents are usually found along with the pathogen, which show on antagonistic reaction. Most species of the genus *Trichoderma* are frequently isolated from plant ecosystems (Harman *et al.*, 2004), and able to antagonize many plant pathogenic fungi (Sivan and Chet, 1989; Ibrahim *et al.*, 2001; Rasmy, 2002; Dubey *et al.*, 2007 and Rojo *et al.*, 2007). The use of *Trichoderma* spp. in agriculture can improve the plant health and stimulate root growth through colonization of root surfaces and cause substantial changes in plant metabolism (Harman *et al.*, 2004). It is not only able to control the pathogens that cause plant disease, but also able to promote plant growth and development (Vinale *et al.*, 2004). The inoculation of plant defence responses mediated by the antagonistic fungus has been well documented (De Meyer *et al.*, 1998; Yedidia *et al.*, 1999; Hanson and Howell, 2004

and Harman *et al.*, 2004). Various plants showed increased resistance to pathogen attack when pre-treated with *Trichoderma*. During the interaction of *Trichoderma* with the plant, different classes of metabolites may act as elicitors or resistance inducers (Harman *et al.*, 2004; Woo *et al.*, 2006 and Woo and Lorito, 2007). Moreover, its application is safe, unhazardous for human, farm animals and avoids the environmental pollution. Certain biochemical changes occur after application of the inducing agents and act as markers for induced systemic resistance (Schönberck *et al.*, 1980).

The present investigation aimed to evaluate the effectiveness *Trichoderma* spp. isolates as antagonistic agents against the mycelial growth of *Fusarium oxysporum* f.sp. *fabae in vitro*. Furthermore, the efficacy of *Trichoderma* spp. isolates for controlling wilt incidence of faba bean under greenhouse and field conditions were also evaluated. In addition; the contents of lignin, cellulose and pectin in treated plant tissues were analyzed to study one of the pathways of biological control.

Materials and Methods

Source of the causal pathogen:

Isolate of *Fusarium oxysporum* f.sp *fabae* (Schlecht), the incitant of wilt disease of faba bean, used in this study was isolated from naturally infected faba bean roots showing root rot and wilt symptoms grown in the fields of Assiut Governorate, Egypt. Fungal isolate was identified by using the morphological features of mycelia and spores as described by Nelson *et al.* (1983). The identification was confirmed by Assiut University Mycological Centre (AUMC).

Pathogenicity and inoculum preparation of the causal pathogen:

The isolate of *Fusarium oxysporum* f.sp *fabae* was isolated from naturally infected faba bean. The isolate was maintained on potato dextrose agar (PDA) medium at 25±1°C. Inocula of the pathogen isolate was prepared on autoclaved barley medium (75g washed dried barley grains, 100 washed dried coarse sand and 75 ml tap water) in 500 ml glass bottles. Each bottle was inoculated with five discs (0.7 cm in diameter) of 14-day-old culture of Fusarium isolate. Bottles were incubated at 25±1°C for 15 day. The contents of all bottles were thoroughly mixed in a plastic container and used as a source of inoculum. Inocula of isolate was added to pots (30 cm. in diameter), each pot contain 3 kg sterile soil at the rate of 3% w/w ($\sim 10^6$ cfu g^{-1}) two weeks before planting. Pots containing non infested soil were used as control.

Assessment of disease severity:

Severity of wilt was determined 90 days after planting according to El-Mougy (2004) using a rating scale of 0 to 5 on the basis of root the discoloration or leaf yellowing as follows, 0 = neither root discoloration nor leaf yellowing, 1= 1-25% root discoloration or one leaf yellowed, 2= 26-50% root discoloration or more than one leaf yellowed, 3= 51-75% root discoloration plus one leaf wilted, 4= up to 76% root discoloration or more than one leaf wilted, and 5 = completely dead plants For each replicate a disease index (DI %) similar to that described by Liu *et al.* (1995) was calculated as follows:

DI %= $\sum$(1A+2B+3C+4D+5E) / 5T X 100 where, A, B, C, D and E are the number of plants corresponding to the numerical grade, 1, 2, 3, 4 and 5, respectively, and 5T is the total number of plants (T) multiplied by the maximum discoloration grade 5, where T= A+B+C+D+E.

Isolation and inoculum preparation of antagonistic fungi:

Trichoderma viride (T1 and M4) *T. harzianum* (T15 and Tz1) and *T. spirale* (T32 and Tm6) were isolated in this study from the rhizosphere of healthy faba bean plants using dilution plate techniques (Timonin, 1940) on PDA and were purified by the hyphal tip technique. The isolated fungi were identified on the basis of their cultural, morphological and microscopical characteristics (Gams and Bissett, 1998 and Rifai, 1969). Inocula of *Trichoderma* spp. isolates were prepared on previously mentioned autoclaved barley medium in 500 ml glass bottles. Each bottle was inoculated with five discs (0.7 cm in diameter) of 4 days old cultures of a desired antagonist and bottles were incubated at 25±1°C for 15 day. Twenty bottles were used for each antagonist. The content of bottles were thoroughly mixed in plastic container and used as a source of antagonist inoculum. Inoculum of each antagonist was added to infested soil in pots at the rate of 3% w/w ($\sim 10^6$ cfu g^{-1}) at the time of planting.

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 site in Petri plate and the opposite site was inoculated by *FO* isolate inocula. Five replicates were used for these experiments. The inoculated plates with *FO* only were used as control. The percentage of radial growth inhibition of the pathogen was calculated after 4 to 7 days incubation periods at 27°C. Each treatment was replicated three times with five plates per replicate.

Greenhouse Experiments:

Trials were carried out in the greenhouse of Plant Pathol. Dept., Fac. of Agric., Assiut Univ. Pots (30 cm in diameter) were sterilized by immersing in 5% formalin solution for 15 min., and then air drayed. Each pot was filled of autoclaved sterilized soil (3kg). Pathogen inocula were added to the potted soil at a rate of 3% (w/w) and mixed thoroughly with the soil 2 week before planting. Antagonists as soil treatment separately were added. Antagonists were applied at 3% (w/w) at the time of planting (Abo-Elyousr *et al.*, 2009). Each pot was seeded with six seeds of bean cv Misr 1 and five replicates were used for each particular treatment. Pots containing only infested soil were used as control. After 90 days, disease index was determined and the percentage of disease reduction was calculated.

Field Experiments:

Field trials were conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2009 and 2010 growing seasons. Field plots (2 x 2.5 m) comprised three rows and 15 holes row^{-1} arranged in a completely randomized block design. Three plots were used as replicates for each treatment as well as for control treatment. Each row was infested with 150 g of the *FO* isolate

desired pathogens separately two week before planting (~ 10 g hole^{-1}). Faba bean seeds Misr 1 cv., were sown (3 seeds hole^{-1}). Application of antagonists was carried out as in greenhouse experiments. Disease index was evaluated after two months from planting. From each row, 10 plants were used (30 plant replicate^{-1}) for evaluating disease index (DI %).

Determination of pectin, cellulose and lignin:

Ten diseased plants from each replicate were selected randomly from field experiment, and the main fractions of the cell wall including, pectin, cellulose and lignin were determined following the methods described by Galbriath and Shields (1981) and Selvendran and O'Neill (1987).

Pectin fraction:

Wall preparations were first extracted twice with 0.5% ammonium oxalate-oxalic acid (pH 4) at 90°C for 24 hours. Ammonium oxalate solution precipitates calcium ions, which connect the glycosidic bounds in pectin molecules, and thus it becomes water-soluble. Extractions were combined and designated as "pectin fraction".

Pectin determination:

This procedure was conducted essentially according to Selvendran and O'Neill (1987). Pectin and cellulose fractions were estimated by anthorne-sulphuric acid method according to Badour (1959). The reaction mixture consists of 0.2 gm. Anthrone, 8ml. absolute ethyl alcohol, 30 ml. distilled water and 100 ml. concentrated H_2SO_4. They were well mixed in a conical flask under continuous cooling in ice bath. This reagent should be always freshly prepared. Half ml. sample + 4.5 ml. anthrone reagent were thoroughly mixed and boiled for exactly 7.0 min. After cooling the absorbency against blank was photometrically at 620 nm. using spectrophotometer (model SPECIRONIC20D). A standard curve was prepared using pure pectin.

Cellulose determination:

Cellulose content was determined according to method of Linskens and Jackson (1999). Two gm of root fractions were boiled with ethanol for 15 min four times. Resultant was kept in oven at 40°C overnight, then treated with 30 ml 1% diastase enzyme for 30 min. and washed thoroughly with distilled water. The material obtained was kept in oven at 40°C for drying. The residue was treated with 24% KOH for 4h. at 25°C, washed thoroughly with distilled water and kept overnight in oven at 80°C overnight and then the dry weight was recorded as (B fraction). B fraction treated with 72% H_2SO_4 for 3h. to hydrolyze cellulose. Sulfuric acid was removed, the residue was kept in oven at 80°C and the dry weight was recorded as (C fraction). Cellulose content was calculated as follows: Cellulose content = B-C.

Lignin determination:

Lignin was determined by the method described by Liyama and Wallis (1988), as follows: 10-15 mg of samples was weighed into 4 ml brown vials and 20 ml of acetyl bromide in glacial acetic acid (1:3 v/v) containing perchloric acid (70%, 0.08ml) was added. After digestion, the samples were transferred, with the aid of acetic acid, to 50 ml volumetric flasks containing 2 M sodium hydroxide (5ml) and

acetic acid (12 ml). The flasks were carried out in triplicate on duplicate sample of root material. The lignin contents were calculated according to the following equations:

Absorbance value = ODs- ODb /concentration of sample (gl^{-1})
Lignin content = 33.6 absorbance -11.1 g kg^{-1} dry matter
Whereas: ODs is optical density of sample and ODb is optical density of blank.

Statistical analysis

All experiments were repeated twice. Analysis of variance (ANOVA) was carried out using MSTAT-C program version 2.10 (Anonymous, 1991). The Least Significant Difference (LSD) at $P \leq 0.05$ was applied to detect differences among treatments (Gomez and Gomez, 1984).

Results

The antagonistic capability of the Trichoderma spp. isolates against F. oxysporum f.sp fabae isolate in vitro.

The antagonistic capability of 6 *Trichoderma* spp. isolates against growth of *F. oxysporum* f.sp *fabae* isolate on PDA was tested. Data illustrated in Fig. (1) indicate that all *Trichoderma* spp. isolates gave positive reaction of antagonism to the pathogen *in vitro*. Data also revealed that the highest inhibition of *FO* linear growth was observed with *T. harzianum* isolate (Tz1), *T. harzianum* (T15) and *T. viride* (T1) and *T. viride* (M4). The lowest inhibition of *FO* linear growth was observed with *T. spirale* isolates (T32 and TM6).

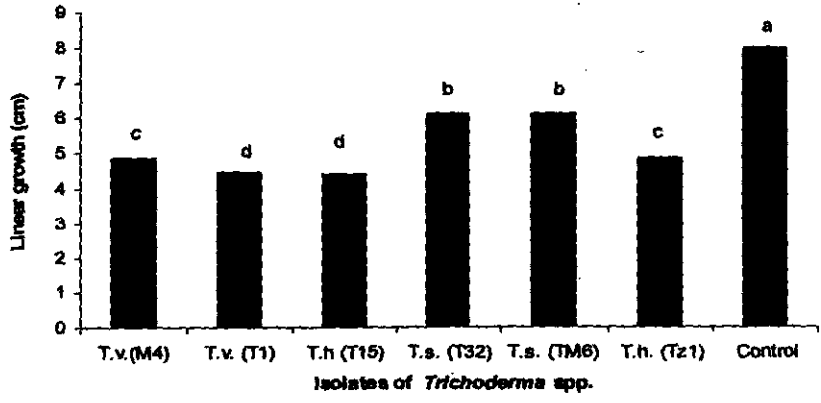

Fig. 1. Effect of certain *Trichoderma* spp. isolates on the linear growth of *F. oxysporum*. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

Effect of certain Trichoderma spp. isolates on faba bean wilt disease: Greenhouse experiments:

Data represented in Fig. (2) indicate that, in general, the different treatments were effective in controlling faba bean wilt caused by *F. oxysporum* f.sp *fabae* isolate. The treatments differed significantly in controlling disease symptoms in faba bean cv. Misr 1 in the treated plants during 2008 growing season under greenhouse conditions. In the tested season, while soil treatment with *T. viride* (T1) showed the lowest reduction in disease incidence, the highest effective treatment was *T. harzianum* (Tz1) followed by *T. harzianum* (T15).

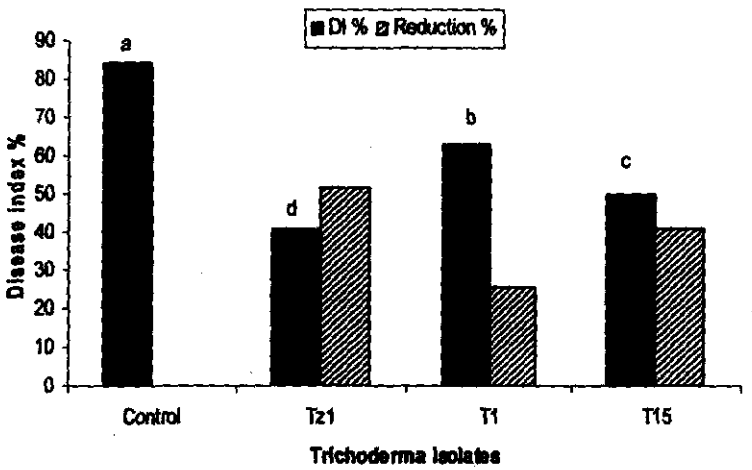

Fig. 2. Effect of certain *Trichoderma* spp. isolates on faba bean wilt as disease index and reduction under greenhouse conditions during 2008 growing season. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

Field experiments:

Results presented in Table (1) indicate that treatments with different *Trichoderma* spp. isolates significantly reduced wilt disease. Soil treatments by *Trichoderma* spp. isolates Tz1, T1 and T15 have reduced disease index. The most effective treatments were isolates Tz1 and T15 followed by isolate T1 in both tested season. The highest reduction was 65.17 and 67.19% with soil treatment by *T. harzianum* (Tz1) in 2009 and 2010 growing seasons, respectively, while the lowest reduction was 49.59 and 52.65% with soil treatment by *T. viride* (T1) in 2009 and 2010 growing seasons, respectively.

Table 1. Incidence of wilt disease on faba bean plants as affected by certain *Trichoderma* spp. under field conditions during 2009 and 2010 growing seasons

Treatment	2009 season		2010 season	
	Disease index (%)	Reduction (%)	Disease index (%)	Reduction (%)
Control	60.17 a	- - - -	67.58 a	- - - -
Trichoderma spp. *				
Tz1	20.66 c	65.17	22.17 c	67.19
T1	30.33 b	49.59	32.00 b	52.65
T15	23.17 c	61.49	27.50 c	59.31

* (Tz1) *T. harzianum*, (T1) *T. viride* and (T15) *T. harzianum*. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

Contents of cellulose, pectin and lignin in treated faba bean plants:

Results in Table (2) indicate that, all treatments significantly increased cellulose, lignin and pectin contents in treated plants compared with untreated plants. The highest increase was obtained by treated Tz1. Lignin contents were increased after all treatments. The highest increase was achieved after treated with Tz1 and T1 while, the lowest levels of lignin (32. 83) were found in host tissue treated with T15. In case of pectin contents, all treatments increase pectin content compared with untreated ones but had no significant effects.

Table 2. Contents of cellulose, pectin and lignin in faba bean roots of cv. Misr 1 growing in soil treated by *Trichoderma* spp. agents

Treatment	Cell wall contents (% of root dry weight)		
	Cellulose	Pectin	Lignin
Control	30.10 b	2.67 b	30.50 c
Trichoderma spp. *			
Tz1	40.19 a	3.30 a	37.47 a
T1	39.13 a	3.30 a	37.47 a
T15	39.00 a	3.20 a	32.83 b

* As described in footnote of Table (1).

Discussion

In vitro testing, antagonistic capability of *Trichoderma* spp. obtained from rhizosphere of faba bean showed inhibitory effect against growth of *FO* with differed degrees. All *Trichoderma* spp. isolates gave positive antagonism reaction to the pathogen isolate. Reduction in *FO* mycelial growth may be due to production of some toxic substances and hydrolytic enzymes like proteases, which could degrade the cell wall of many fungi (Lorito *et al.*, 1993; Elad, 2000 and Kapat *et al.*, 1998).

Trichoderma spp. isolates were applied individually to test their efficacy in controlling faba bean wilt disease caused by *FO* under greenhouse and field

conditions. In greenhouse experiments, all *Trichoderma* spp. protected faba bean seedlings against *FO* and the most effective treatments was *T. harzianum* (Tz1). In field experiments soil treatments by *T. harzianum* (Tz1) and *T. viride* (T1) reduced the disease index.

Many theories have been developed to explain the mechanisms which the antagonistic fungi could suppress diseases. Their action could be explain through antibiosis (Ghisalberti and Rowland, 1993); mycoparasitism (Haran *et al.*, 1996); competition for nutrients and/or space (Simon and Sivasithamparam, 1989 and Inbar *et al.*, 1994). This fact was already observed in the interaction among Trichoderma and other pathogens (Papavizas, 1985 and Melo and Sliva, 1991), the other mechanisms involved in Trichoderma is induction of resistance in plants (Yedidia, *et al.*, 1999) or by the ability of these fungi to induce systemic resistance in plant by tiger some host-biological changes in plants like create more defence barrier and encourage the production of many compound like phytoalexins (De Cal *et al.*, 1997 and Kilic-Ekici and Yuen, 2003). Different classes of compounds may act as elicitors or resistance inducers during the interaction of Trichoderma with plants (Harman *et al.*, 2004). These molecules may include low-molecular-weight compounds released from fungal or plant cell walls by the activity of Trichoderma enzymes (Harman *et al.*, 2004). Determination the content of cellulose, pectin and lignin was very important.

The basic physiology of the changes in the plants introduced by *Trichoderma* spp. is beginning to be understood. For example, it appears that there are a wide range of chemical communication factors and that the particular response may be altered as these factors change. In many cases, these factors are extracellular proteins, or chemicals produced by action of these proteins, that are produced by *Trichoderma* spp. within plant cells (Hanson and Howell, 2004 and Harman and Shoresh, 2007).

Cell wall composition plays an important role in the outcome of host–pathogen interactions. Recently, several genetic studies have provided new evidence implicating cell wall polysaccharides as factors in host–pathogen interactions (Vorwerk *et al.*, 2004), since all plant pathogens interact with plant cell walls. Present study indicated that lignin, cellulose and pectin contents were increased after all treatments in host tissue treated. These results are in agreement with results of Blee *et al.* (2001) who suggested a role for plant peroxidase that participate in the lignifications process leading to increase resistance follow up to the degrading enzymes secreted by invading pathogens. Yedidia *et al.* (1999) found that electron microscopy of ultra thin sections from Trichoderma-treated cucumber roots revealed penetration of Trichoderma into roots, restricted mainly to the epidermis and outer cortex. Strengthening of the epidermal and cortical cell walls was observed, as well the deposition of newly formed barriers. These typical host reactions were found beyond the sites of potential fungal penetration. Wall appositions contained large amounts of cellos and infiltrations of cellulose. From the present experiments it may be concluded that application of *Trichoderma* spp. provide a reasonable level of protection against Fusarium wilt of bean especially in organic farming system, where plant nutrition and disease control are the main limiting factor.

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استحثاث المقاومة الدفاعية ضد الذبول الفيوزارمى فى الفول البلدى عن طريق فطريات الترايكودرما

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أجري هذا البحث بهدف دراسة مدى قدرة الفطر ترايكودرما على استحثاث المقاومة فى نباتات الفول البلدى ضد مرض الذبول الفيوزارمى المتسبب عن الفطر فيوزاريوم أوكسيسبورم فابى. وقد تم عزل الفطر المسبب للمرض من جذور نباتات الفول المصابة بالذبول من مناطق مختلفة فى محافظة أسيوط.

وقد أظهرت الاختبارات الأولية أن عزلات الفطر ترايكودرما هارزيانيم (T15) , (Tz1) وعزلة من الفطر ترايكودرما فيردى (T1) لهم القدرة على تضاد النمو الميسليومى للفطر فيوزاريوم أوكسيسبورم فابى فى المعمل على بيئة البطاطس.

فى تجارب الصوبة والحقل أوضحت النتائج أيضاً أن إضافة عزلات الفطر ترايكوديرما للتربة الملقحة بالفطر المسبب للمرض أدت إلى مقاومة المرض وتقليل شدة الإصابة فى الصوبة وكذلك خلال موسمي الزراعة فى الحقل. كما أظهرت نتائج التحليلات الفسيولوجية لجذور نباتات الفول البلدى المعاملة بعزلات الفطر ترايكوديرما زيادة فى محتوى النباتات من السيلولوز والبكتين واللجنين مقارنة بالنباتات غير المعاملة، وقد أظهرت العزلة (Tz1) من الفطر ترايكوديرما هارزيانيم أعلى زيادة فى محتوى النباتات من البكتين.