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Compatibility between Antagonistic Fungi and Bacteria and their Influence in Controlling Sunflower Charcoal Rot

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Trichoderma harzianum, *T. viride* antagonistic fungi compatible with the antagonistic *Pseudomonas fluorscens*, *P. putida* and *Basillus subtilis* were evaluated as biocontrol agents against *Macrophomina phaseolina*, the causative pathogen of sunflower charcoal rot. No adverse interaction could be recognized among the tested *Trichoderma* isolates and both *P. fluorescens* and *B. subtilis*, in *In vitro* studies. The highest antagonistic effect against *M. Phaseolina* growth was revealed by *B. subtilis* as shown by decrease in mycelia growth and reduction in sclerotia numbers. Similar effect was recorded for *T. viride* and *P. fluorscens*. In greenhouse and field experiments *T. viride* and *P. fluorscens* and *B. subtilis*, along with the biocide (Rhizo-N) and fungicide (Rizolex-T) decreased the disease incidence. In this respect, *T. viride* mixed with either *B. subtilis* or *P. fluorscens* revealed greater effect in disease control compared to the single application by any alone, especially treatment with mixture of *T. Viride* and *B. subtilis*. The effect of the later showed disease control approximately similar to that of Rizolex-T treatments, as shown by healthy survivals and seed yield over two years study.

Keywords: *Basillus subtilis,* bioagents, biocides, biocontrol, *Macrophomina phaseolina, Pseudomonas fluorscens, P. putida, Trichoderma harzianum* and *T. viride.*

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops in the world. In Egypt, there is a great effort to increase the devoted area for its cultivation to increase the local production of edible oil (Anonymous, 2006).

Sunflower is attacked by many pathogens, which cause great losses in yield and quality (Ibrahim, 2006). *Macrophomina phaseolina* (Tassi) Goidanich is an important soil borne pathogen with an exceptionally broad host range that includes over 500 species of monocots and dicots (Mihail, 1992). In Egypt, *M. phaseolina* has been reported more frequently from sunflower, inducing the charcoal rot disease (El-Deeb *et al*., 1985, Sadik and Fayzalla, 1989 and Ibrahim, 2006). The early-infection reported to reduce the yield by 50% (Hilal, 1981). Thus causing negative effects on seed quality in terms of the oil content, fat, protein and ash (El-Deeb *et al*., 1985 and Ibrahim, 2006).

Fungicidal application causes enormous environmental hazards to human health, thus the eco-friendly approaches for plant diseases control because essential and tried as biological control (Cook, 1993).

In general, fungal antagonists depend mainly on physical contacts with their pathogen while, bacteria mainly use antimicrobial agents as weapon for killing of the pathogens (Howell, 2003 and Mohiddin *et al*., 2010).

Most of the studies on biological control of plant pathogens deal with single biocontrol agent as antagonist to a single pathogen. Considering the fact that there is some degree of host-specificity in biocontrol agents even at subspecies level, this may partially account for the reported inconsistent performance of biocontrol agent preparations. Single biocontrol agent is not likely to be active in all soil environments. Mixtures of antagonists are considered to account for protection in disease suppressive soils (Bin *et al*., 1991). Consequently, application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of biological control (Mishra *et al*., 2011 and 2013). Previous studies on combinations of biocontrol agents for plant diseases have included mixtures of fungi (Datnoff *et al*., 1995), mixtures of fungi and bacteria (Hassan *et al*., 1997 and Mishra *et al*., 2013), and mixtures of bacteria (Raupach and Kloepper, 1998).

The present study was conducted to evaluate the effect of mixing compatible efficient antagonists of fungi (Trichoderma) and bacteria (Pseudomonas & Bacillus) agents and testing their efficacy against charcoal rot disease on sunflower.

Materials and Methods

1. Source of fungal isolates:

The fungal isolates used throughout this study were previously isolated by the authors from diseased sesame and sunflower roots and their pathogenic capabilities were also confirmed (Ibrahim *et al.,* 2006 and Mahmoud *et al.,* 2009).

2. Preparation of fungal inoculum:

Inocula of *Macrophomina phaseolina*, were prepared using sorghum-coarse sand -water (2:1:2 v/v) medium. The medium was inoculated using agar discs, obtained from the periphery of 5-day-old colony of each of the tested fungi. The inoculated bottles were incubated at 28°C for 15 days.

3. Soil Infestation:

Inoculum of *M. phaseolina*, was mixed thoroughly with potted soil surface at the rate of 2% (w/w), and was covered with a thin layer of sterilized soil. The infested soil were irrigated and kept for 7 days before sowing.

4. Disease assessment

Disease assessment was made 15 and 45 days after planting for pre- and post-emergence damping-off, respectively. The percentage of charcoal rot was estimated, at harvest,(90 days after sowing).

5. The sources of biocontrol agents tested:

Two known isolates of *Pseudomonas fluorescens*; Pf5 (Howell and Stipanovic, 1979), *P. putida;* PP and *Basillus subtilis*; Bs1 (El-Hadidy, 2003) were obtained from Culture Collection, Plant Pathol. Dept., Fac. Agric., Ain Shams Univ. Egypt. Their efficacy against *M. phaseolina* was tested by Mahmoud (2014). However, the

tested bioagents included antagonistic fungal isolates, *i.e. T. viride* and *T. harzianum*, were obtained from Onion, Garlic and Oil Crops Dis. Res. Dept., Plant Pathol. Res. Inst., Agric. Res. Centre, Giza, Egypt.

6. Testing of compatibility of fungal and bacterial biocontrol agents:

The method described by Nikam *et al.* (2007) with slight modifications was used for *in-vitro* testing. Sterilized filter paper (Whatman paper No.1) discs(5mm) impregnated with bacterial suspension containing 10^6cfu/ ml(prepared in 0.1M $MgSO_4$) of individual isolates were placed at 5 mm apart from one side of a Petri plate filled with the PDA growth media. The bacterial isolates were allowed to grow for 24 hr at 26±2°C. A 5 mm diameter plug from a 5-day-old culture of *Trichoderma* isolate was placed in the opposite side of the plate. After 5 days incubation at 26±2°C the zone of inhibition, if any, was estimated. Three replications were considered for each treatment.

7. Evaluation of biocontrol agents in vitro:

7.1. Effect of antagonistic fungi:

Two discs (5-mm-diam.) of plain agar culture (4-day-old) of both antagonistic fungi and *M. phaseolina* were placed in opposite to each other 1 cm apart from the dish edge (9-cm-diam.) containing 10 ml PDA medium. The dishes only inoculated with the mycelial growth of *M. phaseolina* were served as control treatment. Four replicates were used for each particular treatment, then incubated at 26±2°C for 5-7 days. Percentage of the fungal growth reduction (X) was calculated using the following formula:

$$X = (G1-G2 / G1) \times 100$$

Whereas: G1: linear growth of the pathogen inoculated alone (control treatment).
G2: linear growth of the pathogen inoculated against the antagonistic fungus.

7.2. Effect of antagonistic bacteria:

Bacillus subtilis and *Pseudomonas fluorescens* antagonists were tested in this study. Plats of PDA medium were streaked 1 cm apart at one side of the dish edge with a given antagonistic bacteria and incubated for 24 hrs at 26±2°C. Then, the same plate was inoculated at the opposite side, 1 cm apart from the dish edge, with a disc(5-mm-diam.) of *M. Phaseolina* of 4-day-old plain agar culture. Plates inoculated with one disc of mycelial growth of *M. phaseolina* in the absence of bacteria were prepared as control. The inhibition zone between bacteria and the pathogen was measured as described by Maurhofer *et al.* (1995). Sclerotial formation was determined after 14 days by counting average number of sclerotia in at least 4 microscopic fields (10x).

8. Preparation inocula of biocontrol agents:

Bacterial suspensions ($1x10^6$cfu/ml) were prepared by dilution plate assay as described by Callan *et al.* (1990). The tested antagonistic fungal isolates were prepared as adjusted suspension with approx. $5x10^8$ condia/ml as described by Khalifa (2003).

9. Methods of application:

The biocontrol agents were applied either alone or mixed before sowing blended with 0.1% Arabic gum as a sticker and fungicide Rhizolex-T 50% (Tolclofos-methyl 20% + thiram 30%) and, Rhizo-N (*Bacillus subtilis* $3x10^6$ cell/gm) were applied on sunflower seeds at the rate of 5 ml and 3g and, 5g/Kg seeds respectively .

10. Evaluation of biocontrol agents under greenhouse conditions:

Pot experiments were carried out during 2013 season in order to study the effect of biocontrol agents in controlling charcoal rot incidence (%). The experiment was carried out at Agric. Res. Centre, Giza. Sunflower var. (Giza102) seeds coated with the biocontrol agents, were sown in 50 cm-diameter pots containing sterilized soil previously infested with *M. phaseolina* (2% w/w). Ten seeds were sown in each pot and five replicates (pots) were used for each treatment. Disease assessment was recorded as a percentage of pre-, post-emergence along with the percentage of charcoal rot incidence.

11. Evaluation of biocontrol agents under field conditions:

The field experiments were performed at Nubaria, during 2014 and 2015 growing seasons to study the effect biocontrol agents in controlling charcoal rot incidence. The selected fields had aback histort of natural infestation with charcoal rot pathogens. The biocontrol agents were applied as seed dressing at sowing and were foliar sprayed after 15 days. Seeds were sown on the first week of April with 10 cm spacing between hills. Cultural practices and fertilization for the sunflower crop were applied as recommended. The fungicide Rhizolex-T50% was applied as previously mentioned under the experimental unit area was 21 m^2 (1/200 fed.).The treatments were arranged in completely randomized block design with four replicates. Disease assessment was recorded after days of sowing as percentage of charcoal rotted plants. In addition, the effect of biocontrol agents on crop yield was determined at the end of the experiment as seed yield(kg/plot)

12. Statistical analysis:

The data were statistically analysed by analysis of variance (ANOVA) using the Statistical Analysis System (Anonymous, 1996). Means were separated by least significantdifference(LSD) test at $P \leq 0.05$ levels.

Results

1. Compatibility of fungal and bacterial biocontrol agents:

Table (l) shows that the compatibility of Trichoderma isolates with bacterial biocontrol agents varied greatly according to the isolates. In this respect,*T. harzianum* isolates Th1, Th2 and *T . viridy* isolates Th6, Tv1, Tv3, Tv4 and Tv5, were compatible andexhibited no antagonistic interaction against *P. fluorescens* (Pf5). Moreover, isolates Th1, Th3, Th5, Th6, Tv3, Tv4 and Tv5, showed similar trend with *B. subtilis* (Bs1). Only Tv2 and Tv6 gave a little antagonism against *P. putida* (PP).Based on these results, Trichoderma (Th1,Th6, Tv3, Tv4 and Tv5), fluorescent pseudomonads (Pf5) and *B. subtilis*(Bs1) were evaluated *in vitro* for their antagonistic potential against *M. phaseolina* (Table 2).

Table 1. Test of compatibility of biocontrol agents

Biocontrol agent	*P. fluorescens* (Pf5)	*P. putida* (PP)	*B. subtilis* (Bs1)
Trichoderma harzianum (Th1)	+	-	+
Trichoderma harzianum (Th2)	+	-	-
Trichoderma harzianum (Th3)	-	-	+
Trichoderma harzianum (Th4)	-	-	-
Trichoderma harzianum (Th5)	-	-	+
Trichoderma harzianum (Th6)	+	-	+
Trichoderma viride (Tv1)	+	-	-
Trichoderma viride (Tv2)	-	+	-
Trichoderma viride (Tv3)	+	-	+
Trichoderma viride (Tv4)	+	-	+
Trichoderma viride (Tv5)	+	-	+
Trichoderma viride (Tv6)	-	+	-

Compatible= Inhibition zone < 1 mm (+) Non-compatible= Inhibition zone > 1 mm (-)

2. Evaluation of biocontrol control agents in vitro:

Five isolates of *Trichoderma* (Th1, Th6, Tv3, Tv4 and Tv5) in addition two bacteria (*B. subtilis* and *P. fluorescens*) were evaluated *in vitro* for their antagonistic effect against *M. phaseolina* (Table 2). *B.subtilis* (Bs1) gave the high significant antagonistic effect against the tested pathogen whether on growth reduction or number of sclerotial formation followed by *T. viride* (Tv 3) and *P. fluorscens* (Pf5) in comparison with control treatment. In this regard,*T. harzianum* (Th6) and *T. harzianum* (Th1) gave moderate effect in their inhibition of tested pathogen growth and sclerotial formation while, both of *T. viride* (Tv4) and *T. viride* (Tv5) had a little effect.

Table 2. Antagonistic effect of biocontrol agents on the percentage of linear growth reduction and number of sclerotial formation of *M. phaseolina*

Biocontrol agent	*Macrophomina phaseolina*	
	Growth reduction (%)	No. of sclerotia
Trichoderma harzianum (Th1)	14.25	50
Trichoderma harzianum (Th6)	15.5	54
Trichoderma viride (Tv3)	20.25	44
Trichoderma viride (Tv4)	9.25	52
Trichoderma viride (Tv5)	10.5	59
Basillus subtilis(Bs1)	23.25	39
Pseudomonas fluorscens (Pf5)	18.5	47
Control	--	72
L.S.D. 5%	1.45	3.34

3. Evaluation of biocontrol agents under greenhouse conditions:

One selected fungal isolate and two bacterial isolates beside standard consisting of Rhizo-N (biocide) and Rizolex-T (fungicide) were evaluated for charcoal rot control under greenhouse conditions. Table (3) shows that all tested biocontrol agents and their mixture had significant effect in reducing damping-off and sunflower charcoal rot compared to the control. *Trichoderma viride*(Tv3) alone was superior over *B. subtilis* and *P. fluorscens* in reducing of damping-off and charcoal rot incidence, *T. viride*(Tv3) mixed with *B. subtilis* gave better effect in reducing of damping-off and charcoal rot compared to results of mixing with *P. fluorscens*. Data also showed that, the mixture of *T. Viride*(Tv3) and *B. subtilis* was the nearest one to Rizolex-T effect in reduction of damping-off and charcoal rot and comparatively superior over the biocides Rhizo-N effect (Table3).

Table 3. Effect of biocontrol agents on damping-off and charcoal rot incidence on sunflower seedlings under greenhouse conditions

Biocontrol agent	Disease incidence (%)			
	damping-off		Charcoal rotted plants	Survived healthy plants
	Pre-emergence	Post-emergence		
Trichoderma viride (Tv3) A	8	6	14	72
Basillus subtilis B	6	8	16	70
Pseudomonas fluorscens C	8	10	20	62
A+B	6	8	10	76
A+C	8	10	18	64
B+C	10	10	20	60
A+B+C	8	10	24	58
Rhizo-N	6	6	14	74
Rizolex-T 50%	4	6	10	80
Control	22	18	28	32
L.S.D at 5%:	1.73	1.94	2.89	3.86

4. Evaluation of biocontrol agents under field conditions:

Data in Table (4) indicate that, all tested biocontrol agents and their mixtures had significant effect in reducing charcoal rot incidence during the two successive seasons, 2014 and 2015. In general *T. viride* (Tv3) showed greater influence in reducing the diseases either as single or as mixed application. Moreover, *T. viride*(Tv3) mixed with *B. subtilis* recorded the highest effect in reducing percentage of charcoal rotted plants compared with that being mixed with *P. fluorscens* along with increasing the percentage of healthy plants. The mixture of *T. viride* and *B. subtilis* also showed similar effect to that of Rizolex-T in reducing percentage of charcoal rot and was superior over biocides Rhizo-N effect during 2014 and 2015 (Table4).

5. Effect of biocontrol agent on sunflower seed yield under field conditions:

Data presented in Table (5) demonstrate that all tested biocontrol agents either single or in combination caused a significant increases in seed yield. Percentage of increases however ranged 26-56% and 10-51% in the first and second seasons respectively. The highest seed yield in the two seasons obtained when *T. viride* (Tv3) mixed with *B. subtills* (Bs1), followed by *T. viride* (Tv3) alone compared with other biocontrol agents. While Rizolex-T followed by Rhizo-N gave the highest seed yield/plot in the two successive seasons 2014 and 2015

Table 4. Effect of biocontrol agents on charcoal rot incidence on sunflower seedlings under field conditions

Biocontrol agent	Disease incidence (%)			
	Season 2014		Season 2015	
	Charcoal rotted plants	Survived healthy plants	Charcoal rotted plants	Survived healthy plants
Trichoderma viride (Tv3) A	16.9	83.1	17.2	82.8
Basillus subtilis B	17.6	82.4	18.2	81.8
Pseudomonas fluorscens C	21.5	78.5	21.5	78.5
A+B	14.9	85.1	16.1	83.9
A+C	18.9	81.1	20.5	79.5
B+C	21.6	78.4	21.6	78.4
A+B+C	21.7	78.3	22.6	77.4
Rhizo-N	18.3	81.7	17.5	82.5
Rizolex-T50%	13.1	86.9	15.2	84.8
Control	39.8	60.2	42.2	57.8
L.S.D at 5%:	1.96	2.05	1.63	1.89

Table 5. Effect of biocontrol agents on sunflower seed yield under field conditions

Biocontrol agent	Disease incidence (%)			
	Season 2014		Season 2015	
	Total seed yield (Kg/plot)	Increase of yield (%)	Total seed yield (Kg/plot)	Increase of yield (%)
Trichoderma viride (Tv3) A	2.28	36.00	1.99	25.00
Basillus subtilis -B	2.23	31.00	1.90	16.00
Pseudomonas fluorscens C	2.18	26.00	1.84	10.00
A+B	2.38	46.00	2.12	38.00
A+C	2.30	38.00	2.00	26.00
B+C	2.25	33.00	1.97	23.00
A+B+C	2.20	28.00	1.86	12.00
Rhizo-N	2.40	48.00	2.14	40.00
Rizolex-T50%	2.48	56.00	2.25	51.00
Control	1.92	0.00	1.74	0.00
L.S.D at 5%:	0.28		0.21	

On the other hand, the mixture of *Trichoderma viride*(Tv3) and *B. subtilis* was the nearest biocontrol agent to fungicides (Rizolex-T) effect in increasing of seed yield in the two successive seasons 2014 and 2015 compared with other biocontrol agents (Table 5).

Discussion

Biological control of plant pathogens by microorganisms decrease use effect of fungicides hazardous to humans and environment (Cook and Baker, 1983).

In the present study results indicated that the tested biocontrol agents and biocides, as well as, Rhizolex fungicide significantly reduced damping-off and charcoal rot disease of sunflower plants. In this respect, *Bacillus subtilis* (Bs1) followed by *Trichoderma viride*(Tv3) and *Pseudomonas fluorescens* (Pf5)gave the highest significant antagonistic effect against the tested pathogen growth and reduction in number of sclerotia formation. These results are in harmony with those reported by Ibrahim, (2006), Khalifa *et al*. (2007), Ullah *et al.* (2011), Mishra *et al.* (2013) and Reetha *et al.* (2014). Positive antagonism of fungal and bacterial bioagents against *M. Phaseolina* and their respective diseases on several crops including sunflower was recorded. Sreedevi *et al*. (2011), Ullah *et al.* (2011), Reetha *et al.* (2014) and Imarah (2015), recorded significant reductions in charcoal rot of sunflower seed treatments with antagonistic *T. virid* and *T. harzianum* fungi. Moreover, Karunanithi, *et al*. (2000), Ibrahim *et al.* (2008), Mahmoud (2014) and Imarah (2015), found that, *B. subtilis* and *P. fluorescens* caused strong reduction in poth growth and infection by *M. phaseolina in vitro* and *in vivo*.

These results strengthen the opinion that control with fungicides can be partially replaced by biological control because biocides effectively protected the roots and stem of sunflower plants from infection by *M. phaseolina* (Ullah *et al.*, 2011). The attractive influence of mixing *T. viride* and *B. subtilis* in decreasing charcoal rot compared to the fungicide Rizolex-T effect, as well as the increase of seed yield and the superior effect over biocides Rhizo-N in greenhouse experiment or in field experiments during the two seasons 2014 and 2015 were obtained in this study.

The present study is in the line of the studies carried out by many workers, where they have reported that increased biocontrol activity might be achieved by combining different isolates of biocontrol agents (Duffy *et al.,* l996; Raupach and Kloepper, l998). The feasibility of combining *Trichoderma* spp. with *pseudomonads fluorescent* initially was questioned by Hubbard *et al.* (l983). They reported that indigenous populations of fluorescent pseudomonads significantly reduced the biocontrol activity of *T. hamatum* applied to control Pythium seed rot of pea and iron competition was the primary mechanism involved. In contrast, Dandurand and Knudsen (l993) reported that the combination of *P. fluorescens* (2-79) and *T. harzianum* neither inhibited nor enhanced the biocontrol activity of the latter agent against root rot of pea caused by *Aphanomyces euteiches* f.sp. *pisi*. Further, Duffy *et al.* (1996) indicated that *P. fluorescent* species and *T. koningii* are compatible when

applied to wheat simultaneously. The performance of all bacterial treatments was greatly enhanced by combination with *T. koningii*, suggesting that the fungus was largely responsible for the take-all suppression. Similarly in field, the bacteria did not adversely affect the activity of *T. koningii*. Appliance of combination of compatible bio-control agents possessing differential mechanisms of pathogen suppression is suggested as a reliable and potential means of disease suppression. Mishra *et al.,* (2013) in their study clearly indicated that, application of compatible mixture of fungi and bacterial biocontrol agents possessing various mechanism of pathogen suppression is suggested as a reliable and potential means of disease suppression high potentiality of mixed formulation of fungal (*T. harzianum*) and bacterial (fluorescent pseudomonads) biocontrol agents against economically important plant diseases.

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التوافق بين الفطريات والبكتريا المضادة ومدي تاثيرها فى مقاومة مرض العفن الفحمى عل دوار الشمس

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تم تقييم العزلات الفطرية المضادة *Trichoderma harzianum* ، *T. viride* والمتوافقة مع عزلات البكتريا المضادة *Pseudomonas putida* و *Pseudomonas fluorescens* و*Bacillus subtilis* كعوامل مقاومة حيوية لفطر *Macrophomina phaseolina* المسبب لمرضى للعفن الفحمي على نباتات دوار الشمس. معمليا لم تسجل بعض عزلات فطر *Trichoderma* أى تأثيرعكسى مع عزلات البكتيريا *P. fluorescens* و*B. Subtilis*. فى تجارب الصوبة و الحقل و كان اعلى تأثير مضاد على نمو الفطر *Macrophomina phaseolina* كان متحصل عليه باستخدام *B. subtilis* حيث ظهر ذلك باحداث نقص فى النمو الميسليومى للفطر وكذلك عدد الاجسام الحجرية المتكونة . هذا وقد تم الحصول على تأثير مشابه عند استخدام *T. Viride* و *Pseudomonas fluorescens* وسجل كل من *T. viride* و *P. fluorescens* و *B. subtilis* الى جانب المبيد الحيوى Rhizo-N و المبيد الكيميائى Rizolex-T نقصا فى حدوث المرض. و فى هذا الصدد كان المخلوط من *T. viride* و*P. fluorescens* او من *T. viride* و *B. subtilis* لهما تأثير عالى فى مقاومة الفطر بالمقارنة بتطبيق اى منهما بمفرده. كما اظهرت المعاملات السابق ذكرها فى المقاومة تشابها كبيرا مع قدرة المبيد الفطرى Rizolex-T على مقاومة المرض و كذلك زيادة عدد النباتات السليمة ومحصول البذور خلال موسمى الزراعة (2014و 2015).