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Biocontrol of Sugar Beet Pathogens *Fusarium* Wilt by *Trichoderma viride*

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ABSTRACT

Six fungal strains were isolated from the rhizosphere of sugar beet cultivars in Egypt. Two fungal isolates were effective against three phytopathogenic fungi: *Fusarium solani, Fusarium oxysporum* and *Fusarium dimerum*. The two strains were identified and belonged to *Trichoderma viride*. *In vivo* the highest percentages of survival plants were 72.5, 75 and 82.5%by *T.viride* code No. (2*)* for the control of *F. solani, F. oxysporum* and *F. dimerum,* respectively while more than *T.viride* code No. (1*)* . *T. viride* isolates produced HCN, chitinase and cellulase, but it failed to produce IAA. The usage of *T. viride* as seed treatment reduced the percentage of damping-off of sugar beet under greenhouse conditions. This result shows that these *T. viride* are very effective biocontrol agents and should be harnessed for further biocontrol applications.
Keywords: *Trichoderma viride*, Biological control, *Fusarium* wilt, Antagonism.

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is one of the most important sugar crops in many countries of the world. In Egypt, due to the great consumption of sugar, the production of sugar beet must be increased to cover the requirements of sugar which depended sugar cane. Under Egyptian conditions, sugar beet plant attacked by numerous foliar and root diseases, which have been recorded in several reports (Mosa and El-Kholi, 1996). The most important of soil diseases are damping-off and root-rot disease caused by different pathogens such as *Rhizoctonia solani* Kuhn, *Sclerotium rolfsii* Sacc., *Phoma* (*Pelospora*) *beta* Berl. Several species of *Fusarium* and *Phythium* were also recorded, i.e. *Fusarium solani* (Mart) Sacc, *F. oxysporum* f. sp. *conglutinans* Wollenw, *F. oxysporum* Snyder & Hans, *F. moniliforium* Sacc. and *F. meresmoides* Corda, *Pythium aphanidermatum*, *P. mamillatum* Meurfd, *P. ultimum* and *P. debaryanum* Hesse (El-Kholi, 2000). Many rhizospheric microorganisms are known to be equipped with antagonistic potential against soilborne pathogens such as *Trichoderma* spp. (Chet and Baker, 1981). *Trichoderma* species is considered biocontrol agents in plant disease control were applied successfully against several plant diseases in commercial agriculture (Howell, 2003). Biocontrol technologies have gained momentum in disease control of crop plants, in recent times as these technologies not only minimize of replace the usages of harmful chemical pesticides but also found to be cheaper and efficient in certain disease control programmers. Yadav *et al*., 2011 reported that *Trichoderm* spp. is the most effective as biocontrol against soil-borne diseases. *Rhizoctonia, Sclerotium, Fusarium, Pythium and Phophthora* caused soil-borne diseases in several crops have been reported. (Cook and Baker, 1983) therefore using biological control such as *T.viride* which is one the efficient biocontrol have been suggested as being responsible for their bio–control activity which includes mycoparasitism, antibiosis, competition for nutrients and space. These mechanisms are not mutually exclusive and the overall biocontrol activity is due to the synergistic effects of different modes of antagonism (O'Sullivan and O'Gara 1992). Cyanide is a toxic and dreaded chemical. It produced by many rhizobacteria. Some bacteria synthesis it, others excrete it and yet others metabolize it in other to avoid predation and competition. According to Etesami *et al*., 2009, production of IAA in plants help to increase root dry weight and thereby increase the plants ability to take up N, P, K compared to untreated control. It caused increase in vegetables especially pepper, cucumber and tomato (Kidoglu *et al*., 2007). Many microorganisms produce and release lytic enzymes. Polymeric compounds hydrolyze by enzymes including chitinase, proteinase, cellulase, hemicellulase, and DNAase. Nonetheless, microbes that know a preference for colonizing and lysing plant pathogens should be classified as biocontrol agents.This work aims at evaluating the antagonistic effect of some selected fungal isolates against sugar beet pathogens *Fusarium* wilt and their effects on damping-off and survival sugar beet plants infected with the pathogenic fungi.

MATERIALS AND METHODS

A total of fifteen fungal isolated from rhizosphere of sugar beet cultivars at different governorates of Egypt and selected six strains for it according to differences between growth on PDA medium during isolation and incubation. These six fungal strains were screening to controlling *Fusarium* wilt fungi by *in vitro* antagonism.

Pathogens:

Fusarium solani, F. oxysporum and *F. dimarium* were obtained from Plant Pathology Research Institute, Agric. Res. Center (A.R.C.), Giza, Egypt.

Isolation and purification of Fungi

Ten grams of soil samples was suspended in 90 ml of sterile tap water and serial dilution were made. One ml from each dilution was transferred to Petri-dishes. Thereafter, potato dextrose agar (PDA) was added and mixed thoroughly. Three replicates were prepared from each dilution. Colony units were obtained after five days of incubation at 30^0C and purified on PDA medium. The fungall isolates were maintained on PDA medium at 4^0C for further use.

Antagonistic effect of the fungal isolates against pathogenic fungi:

This work was carried out to manage the causes damping-off diseases of sugar beet *Fusarium* spp. by applying bioagents. Six antagonistic fungi were screened for their efficiency in inhibiting the linear growth (LG) of the target fungi. PDA plates (9 cm diam.) were inoculated peripherally with each of the

pathogens and the bioagents under study at the opposite side to each other and incubated at 30^0C for 7 days. After the elapse of the incubation period, the colony diameter of pathogens were measured and recorded as the percentage of reduction over the control. Three replicates plates were used. A complete randomized design was used in this experiment. The percentage of reduction (R %) in the mycelial growth was calculated according to the following formula adopted by Ferreira *et al.* (1991) as follows:

$$\mathbf{R\ \% = \frac{A - B}{A} \times 100}$$

where

R%=Percentage of growth reduction.

A=The distance of mycelial growth of the pathogenic fungi in the control.

B =The distance of mycelial growth of the pathogenic fungus towards the tested bioagents.

Identification of fungal isolates:

The best antagonistic fungal isolates were identified by microscopic observation according to (Domsch *et al*., 1993 and Samson *et al*., 2000) in the Regional Center for Mycology and Biotechnology Culture Collection on Identification Unit. Faculty of Science. Al-Azhar University, Cairo, Egypt.

***In vivo* pot experiments:**

This experiment aims at investigate the role of isolate No. (2) and (3) were the most effective *in vitro* with pathogen fungi (*Fusarium* spp.) to control damping-off of sugar beet.

Pots (35 cm. in diameter) were sterilized by immersing in 5% formalin solution for 10 mins. and left to dry in open air. Soils were sterilized by formalin. 4 kg of sterilized soil were put in each pot. Five mm mycelial disk from a 5 days old culture of *Fusarium* spp. grown on PDA were prepared by growing in sterilized glass bottles (500ml) containing 150g sorghum seeds, 50g clean sand, 4g glucose and 200 ml water and incubated at 27 ± 1^0C for 15 days.

Fungi surface sterilized seeds were soaked for one hour before sowing in a 5-day-old culture of *Trichoderma* spp. (10^8-10^9 spores /ml) grown in liquid potato dextrose broth medium. The supernatants were taken and used for seed treatment. Suspension was prepared by mixing of each bioagent with soaking (2:1v/w) .

Ten seeds were sown in each pot and four replicates were used for each treatment. Surface sterilized un-soaked seeds were served as a control check.

Assessment of disease incidence was calculated as a percentage of pre- and post-emergence damping-off after 15 and 45 days of sowing. These experiments were carried out under greenhouse conditions for growing season 2014/2015.

Percentages of pre and post-emergence damping-off as well as survival plants were calculated up to 45 days from planting as follows:

1. % of pre-emergence damping-off = (No. of non-emerged seeds/No. of sown seeds) x 100
2. % of post-emergence damping-off = (No. of killed seedlings/ total No. of emerged seedlings) x 100
3. % of survival plants = (No. of un-infected plants/total No. of plants) x 100

Antagonistic parameters produced by *T. viride*

Detection of Hydrogen Cyanide (HCN)

The production of hydrocyanic acid (HCN) was tested according to Bakker and Schippers (1987). Tryptone soy agar containing 4.4 g per liter of glycine was prepared and inoculated with fungal cultures. Filter paper soaked in 0.5 % picric acid solution and 2.0 % sodium carbonate was placed inside the lid of the plate. Plates were incubated at 28 °C for 3 days inversely. Development of yellow to orange–brown color referred to HCN production.

Determination of Indole Acetic Acid (IAA)

The amounts of IAA were determined according to (Ehmann, 1977). The isolates were grown in yeast malt dextrose broth medium (YMD broth) and incubated at 28 °C for 4 days. After incubation, the broth was centrifuged. 50 µl of 10 mM orthophosphoric acid were prepared and a 2 ml of Salkowski reagent consist of 1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$ were prepared and added to the mixture. Development of a pink colour referred to the presence of indole acetic acid production.

Cellulase production

Yeast extract peptone agar medium were prepared. This medium consist of (yeast extract, 0.1g; peptone 0.5g; agar, 20g and distilled water, 1000 mL; pH, 6.5) completed with 0.5% Na-carboxymethyl cellulose (CMC). After incubation, Congo Red was added to the plates. The colony that surroundded by clear zone refered to cellulase production. (Lingappa and Lockwood, 1962)

Chitinase production

Colloidal chitin was made according to Faramarzi *et al*., (2009). Chitinase detection medium was made according to Wirth and Wolf 1990.

Statistical analysis

The obtained data were subjected analysis of variance (ANOVA) (Steel and Terrie 1960). Least significant differences (L.S.D.) and Duncan's multiple range test (DMRT) were applied for comparing means under study (Duncan, 1955).

RESULTS AND DISCUSSION

Isolation and purification of fungi

After chosen, six fungal isolates were isolated and purified from different types of soil and were examined in this study to control damping-off of sugar beet

Antagonistic effect of fungal isolates against fungal pathogens *in vitro*

Data presented in Table (1) indicated that all bioagents actively affected the growth of the three pathogens under study and slight differences between them were observed. Isolate No.3 was the most potent inhibitors to the growth of *F. solani* compared to the other used fungi, followed by isolate No.2. The least effect was happened by the isolates No 5 and No. 6. The

rest antagonists distributed between these two extremists.

Results shown in Table (1) indicated that, isolates No.3 reduced growth of *F. oxysporum* (72.22%) more than No. 2 (71.44%). Isolates No. 1 and No.4 have moderately effect in reducing the radial growth of *F. oxysporum* compared with the control. While isolates No. 5and No. 6 were the least effective against the same fungus, it gave 34.11 and 49.22% reduction, respectively.

In the case of *F. dimerum*, as shown in Table (1), results showed that isolates No.3 and No. 2 were the most effective (69.43%) and (67.86%) respectively. While isolates No. 6 and No. 4 were the least effective against *F. dimerum* compared with the other bioagents.

Table 1. Effect of fungal isolates on the reduction (R %) of linear growth *F. solani*, *F. oxysporum* and *F. dimerum*.

Fungal isolate No.	*F. solani*		*F. oxysporum*		*F. dimerum*	
	L.G "cm"	Reduction %	L.G "cm"	Reduction %	L.G "cm"	Reduction%
1	4.10^{d}	54.09	4.17^{d}	53.67	4.37^{c}	51.06
2	2.23^{e}	75.03	2.57^{e}	71.44	2.73^{d}	69.43
3	1.93^{f}	78.39	2.50^{e}	72.22	2.87^{d}	67.86
4	4.33^{cd}	51.51	4.43^{c}	50.78	5.30^{b}	40.65
5	6.23^{b}	30.24	5.93^{b}	34.11	4.07^{c}	54.42
6	4.47^{c}	49.94	4.57^{c}	49.22	5.40^{b}	39.53
Control	8.93^{a}	0.00	9.00^{a}	0.00	8.93^{a}	0.00

Values with different letters are significantly different L.G: Linear growth of the pathogen (cm)

Identification of fungal strains:

Of the six fungal isolates, two were chosen for identification because they showed very high antagonism against *Fusarium solani, F. oxysporum* and *F. dimarium*. These two fungal isolates No.(3) and No.(2) were identified as *Trichoderma viride* code No.(1) and *Trichoderma viride* code No.(2) according to source of soil samples. Table(2) and Photo (1).

Table 2. Microscopic and Culture examination for fungal isolates

Growth of culture	Microscopic examinations	
	Phialides	Conidia
Colonies reaching 5-7 cm diameter in ten days at 28°C, on Malt, whitish green color.	In whorls of 2-4 flask shaped, 8.8X2.5 µm.	Conidia sub - spherical, green in mass 3.8X2.0 µm.

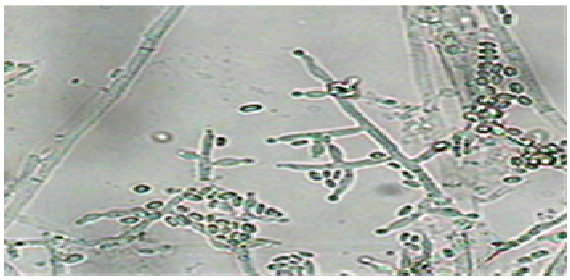

Photo 1. Microscopic observation of *Trichoderma* spp. using an image analysis system .

Effect of *T. viride* inoculation on damping-off and survival plants infected with *Fusarium* spp.

T. viride code No. (1) and *T. viride* code No. (2) were the most effective *in vitro*, so they were selected to test their antagonistic activity in the greenhouse. Data in Table (3) indicated that all treatments decreased damping-off and increased healthy plants compared with the control treatment. Afify and Ashour (1995) recommended that *T. hrazianum* bio–controlling agents as combination in integrated control system of damping-off disease. Also, Tondje *et al.* (2007) reported that *Trichoderma* spp. has been widely used in biological control studies against several commercial phytopathogens.

a) Effect of *T. viride* isolates on damping-off and survival plants infected with *F. solani.*

Data in Table (3) indicate that all treatments decreased damping-off and increased healthy plants compared with the control treatment. *T. viride* code No. (2) *(75%)* was the most effective survival plants, followed by *T. viride code* No. (1) (72.5%).

Table 3. Evaluation of the efficacy of *T. viride* isolates against *F.solani* in controlling damping- off of sugar beet seedling in the greenhouse

Treatments	Damping – off (%)		survival plants (%)
	pre emergence 15 days	post emergence 45 days	
Trichoderma viride code No. (1).+ *F.solani*	12.5^{b}	27.5^{b}	72.5^{b}
Trichoderma viride code No. (2).+ *F.solani*	12.5^{b}	25^{b}	75^{a}
Un treated control(*F.solani*)	35^{a}	55^{a}	45^{b}

Values with different letters are significantly different

Ushamalini *et aI.* (1997) studied the inhibitory effects of antagonists *T. viride, T. hrazianum T. hamatum* and *T. koningii* against *M. phaseolina* and *F.*

spp. and they found that all the antagonists significantly inhibited the growth of *M. phaseolina. T. viride* and *T. hrazianum* were the most effective but in case of *F. oxysporum. T. hrazianum* was also , the most effective.

b) Effect of *T. viride* isolates on damping-off and survival plants inoculated with *T. viride* and infected with *F. oxysporum*.

While *F oxysporum*, data in Table (4) indicated that *T. viride* code No. (2) (75%) was the most effective survival plants, followed by*T. viride* code No. (1) 70% Gouda (2001) showed the possibility of controlling sugar beet damping-off and root rot by certain bioagants *T. hamatum, T. harzianum*, *Pseudomonas* and *Bacillus subtilis* under greenhouse and field condition, *Trichoderma* spp. and *P. flurescense* the most effective against all soil borne pathogens.

Table 4. Evaluation of the efficacy of *T. viride* isolates against *F.oxysporum* in controlling damping-off of sugar beet seedling in the greenhouse

Treatments	Damping – off (%)		Survival plants (%)
	pre emergence 15 days	post emergence 45 days	
Trichoderma viride code No. (1).+ *F.oxysporum*	7.5^{b}	30^{b}	70^{a}
Trichoderma viride code No. (2).+ *F.oxysporum*	10^{b}	25^{b}	75^{a}
Untreated control (*F.oxysporum)*	35^{a}	60^{a}	40^{b}

Values with different letters are significantly different

c) Effect of *T. viride* isolates on damping-off and survival plants inoculated with *T. viride* and infected with *F. dimerum*.

In the case of *F. dimerum* data in Table (5) indicated that *T. viride* code No. (2) was the most effective (82.5%) survival plants followed by *T. viride* code No. (1) 70%. Esh (2000) *in vivo* studies showed that high significant differences between the bioagents in controlling damping- off diseases in the greenhouse the highest effect showed by the bioagent *T. hamatum* followed by *T. viride*.

Table 5. Evaluation of the efficacy of *T. viride* isolates against *F.dimerum* in controlling damping- off of sugar beet seedling in the greenhouse

Treatments	Damping – off (%)		survival plants (%)
	pre emergence 15 days	post emergence 45days	
Trichoderma viride code No. (1).+ *F.dimerum*	10^{b}	30^{b}	70^{b}
Trichoderma viride code No. (2).+ *F.dimerum*	5^{b}	17.5^{c}	82.5^{a}
Un treated control *F.dimerum*	35^{a}	52.5^{a}	47.5^{c}

Values with different letters are significantly different

Mechanism of biocontrol *T. viride* against *Fusarium* spp.

The Mechanism of antagonist of the two *T. viride* isolates was investigated. Results presented in Table (6) showed that *T.viride* code No. (1) and *T. viride* code No. (2) were all positive for HCN. *T.viride* code No. (1) and *T.viride* code No. (2) were all negative for IAA, while both *T. viride* code No. (1) and code No. (2) were produced cellulase and chitinase.Similar results were reported by Allpress *et al*., 2002. The production of hydrolytic enzymes has been described for the biocontrol activity (Sidhu and Dadarwal 2001).

Hoyos-Carvajal *et al*., (2009) showed that *Trichoderma* spp. establishes a benefical interaction within roots and have a similar result with symbioses to enhance crops growth and health. Zheng and Shetty (2000) reported that *Trichoderma* species improve seedling vigor and increase root length, thus helping host plants absorb more nutrients (Altomare *et al.*, 1999). Hydrogen cyanide is a microbial metabolite that promotes plant nitrogen accumulation and root elongation (Marques *et al*., 2010).

Table 6. Production of some antagonistic properties by *T. viride* isolates:

Fungal isolates	HCN	IAA	Enzymes production	
			Cellulase	Chitinase
T. viride code No. (1).	+	-	+	+
T. viride code No. (2).	+	-	+	+

Finally, this shows that *T. viride* are quite important and effective as biocontrol agents.

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المقاومة الحيوية لمسببات الذبول الفيوزاريومى لبنجر السكر بواسطة التريكودرما فردى

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تم فى هذه الدراسة عزل ٦ عزلات فطرية من المجال الجذرى لنبات بنجر السكر في مصر وهذه العزلات مختلفة فى شكل النمو على بيئة اجار البطاطس والدكستروز وعند اختبارهذه العزلات الستة بالتضاد الحيوى فى المعمل ضد ثلاثة فطريات مسببة للذبول الفيوزاريومى للنبات: فيوزاريوم سولاني فيوزاريوم أوكسيسبوريوم، و فيوزاريوم ديمريم. اشارت النتائج في المختبر ان عزلتين فقط من العزلات الفطرية اعطيا اعلى كفاءة لذلك تم تعريف العزلات الفطرية على انهما سلالتين للتريكودرما فردى وهي:التريكودرما فردى كود رقم ١ والتريكودرما فردى كود رقم٢. وقد اختبرت النتائج ايضا فى الصوبة فكانت كما يلى: نسبة النباتات السليمة بعد المعاملة بالتريكودرما فردى كود رقم٢ (٧٢,٥%) فى حالة المقاومة لفطر الفيوزاريم سولانى بينما كانت النسبة(٧٥ ،٨٢,٥)%فى مقاومة كل من الفيوزاريم اوكسبوريم والفيوزاريم دايمريم على التوالى .وايضا اظهرت السلالات عند اختبار المقاومة الحيوية تحت ظروف الصوبة الزراعية ان التريكودرما قد خفضت فى نسبة موت البادرات قبل وبعد ظهورها فوق سطح التربة، أظهرت السلالتين فى ميكانيكية المقاومة ايجابية فى اختبار سيانيد الهيدروجين ،بينما فشلت فى انتاج اندول حمض الخليك .اما بالنسبة للانزيمات فقد انتجت التريكودرما فردى كل من السليليز والكيتينيز• وبالتالى استخدام التريكودرما لمعاملة بذور بنجر السكر أدى الى تخفيض النسبة المئوية للذبول الفيوزاريومى تحت ظروف الصوبة الزراعية. وبهذا تعتبر التريكودرما من العوامل الفعالة جدا فى المكافحة الحيوية وينبغي استخدامها من أجل المزيد من تطبيقات المقاومة الحيوية.