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

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Trichoderma *harzianum* and *T. viride* as well as *Pseudomonas fluorescens* , *P. putida* and *Bacillus subtilis* were evaluated as bioagents against *Macrophomina phaseolina*, the causative pathogen of sunflower charcoal rot. No *in vitro* adverse interaction could be recognized among the tested Trichoderma isolates and both *P. fluorescens* and *B. subtilis*. The highest antagonistic effect against *M. phaseolina* was recorded by *B. subtilis* as decrements in mycelial growth and sclerotial numbers. Similar effect was recorded for *T. viride* and *P. fluorescens*. In greenhouse and field experiments, *T. viride* and *P. fluorescens* 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. fluorescens* revealed greater effect in disease control compared to the single application of any of them, especially with mixed *B. subtilis* treatment. The effect of the later showed disease control approximately similar to that of Rizolex-T treatment, as shown by healthy survivals and seed yield over two years study.

Keywords: *Bacillus subtilis*, charcoal rot, *Macrophomina phaseolina*, *Pseudomonas* spp., *Trichoderma* spp. and sunflower.

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.

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 bioagent 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 bioagent preparations. Single bioagent 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 bioagents 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 mixed 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. Isolation of causal organisms:

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.,* 2008 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 time (90 days after sowing).

5. Source of tested bioagents:

Two isolates of *Pseudomonas fluorescens* (Pf5) (Howell and Stipanovic, 1979) and *P. putida* (PP) as well as one isolate of *Bacillus subtilis* (Bs1) (El-Hadidy, 2003) were obtained from Culture Collection, Plant Pathol. Dept., Fac. Agric., Ain Shams Univ., Egypt, and 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 bioagents:

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

7. In vitro evaluation of bioagents:

7.1. Evaluation of antagonistic fungi:

Two discs (5-mm-diam.) of plain agar culture of both antagonistic fungi and *M. phaseolina* (4-day-old) were inoculated in opposite to each other 1 cm apart from the dish edge (9-cm-diam.) containing 10 ml PDA medium. The dishes were inoculated with one disc of mycelial growth of *M. phaseolina* in control treatment. Four replicates were used for each particular treatment and then incubated at 26±2ºC 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. Evaluation of antagonistic bacteria:

Bacillus subtilis and *P. fluorescence* 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 24hr at 26±2ºC. Then, the same plate was inoculated at the opposite side, 1 cm apart from the dish edge, with a disc (5-mm-diam.) of 4-day-old *M. phaseolina* plain agar culture. Control plates were inoculated with one disc of *M. phaseolina* mycelial growth in the absence of bacteria. The inhibition zone between bacteria and the pathogen was measured as described by Maurhofer *et al.* (1995). Sclerotial formation was determined 14 days after incubation by counting average number of formed sclerotia in at least 4 microscopic fields (X10).

8. Preparation of bioagents:

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

9. Methods of application:

Tested bioagents were applied either alone or mixed with before sowing with 0.1% Arabic gum as a sticker with sunflower seeds (5 ml/kg seed). While fungicide Rizolex-T 50% (Tolclofos-methyl 20% + thiram 30%) were applied as seed treatment at the rate of 3g/kg seed and Rhizo-N (*B. subtilis* $3x10^6$ cell/gm) were applied as seed treatment at the rate of 5g/kg seed.

10. Evaluation of bioagents under greenhouse conditions:

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

11. Evaluation of bioagents under field conditions:

The field experiments were performed at Nubariya, during 2014 and 2015 seasons to determine the effect of bioagents in controlling charcoal rot incidence. The selected fields considered were known to have natural infestation with charcoal rot pathogens. Tested bioagents 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 as mentioned before.

12. Statistical analysis:

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

Results

1. Compatibility of fungal and bacterial bioagents:

Table (l) shows that the compatibility of Trichoderma isolates with bacterial agents varied greatly according to the isolates. In this respect, Th 1, Th 2, Th 6**,** Tv 1, Tv 3, Tv 4 and Tv 5, were compatible and exhibited no antagonistic interaction against *P. fluorescens* (Pf5). Moreover, Th1, Th 3, Th 5, Th 6**,** Tv 3, Tv 4 and Tv 5, showed similar trend with *B. subtilis* (Bs1). Only Tv 2 and Tv 6 gave a little antagonism against *P. putida* (PP). Based on these results, Trichoderma (Th 1, Th 6**,** Tv 3, Tv 4 and Tv 5), fluorescent pseudomonads (Pf5) and *B. subtilis* (Bs1) were *in vitro* evaluated for their antagonistic potential against *M. phaseolina* (Table 2).

2. In vitro screening of bioagent potential:

Five isolates of Trichoderma (Th 1, Th 6**,** Tv 3, Tv 4 and Tv 5) as well as two bacteria (*B. subtilis* and *P. fluorescens*) were *in vitro* evaluated for their antagonistic effect against *M. phaseolina* (Table 2). *Bacillus 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. fluorescens* (Pf5). In this regard, *T. harzianum* (Th 6) and *T. harzianum* (Th 1) gave moderate effect in their inhibition of tested pathogen growth and sclerotial formation. While, both of *T. viride* (Tv 4) and *T. viride* (Tv 5) had a little effect.

Table 1. Compatibility the tested bioagents

Tested bioagent	*P. fluorescens* (Pf5)*	*P. putida* (PP)	*B. subtilis* (Bs1)
T. harzianum (Th 1)	+	-	+
T. harzianum (Th 2)	+	-	-
T. harzianum (Th 3)	-	-	+
T. harzianum (Th 4)	-	-	-
T. harzianum (Th 5)	-	-	+
T. harzianum (Th 6)	+	-	+
T. viride (Tv 1)	+	-	-
T. viride (Tv 2)	-	+	-
T. viride (Tv 3)	+	-	+
T. viride (Tv 4)	+	-	+
T. viride (Tv 5)	+	-	+
T. viride (Tv 6)	-	+	-

* (+)= Compatible (Inhibition zone ≤ 1 mm).
(-)= Un-compatible (Inhibition zone > 1 mm).

Table 2. Antagonistic effect of bioagents on the linear growth reduction (%) and number of sclerotial formation of *M. phaseolina*

Tested bioagent	*Macrophomina phaseolina*	
	Growth reduction (%)	No. of sclerotia
T. harzianum (Th 1)	14.3	50
T. harzianum (Th 6)	15.5	54
T. viride (Tv 3)	20.3	44
T. viride (Tv 4)	9.3	52
T. viride (Tv 5)	10.5	59
B. subtilis (Bs1)	23.3	39
P. fluorescens (Pf5)	18.5	47
Control	----	72
L.S.D. 5%	1.45	3.34

3. Evaluation of bioagents under greenhouse conditions:

One selected fungal isolate and two bacterial ones beside standard consisting of Rhizo-N (biocide) and Rizolex-T (fungicide) were evaluated for charcoal rot control under greenhouse conditions. Data in Table (3) show that all tested bioagents, either individual or in mixture, had significant effect in reducing damping-off and sunflower charcoal rot compared to the control. *Trichoderma viride* alone was superior over *B. subtilis* and *P. fluorescens* in reducing of damping-off and charcoal rot incidence, and when mixed with *B. subtilis* gave better effect in reducing damping-off and charcoal rot compared to results of mixing with *P. fluorescens*. Data also show that the mixture of *T. viride* 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.

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

Tested bioagent	Disease incidence (%)			
	damping-off		Charcoal rotted plants	Survived healthy plants
	Pre-emergence	Post-emergence		
T. viride (Tv3) A	8	6	14	72
B. subtilis B	6	8	16	70
P. fluorescens 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 bioagents under field conditions:

Data in Table (4) indicate that, all tested bioagents and their mixtures had significant effect in reducing of damping-off and charcoal rot incidence during the two successive seasons of 2014 and 2015.

In general *T. viride* (Tv3) showed greater influence of tested bioagents, either individual or in mixture application, in reducing the diseases. Moreover, *T. viride* mixed with *B. subtilis* recorded the highest effect in reducing damping-off and charcoal rot compared with that being mixed with *P. fluorescens* along with increasing the number of survived plants. The mixture of *T. viride* and *B. subtilis* also showed similar effect to that of Rizolex-T in reducing damping-off and charcoal rot and was superior over biocides Rhizo-N effect during 2014 and 2015 (Table4).

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

Data presented in Table (5) demonstrate that all tested bioagents, either individual or in combination, caused significant increases in seed yield. However, yield increase (%) reached (26-56%) and (10-51%) in the first and second seasons, respectively.

The highest seed yield in the two seasons obtained with mixed *T. viride* (Tv 3) and *B. subtilis* (Bs1), followed by *T. viride* (Tv 3) alone compared with other tested bioagents. While, Rizolex-T followed by Rhizo-N gave the highest seed yield at all as well as their effect on increase yield in the two successive seasons 2014 and 2015 compared with other treatments. On the other hand, mixture of *T. viride* and *B. subtilis* recorded the nearest effect as the fungicides (Rizolex-T) on seed yield increment in the two successive seasons of 2014 and 2015 compared to the other tested bioagents (Table 5).

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

Tested bioagent	Disease incidence (%)			
	Season 2014		Season 2015	
	Charcoal rotted plants	Survived healthy plants	Charcoal rotted plants	Survived healthy plants
T. viride (Tv 3) A	16.9	83.1	17.2	82.8
B. subtilis B	17.6	82.4	18.2	81.8
P. fluorescens 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 bioagents on sunflower seed yield under field conditions

Tested bioagent	Disease incidence (%)			
	Season 2014		Season 2015	
	Total seed yield (kg/plot)	Yield increase (%)	Total seed yield (kg/plot)	Yield increase (%)
T. viride (Tv 3) A	2.28	36.00	1.99	25.00
B. subtilis B	2.23	31.00	1.90	16.00
P. fluorescens 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	

D i s c u s s i o n

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 bioagents and biocides, as well as, Rizolex fungicide significantly reduced damping-off and charcoal rot disease on 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 treated with antagonistic fungi, *i.e. T. viride* and *T. harzianum.* Moreover, Karunanithi *et al*. (2000); Ibrahim *et al.* (2008); Mahmoud (2014) and Imarah (2015) found that *B. subtilis* and *P. fluorescens* caused strong reduction on *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 with *M. phaseolina* (Ullah *et al.*, 2011). The attractive influence of mixing *T. viride* and *B. subtilis* in decreasing charcoal rot compared to the fungicides 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.

The present study are in agreement with the studies conducted by different workers, where they have reported that increased biocontrol activity might be achieved by combining different isolates of bioagents (Duffy *et al.*, l996 and Raupach and Kloepper, l998). The feasibility of combining Trichoderma spp. with fluorescent pseudomonads 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*. Furthermore, 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 mechanism 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 fungal and bacterial bioagents 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) bioagents against economically important plant diseases.

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

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تم تقييم العزلات الفطرية المضادة *Trichoderma harzianum* ، *T. viride* والمتوافقة مع عزلات البكتريا المضادة *Pseudomonas fluorescens* ، *P. putida* و *Bacillus subtilis* كعوامل مقاومة حيوية لفطر *Macrophomina phaseolina* المسبب لمرض للعفن الفحمي على نباتات دوار الشمس. معمليا لم تسجل بعض عزلات الفطر *Trichoderma* أى تأثير عكسى مع عزلات البكتيريا *P. fluorescens* و *B. Subtilis*. وفى تجارب الصوبة والحقل سجل كل من *T. viride* و *P. fluorescens* و *B. subtilis* الى جانب المبيد الحيوى Rhizo-N والمبيد الكيميائى Rizolex-T اقل نسبة من المرض. وفى هذا الصدد كان المخلوط من *T. viride* و *P. fluorescens* او من *T. viride* و*B. Subtilis* لهما تأير عالى فى مقاومة الفطر بالمقارنة بتطبيق اى منهما بمفرده. كما اظهرت المعاملات السابق ذكرها فى قدرتها على المقاومة تشابه كبير فى قدرة المبيد الفطرى Rizolex-T على مقاومة المرض وكذلك زيادة عدد النباتات السليمة ومحصول البذور خلال موسمى الزراعة .