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Field Application with Biocontrol Agents for Controlling Root-rot Disease of Grapevine (*Vitis vinifera* L.)

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ABSTRACT

Grapevine plants, grown under field conditions, highly infected with root-rot disease caused by the soilborne pathogens, *i.e. Fusarium solani*; *M. phaseolina* and *R. solani*. The *in vitro* experiments revealed that biocontrol agents of *T. harzianum, T. viride* and *B. subtilis*, caused the highest reduction (%) in mycelial growth of the three pathogenic fungi. Field application with *T. harzianum, T. viride* and *B. subtilis* for controlling root-rot disease of grapevine (cv. Thompson seedless) were investigated. Three applicable methods were conducted, *i.e.* soil drench treatments in root region (75 cm away from the trunk and 30 cm depth), soil drench treatments in trunk region around the main stem and a combination treatment. Obtained data indicate that the combination treatment of *B. subtilis* highly reduced grapevine root-rot percentage, severity and recorded higher of yield components than single treatment. The treatment of *T. harzianum* was significantly ranked the second, while *T. viride* occupied significantly the third one. Obtained data recommended that the combination treatment by releasing the biocontrol agents in root region plus in trunk region could be considered as a promising technique for field application to control grapevine root-rot disease.

Key words: Grapevine root-rot, biological control, field application, *T. harzianum, T. viride* and *B. subtilis*.

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most widely distributed fruit crop in the world yielding berries, wine products as well as derivates. In Egypt, grapevine ranks the second after citrus among fruit crops. Soilborne fungal diseases are among the most important factors limiting the yield production of grapevine, resulting in serious economic losses. Several soil-pathogens including *Fusarium solani* (Mart.) App. & Wr.; *Macrophomina phaseolina* (Maubl) Ashby and *Rhizoctonia solani* Kühn attack the roots of grapevine (Marais 1979; Walker, 1992 ; Gugino *et al*. 2001; Gubler *et al*. 2004; Ziedan, 2005 and Ziedan *et al*. 2010 and 2011). Some chemicals are effective in controlling these diseases but these chemicals are expensive and not environmental friendly. Therefore, alternative control methods are needed for managing these pathogens. Biological control is a strategy that was proposed half a century ago. The application of biological control using antagonistic microorganisms *i.e*., *Trichoderma harzianum*, *T.viride* and *Bacillus subtilis*, proved to be successful for controlling various soilborne plant diseases in many countries (Leifert *et al*., 1995; Asaka and Shoda 1996; Pinchuk *et al*., 2002; Benitez *et al*., 2004; Singh *et al*., 2008; Tran, 2010; Killani *et al*., 2011; Abdel-Kader *et al*., 2012 and Compant *et al*., 2013). The objective of this study was to investigate the efficacy of *T. harzianum*, *T. viride* and *B. subtilis* applications for controlling root-rot disease of grapevine under field conditions.

Materials and Methods

In vitro experiments

Grapevine root-rot pathogens:

Fusarium solani; *Macrophomina phaseolina* and *Rhizoctonia solani*, which considered the most virulent pathogens causing root-rot disease of grapevine Ziedan (2003), were obtained from Plant Pathol. Dept., National Research center (NRC).

Biocontrol agents:

The antagonistic strains of *T. harzianum* , *T. viride* and *B. subtilis* was obtained from Plant Pathol. Dept., NRC. The strains were previously isolated from grapevine rhizosphere.

Antagonistic potential of biocontrol agents:

The antagonistic activity of *T. harzianum* and *T. viride* isolates against *F. solani*, *M. phaseolina* and *R. solani* was studied via the dual culture technique using the method described by Amel *et al*. (2007). The method

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consists of placing an active mycelial disc (5-mm in diameter) of the pathogen, 1cm from the edge of a 9-cm-diameter Petri plate containing freshly prepared PDA medium. Another disc (5-mm in diameter) of the antagonist fungus was deposited in a diametrically opposed position 1cm away from the other set of the plate. For untreated plates, an agar disc (5-mm in diameter) of pathogenic fungi only was placed at 1cm from the edge of a 9-cm-diameter Petri plate containing freshly prepared PDA medium.

The bacterial isolate of *B. subtilis* was also screened for their antagonistic ability against cucumber root-rot pathogens *in vitro* via the dual culture technique using the method described by Estrella *et al.* (2001). Therefore, the bacterial isolate was cultured (by streaking) at 1cm from the edge of a 9-cm diameter Petri plate containing freshly prepared PDA medium. On the opposed position 1cm away from the other set of the plate a 5-mm plug from the leading edge of a 5-days old culture of *F. solani*, *M. phaseolina* and *R. solani*, cultured on PDA medium were inoculated individually. For untreated plates, an agar disc (5-mm in diameter) of pathogenic fungi only was placed at 1cm from the edge of a 9-cm diameter Petri plate containing freshly prepared PDA medium.

Five plates were used as replicates for each treatment as well as the control. Inoculated plates were incubated at 25 ± 1°C until the fungal growth of the control plates reached the edge of the plate.

The reduction in mycelial growth of the pathogenic fungi was calculated using the formula suggested by Fokemma (1973) and Pandy *et al*. (2000) as follows: R = C – T / C × 100, whereas: R = Mycelial growth reduction (%) of the pathogen, C = Radial growth of the pathogen in control plates (cm) and T = Radial growth of the pathogen in dual culture plate (cm).

Field experiments

Biocontrol agents inocula:

T. harzianum and *T. viride* was grown on PDA medium at (25 ± 1°C) for 10 days, afterwards the mycelium with the spores was scraped from Petri plate and mixed with sterilized distilled water (20 ml / plate) in a blender. The suspension was adjusted by hemocytometer slide to 3×10^6 propagules / ml as described by (Morsy *et al.,* 2009). On the other hand, one loopful of *B. subtilis* isolate was inoculated into Nutrient Broth medium (g/l): peptone 5 g, beef extract 3 g, sodium chloride 5 g, glucose 20 g, pH 7; and incubated on a shaker incubator (125 rpm) at 28 ± 1°C. Antagonistic bacterial cells were then harvested after 48 hours of growth in culture medium by centrifugation at 6,000 rpm for 15 min and resuspended in a phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted by plate count technique to an approximately 3×10^7 colony forming unit CFU / ml as mentioned by Morsy *et al.* (2009).

Application of biocontrol agents:

Field experiments were carried out at Taba vineyard (El-Sadat city, El-Monofia Governorate, Egypt), which have been seriously destroyed by root-rot pathogens. Grapevine orchard 5 years old (cv. Thompson seedless) showing different grades of root-rot symptoms on shoot system were selected in an orchard of 5 feddan. Thirty diseased grapevine trees showed different degrees of disease severity 1, 2 and 3 grades were selected for each treatments as well as untreated one (control). Soil drench treatment in root region (75 cm away from the trunk and 30 cm depth) at the four dimensions of the diseased tree were applied individually with 400 ml inoculum/tree of each of *T. harzianum* , *T. viride* and *B. subtilis* for three application during March, April and May. Soil drench treatments in the trunk region (closely around the main stem of diseased tree) were applied individually with 200 ml inoculum/tree of each of *T. harzianum* , *T. viride* and *B. subtilis* for three application during March, April and May. The combination treatment of soil drench in root region + soil drench in the trunk region were also done. Another groups of thirty trees with the same degrees of disease severity were left without treatment as a control. All treatments were repeated two seasons. The treatments can be summarized as follows:

1-Soil drench with *T. harzianum* in root region.
2-Soil drench with *T. viride* in root region.
3-Soil drench with *B. subtilis* in root region.
4-Soil drench with *T. harzianum* in trunk region.
5-Soil drench with *T. viride* in trunk region.
6-Soil drench with *B. subtilis* in trunk region.
7-Soil drench with *T. harzianum* in root region + trunk region.
8-Soil drench with *T. viride* in root region + in trunk region.
9-Soil drench with *B. subtilis* in root region + in trunk region.
10- Control.

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Root-rot incidence and severity of grapevine:

Root-rot disease incidence and disease severity were registered 4 months after the first treatment with different biocontrol agents according to the disease grades suggested by Ziedan (2003). Disease severity on foliage systems was as follows: 0 = healthy, 1 = yellowish +1/3 plant wilted, 2 = 2/3 plant wilted, 3 = whole plant wilted and 4 = plant dead showed sever wilt.

Assessment of garpvine yield:

At the harvest time, grapevine fruit yield was determined in terms of number of clusters/ tree, average weight of cluster, fruit yield (kg/ tree and ton/feddan) for each treatment as well as the control.

Statistical analysis:

Statistical analyses of all the previously designed experiments were carried out according to (ANOVA) procedures reported by Snedecor and Cochran (1982). Treatment means were compared by the least significant difference test "LSD" at 5% level of probability.

Results

Antagonistic potential of biocontrol agents against grapevine root-rot pathogens:

Data presented in Table (1) indicated that all biocontrol agent isolates showed antagonistic activity against mycelial growth of root-rot pathogens. Among of them, the bacterial isolate *B. subtilis* and *T. harzianum* caused the highest reduction (%) in mycelial growth reached to 77.6 & 75.6; 83.3 & 82.2% and 86.2 &84.4% against *F. solani, R. solan* and *M. phaseolina,* respectively. Data also indicated that *T. viride* isolate showed moderate reduction (%) in mycelial growth reached to 66.7; 72.2 and 75.6 % against *F. solani, R. solan* and *M. phaseolina,* respectively.

Table 1. The antagonistic effect of biocontrol agents isolates against the mycelial growth of the pathogenic fungi.

Biocontrol agent	Linear mycelial growth (mm) and reduction (%) of					
	F. solani		*R. solani*		*M. phaseolina*	
	Linear growth	Reduction (%)	Linear growth	Reduction (%)	Linear growth	Reduction (%)
T. harzianum	22.0 c	75.6	16.0 c	82.2	14.0 c	84.4
T. viride	30.0 b	66.7	25.0b	72.2	22.0 b	75.6
B. subtilis	20.2 c	77.6	15.0 c	83.3	12.4 c	86.2
Control	90.0 a	-	90.0a	-	90.0 a	-

Effect of biocontrol agents application on root-rot of grapevine:

Effect of soil drench in root region, soil drench in trunk region and the combination of the towe treatments with biocontrol agents on the percentage and severity of grapevine root-rot are shown in Table (2). Data show that the most effective treatment is *B. subtilis* followed by the treatment of *T. harzianum* , when applied individually as soil + trunk treatments, which reduced the disease incidence and severity by (83.8 ; 81.3 & 76.4 & 69.6%) and (90%), during the two growing seasons, respectively. Meanwhile, the treatment of *T. viride* had moderate effect.

It is evident that the combination treatment of soil drench in root region + soil drench in trunk region with any of the desired biocontrol agents significantly reduced root-rot percentage and disease severity of grapevine than in the untreated plant (control) followed by soil drench in root region of grapevine only and soil drench in trunk region of grapevine only, respectively. It is evident also that *B. subtilis* treatment caused the highest reduction in grapevine root-rot percentage and severity on shoot system, followed by the treatment with each of *T. harzianum* and *T. viride,* respectively.

Effect of biocontrol agents application on grapveine yield:

Effect of biocontrol agents application on grapevine yield are shown in Table (3). Data revealed that all biocontrol agents treatments significantly improved grapevine yield compared with the untreated trees (control).The highest increase in yield was obtained with *B. subtilis* when applied as soil + trunk treatments which increased the yield by 150.0 and 132.4 % during tow growing seasons respectively. Followed by *T. harzianum* when applied as soil + trunk treatments which increased the yield by 113.9 and 138.2 % during tow growing seasons respectively . Meanwhile, the treatment of *T. viride* had moderate effect.

Table 2. Effect of *T. harzianum*, *T. viride* and *B. subtilis* on root-rot disease incidence and severity of grapevine (cv. Thompson seedless) grown under natural infection conditions.

Biocontrol agent	Application technique	Grapevine root-rot			
		Disease incidence	Reduction %	Disease severity***	Reduction %
First season					
T. harzianum	Soil region*	17.5 e	72.8	1.2	70.0
	Trunk region**	25.3 c	60.7	1.0	75.0
	Soil + Trunk	15.2 f	76.4	0.4	90.0
T. viride	Soil region	21.1 d	67.2	2.0	50.0
	Trunk region	29.2 b	54.6	2.0	50.0
	Soil + Trunk	20.7 d	67.8	1.5	62.5
B. subtilis	Soil region	13.1 g	79.6	0.7	82.5
	Trunk region	21.6 d	66.4	1.3	67.5
	Soil + Trunk	10.4 h	83.8	0.4	90.0
Control	—	64.3 a	—	4.0	—
Second season					
T. harzianum	Soil region	20.0 f	64.4	1.0	75.0
	Trunk region	27.0 c	52.0	1.0	75.0
	Soil + Trunk	17.1 g	69.6	0.4	90.0
T. viride	Soil region	21.5 d	61.7	2.0	50.0
	Trunk region	30.6 b	45.6	2.0	50.0
	Soil + Trunk	19.5 e	65.3	1.0	75.0
B. subtilis	Soil region	12.5 h	77.8	0.6	85.0
	Trunk region	19.3 e	65.7	1.0	75.0
	Soil + Trunk	10.5 i	81.3	0.4	90.0
Control	—	56.2 a	—	4.0	—

Means designated with the same letter in the same column are not significantly different at 0.05 level of probability.
** Soil drench treatments in root region (75 cm away from the trunk and 30 cm depth)*
*** Soil drench treatments in trunk region were applied closely around the main stem and*
**** Root-rot disease severity was determined on shoot system of grape plant according to (Ziedan, 2003) as follows:0= healthy, 1= yellowish +1/3 plant wilted, 2= 2/3 plant wilted, 3= whole plant wilted and 4= plant dead showed sever wilt.*

Table 3. Effect of *T. harzianum* , *T. viride* and *B. subtilis* applications on clusters parameters and yield of grapevine (cv. Thompson seedless) grown under natural infection conditions.

Biocontrol agent	Application technique	Clusters parameters		Grapevine yield		
		No. / tree	Weight (g)	Kg / tree	Ton/ feddan	Increase %
First season						
T. harzianum	Soil region*	6.80 c	853.0 h	5.7 d	4.5 f	25.0
	Trunk region**	5.90 d	945.0 d	5.6 d	4.5 d	25.0
	Soil + Trunk	8.50 b	1125.0 a	9.7 b	7.7 b	113.9
T. viride	Soil region*	5.70 d	920.0 f	5.4 f	4.2 h	16.7
	Trunk region**	5.20 d	992.0 b	5.2 g	4.1 h	13.9
	Soil + Trunk	7.50 b	985.0 c	7.4 c	5.9 c	63.9
B. subtilis	Soil region*	6.50 c	850.0 g	5.5 e	4.4 g	22.2
	Trunk region**	6.20 c	935.0 e	5.8 d	4.6 c	27.8
	Soil + Trunk	10.0 a	1120.0 a	11.2 a	9.0 a	150.0
Control	-	6.50 c	750.0 i	4.5 h	3.6 i	
Second season						
T. harzianum	Soil region*	7.00 c	820.0 d	5.7 d	4.6 d	35.3
	Trunk region**	6.20 c	965.0 c	6.0 d	4.8 d	41.2
	Soil + Trunk	9.00 b	1130.0 b	10.2 b	8.1 b	138.2
T. viride	Soil region*	6.10 d	810.0 d	4.9 e	4.0 d	17.6
	Trunk region**	5.80 d	935.0 c	5.4 d	4.3 c	26.5
	Soil + Trunk	8.30 b	1100.0 b	8.1 c	6.5 c	91.2
B. subtilis	Soil region*	6.30 d	865.0 d	5.5 d	4.4 d	29.4
	Trunk region**	6.10 d	950.0 c	5.8 d	4.6 d	35.3
	Soil + Trunk	10.5 a	1155.0 a	12.1 a	9.7 a	132.4
Control	-	6.70 d	735.0 f	4.9 e	3.4 e	

*Means designated with the same letter in the same column are not significantly different at 0.05 level of probability. * Soil drench treatments in root region (75 cm away from the trunk and 30 cm depth);** Soil drench treatments in trunk region were applied closely around the main stem.*

The combination treatments of soil drench in root region + soil drench in trunk region with each of *B. subtilis* and/or *T. harzianum* gave significantly the highest clusters parameters and grapevine yield, where the No. of cluster / tree; cluster weight (gm); yield/tree (kg) and yield/feddan reached to 10.0 & 10.5 and 8.50 & 9.00; 1120.0 & 1155.0 g and 1125.0 &1130.0g; 11.0 & 12.13 and 9.675 &10.17 kg and 8.960 & 9.702 and 7.740 & 8.135 ton/fed., during the two growing seasons, respectively. The combination treatments of soil drench in root region + soil drench in trunk region with *T. viride* occupied significantly the second degree in the

enhancement of clusters parameters and grapevine yield. It was observed also that the combination treatment of soil drench in root region + soil drench in trunk region with the desired biocontrol agents was significantly the best treatments in enhancement of clusters parameters and grapevine yield followed by soil drench in root region of grapevine only and soil drench in trunk region of grapevine only, respectively.

Discussion

Grapevine plants, grown under field conditions, highly infected with root-rot disease. Omer,1999; Ziedan, 2003; Ziedan *et al*. (2005); Ziedan and El-Mohamedy (2008) and Ziedan *et al.* (2010 and 2011) reported that *Fusarium solani* (Mart.) App. & Wr.; *Macrophomina phaseolina* (Maubl) Ashby and *Rhizoctonia solani* Kühn, are the main pathogens causing root-rot of grapevine.

Results of the present study *in vitro*, revealed that different biocontrol agents isolated from grapevine rhizosphere, *i.e. B. subtilis, T. harzianum* and *T.viride* showed antagonistic potential against root-rot pathogens. These results are in agreement with those recorded by Scherwinski *et al.* (2008), who reported that biocontrol strains can be isolated from rhizospheric soil. The rhizosphere is a region populated by several beneficial microorganisms and is thought to be a region of first line of defense for roots against attack by pathogenic fungi. In this regards, Chakraborty and Chatterjee (2007) found that *T. harzianum* strains produces chitinase protein which showed clear hyphal lysis in *in vitro* observations. Also, *B. subtilis* showed inhibition zones against *R. solani, Colletotrichum truncatum, S. sclerotium, M. phaseolina, Phomopsis* spp., *P. aphanidermatum*, *F. verticilloides, F. equiseti, F. solani, F. oxysporum* and *F. oxysporum* f.sp. *lycopersici* under *in vitro* conditions (Devi *et al*., 2011and Vethavalli & Sudha 2012).

The present study showed that field application with each of *T. harzianum, T. viride* and *B. subtilis* reduced the percentage and severity of grapevine root-rot. The combination technique of soil drench in root region + soil drench in trunk region showed more management efficacy than the individual treatment. This may be due to the high inoculum of the desired biocontrol agent in the root region resulting in a reduction in invaded grapevine roots with root-rot pathogens. The inhibitory effect of biocontrol agents might be related mainly to the antagonistic properties, which involve parasitism and lysis of pathogenic fungi and/or competition for limiting factors in the rhizosphere, mainly iron and carbon (Velazhahan *et al*., 1999 and Wahyudi *et al.*, 2011). Recently however, another possible mechanism has been suggested by Baraka *et al*. (2012) namely, induced resistance in plants to soilborne fungal attack.

Singh *et al*. (2008) and Vethavalli & Sudha (2012) reported that *Bacillus subtilis* isolates exhibited strong antagonistic activity against *M. phaseolina* and other phytopathogens including *F. solani* and *R. solani*. Archana *et al*. (2010) recorded that 38% of *B. subtilis* isolates showed competitive activity against *Fusarium oxysporum*. On the other hand, Chakraborty and Chatterjee (2007) and Tran (2010) reported that *T. viride*, *T. harzianum*, *T. hamatum* had ability to suppress growth of fungal plant pathogens including: *M. phaseolina F. solani* and *R. solani* on several crops.

In the present study, field application with each of *T. harzianum , T. viride* and *B. subtilis* promote clusters parameters and grapevine yield. Growth promotion resulted by the biocontrol agents isolates may be due to thier PGPB effect. Plant growth-promoting bacteria (PGPB) are defined as free-living soil, rhizosphere, rhizoplane, and phylosphere bacteria that are beneficial for plants under some conditions. PGPB promote plant growth in two different ways. First, they directly affect the metabolism of the plants by providing substances that are usually in short supply. These bacteria are capable of fixing atmospheric nitrogen or solubilizing phosphorus and iron and producing plant hormones such as auxins, gibberellins, cytokinins, indole acetic acid (IAA) and ethylene. Additionally, they improve the stress tolerance in plants such as drought, high salinity, metal toxicity, and pesticide load. A second group of PGPB, referred to biocontrol PGPB, indirectly promote plant growth by preventing the deleterious effects of phytopathogenic microorganisms. They produce substances (siderophores, β-1,3-glucanases, chitinases, antibiotics and cyanide) that harm or inhibit other microbes but not plants, by limiting the availability of iron to pathogens (production of siderophores) or by altering the metabolism of the host plant to increase its resistance to pathogen infection (Khalid *et al*., 2004). Fungal biocontrol agents enhanced the plant growth parameters by plant growth-promoting (PGPF) effects. *Trichoderma* spp. influence plant growth through numerous mechanisms, which mainly include enhancing the solubilization of soil nutrients (Kapri and Tewari 2010), increasing root length and number of root hairs to explore larger spaces of soil to absorb nutrients (Samolski *et al.*, 2012) and improving the production of plant stimulatory compounds, such as growth hormones, *i.e.* indole acetic acid, cytokinin, gibberellins, and zeatin (Contreras *et al*., 2009). Promotion of plant growth by *Trichoderma* spp. may also result from their secretion of antifungal substances, such as iron chelators (siderophore) and hydrogen cyanide (Ushamailini *et al.*, 2008), which can protect plants from infection by phytopathogens.

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