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BIOEFFICACY OF MICROBIAL ANTAGONISTS AGAINST *MACROPHOMINA PHASEOLINA* ON SUNFLOWER

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Abstract

Seed treatment with biological control agents were found successful in prevention of fungal diseases of crop plants. In the present study, efficacy of microbial antagonists viz., *Aspergillus flavus* Link, *Paecilomyces variotii* Bainier, *Trichoderma viride* Pers., *Rhizobium meliloti* Dangeard and *Bacillus subtilis* Ferdinand Cohn was evaluated for their effect on plant growth promotion and against *Macrophomina phaseolina* (Tassi) Goid., the cause of root rot of sunflower (*Helianthus annus* L.). In dual culture assays, all antagonists inhibited the growth of *M. phaseolina*. *Rhizobium meliloti* and *Bacillus subtilis* showed maximum inhibition in the growth of *M. phaseolina.* Seed treatments with tested antagonists in blotting paper, test tube and pot experiments, did not show any detrimental effect on germination of sunflower seeds. On the other hand, in all the experiments seeds coating with antagonists proved effective in protecting sunflower seeds from root rot and significantly increased in root length and vigor index.

Introduction

Macrophomina phaseolina (Tassi) Goid., is an important root pathogen and causes dry root rot/stem canker, stalk rot or charcoal rot of over 500 plant species including sunflower (Sinclair, 1985, Shahzad *et al*., 1988, Ghaffar, 1992). Various disease management methods have been implemented to combat and eradicate pathogenic fungi. These include cultural, regulatory, physical, chemical and biological methods. All these methods are effective only when employed well in advance as precautionary measure (Sharma, 1996; Kata, 2000). Once a disease has appeared these methods become impractical / ineffective. In that situation, chemical control offers a good choice to grower to control the disease. Chemical pesticides have been in use since long and they provide quick, effective and economic management of plant diseases. Seed treatment with fungicide does not protect the crop for long periods. Soil drenching with fungicides are not economical and they may establish imbalances in the microbial community unfavourable for activities of beneficial organisms (Jeyarajan *et al*., 1991). In addition, continuous use of the same fungicides for the same pathogen results in the development of resistant strains of the pathogens, besides polluting the environment (Muthukrishnan, 1989). Due to increase in cost of chemical pesticides and environmental hazards involved with their application emphasis is now given on the biological control agent against plant pathogens (Agrios, 2004). Increasing awareness of humankind toward the ecosystem and environment has made a marked shift from synthetic materials to bio-products. Biological control is a potential non chemical means for plant disease control by reducing the harmful effects of a pathogen through the use of other living entities (Ramezani, 2008).

Seed treatment promote seedling establishment, help ensure yield and reduce the quality losses due to many diseases and insects. Seed treatment control the fungi residing on the surface of seed or inside the seed and are affective against pathogen that reside in the soil and cause seed rot, damping off and root rots (Martha *et al.*, 2003). The objective of the present studies was to study the effect of seed coating with biocontrol agents in the control of sunflower root rot disease caused by *M. phaseolina*.

Materials and Methods

***In vitro* test:** Biocontrol fungi and bacteria were obtained from Mycological Culture Collection of Department of Botany, University of Karachi. The biocontrol agents viz., *Aspergillus flavus* Link, *Paecilomyces variotii* Bainier and *Trichoderma viride* Pers., and test pathogen *Macrophomina phaseolina* (Tassi) Goid., were inoculated simultaneously side by side on single Petri plate containing PDA medium supplemented with penicillin and streptomycin @ 200mg /L. Three replications were maintained for each biocontrol agent and were incubated for six days. The efficacy of *Rhizobium meliloti* Dangeard and *Bacillus subtilis* Ferdinand Cohn were tested by streaking the bacteria at one side of the Petri plate opposite to the test pathogen. The growth of the fungus was inhibited when it grew towards the bacterial colony on PDA. The inhibition zone was measured from the edge of test fungal mycelium to the edge of the bacterial colony after 6 days of incubation and expressed as percent inhibition over control as suggested by Lokesha & Benagi (2007):

$$I = \frac{C-T}{C} \times 100$$

where I= Per cent inhibition
C= Growth in control
T= Growth in treatment

Blotting paper: The antagonists (fungi and bacteria) were tested by paper towel (blotter) method, to asses their effect on germination and biomass of sunflower seeds. Seeds were first soaked in the suspension of *M. phaseolina* (hyphae and sclerotia) followed with suspension of the antagonists separately, rolled in moist blotter and incubated at room temperature (28 ± 5 C). The seeds soaked only in *M. phaseolina* suspension served as control. Five replications were maintained. After 10 days, the germination, radicle and plumule lengths were measured. The vigour index was calculated by multiplying germination percentage with the sum of radicle and plumule lengths.

Test tubes: The antagonists (fungi and bacteria) were tested in test tubes, to check their effect on germination and biomass of sunflower seeds. Seeds were first soaked in the suspension of *M. phaseolina* (hyphae and sclerotia) followed with suspension of the antagonists separately, planted in sterilized soil and incubated at room temperature. The seeds soaked only in *M. phaseolina* suspension served as control. Five replications were maintained. Ten days after the germination, shoot and root lengths and weight were measured. The vigour index was calculated by multiplying germination percentage with the sum of shoot and root lengths.

Pots: Seeds of sunflower (*Helianthus annus* L.) were surface sterilized with 1% Ca $(OCl)_2$ for three minutes, rinsed thoroughly in sterilized distilled water and dried aseptically. The seeds were coated with microbial antagonists viz., *Aspergillus flavus, Paecilomyces variotii, Trichoderma viride, Rhizobium meliloti* and *Bacillus subtilis* separately by using 2% gum Arabic as sticker. Ten seeds after treatment with microbial antagonists were transferred in test tube containing 9 ml sterile distilled water. The test tubes were shaken and dilution series was made. One ml suspension was poured on PDA and bacterial cells / seed or fungal conidia / seed was calculated by using the formula: No. of cells or conidia x dilution factor.

Soil used for this experiment was obtained from the experimental field of Department of Botany, University of Karachi and passed through 2 mm sieve to discard particles. The soil used was sandy-loam (sand, silt, clay: 70, 19, 11%), pH range from 7.5-8.1 with 24% moisture holding capacity (Keen & Roczkowski, 1922), total nitrogen 1.5% (Mackenzie & Wallace, 1954), total organic matter 24%. Soil had natural infestation of 1-3 sclerotia of *M. phaseolina* per g of soil as found by wet sieving dilution technique (Sheikh & Ghaffar, 1975).

Seeds of sunflower were treated with 48 hrs old culture of *R. meliloti* and *Bacillus subtilis* and 7 days old culture of *Aspergillus flavus*, *Paecilomyces variotii* and *Trichoderma viride* by coating the seeds in 2% gum Arabic as a sticker. Five seeds/pot were sown in 8 cm diam., plastic pots, each containing 300 g soil. There were three replicates of each treatment and pots without antagonist and without seed coating material served as control. Pots were kept randomized in a green house at the Department of Botany, University of Karachi, where soil was kept @ 50% MHC. Germination was recorded after 10 days and plants were uprooted after 30 days. Plant growth parameters such as root length, shoot length and fresh weight of root and shoot and incidence of root infection caused by *M. phaseolina* were recorded. Data were analyzed and subjected to analysis of variance (ANOVA) following the procedure as given by Gomez & Gomez (1984).

Results

***In vitro* test:** All the biocontrol agents tested were found to be effective in inhibiting the mycelial growth of *M. phaseolina* in dual culture technique (Table 1). Off the antagonists, *R. meliloti* was found significantly better in inhibiting the growth of *M. phaseolina* followed by *B. subtilis* and *Trichoderma viride*. However, *T. viride* showed mycoparasitism and grown over the *M. phaseolina* after 10 days of growth.

Blotting paper: Seed treatment with antagonist did not have any adverse effect on germination of sunflower seeds. There was a significant increase in radicle length of seeds coated with the antagonists compared to the seed coated with *M. phaseolina*. The plumule lengths of the antagonist-coated seeds and control were at par with each other. Seeds treated with the antagonists showed a significantly higher vigour index than that of control. Maximum plant height (radicle length and plumule length) was observed in seed treated with *B. subtilis* followed by *Trichoderma viride*. Maximum biomass was observed in seed treated with *R. meliloti* (Table 2).

Test tubes: Seed treatment with antagonist did not have any adverse effect on germination of sunflower seeds. There was a significant increase in shoot length of seeds coated with the antagonists compared to the seed coated with *M. phaselina*. Maximum plant length (radicle length and plumule length) was observed with *B. subtilis* followed by *R. meliloti.* Seeds treated with the antagonists showed a significantly higher vigour index than seed treated with *M. phaseolina* (Table 3).

Pots: Antagonist-coated seeds in pots containing naturally infested soil gave significant better germination percentage compared to the control (without antagonists) Shoot and root lengths of the seedlings from the antagonist-coated seeds were also significantly better than those of control. Maximum plant length was observed with *B. subtilis* followed by *P. variotii.* Similarly maximum plant biomass was observed with *B. subtilis*. Seed dressing with microbial antagonists efficiently controlled *M. phaseolina* infection on sunflower (Table 4).

Table 1. Growth Inhibition percentage of *M. phaseolina* with antagonists.

S. No.	Test organisms	Inhibition per cent
1.	*Aspergillus flavus*	10 c
2.	*Paecilomyces variotii*	5 c
3.	*Trichoderma viride*	7.3 c
4.	*Rhizobium meliloti*	25 a
5.	*Bacillus subtilis*	25 a
	$LSD_{0.05}$	5.135

Table 2. Response of antagonist-coated seeds against *M. phaseolina* on blotter.

S. No.	Treatment	Germination %	Radicle length (cm)	Plumule length (cm)	Biomass (gm)	Vigour index
1.	Control	80 bc	3.5	1 d	3.2 cd	360 f
2.	*Macrophomina phaseolina*	60 d	1.7	1 d	2.6 d	168 g
3.	*Aspergillus flavus*	77 bc	3.5 e	1.5 cd	1.8 e	385 f
4.	*Paecilomyces variotii*	93 a	4.5 d	1.8 c	3 d	586 d
5.	*Trichoderma viride*	93 a	8.7 b	4.1 b	4 b	1097 b
6.	*Rhizobium meliloti*	80 bc	7.5 c	3.5 b	5.5 a	880 c
7.	*Bacillus subtilis*	73 c	5.3 a	12.3 a	3.7 bc	547 e
	$LSD_{0.05}$	9.695	0.854	0.736	0.641	29.960

Table 3. Response of antagonist-coated seeds against *M. phaseolina* in test tube.

S. No.	Treatment	Germination %	Shoot length (cm)	Root length (cm)	Biomass (gm)	Vigour index
1.	Control	80 a	1.1 c	0.8 c	0.8 cd	152 d
2.	*Macrophomina phaseolina*	60 b	1.1 c	0.5 c	0.5 b	96 f
3.	*Aspergillus flavus*	81 a	1 c	0.6 c	0.9 bcd	130 e
4.	*Paecilomyces variotii*	85 a	1 c	0.8 c	0.5 d	153 d
5.	*Trichoderma viride*	90 a	1 c	0.9 bc	1.2 bc	171 c
6.	*Rhizobium meliloti*	85 a	2.4 b	0.7 c	2 a	263 b
7.	*Bacillus subtilis*	80 a	2.2 b	1.3 ab	2.1 a	280 a
	$LSD_{0.05}$	12.436	0.628	0.45	0.46	13.833

Table 4. Response of antagonist-coated seeds against *M. phaseolina* in pots.

S.No.	Treatment	Germination %	Shoot length (cm)	Root length (cm)	Biomass (gm)	Colonization %
1.	Control	70 ab	7.5 bc	1.8 ab	2.8 a	60 a
2.	*Aspergillus flavus*	65 b	6.6 c	1.8 ab	2.6 a	55 ab
3.	*Paecilomyces variotii*	70 ab	7.3 c	1.9 ab	2.8 a	40 d
4.	*Trichoderma viride*	80 a	7 c	1.5 b	2.4 ab	40 d
5.	*Rhizobium meliloti*	75 ab	6.6 c	1.7 b	1.7 ab	46 cd
6.	*Bacillus subtilis*	75 ab	8.7 a	2.5 a	3 a	50 bc
	$LSD_{0.05}$	9.928	0.933	0.675	0.685	8.160

Discussion

In the present studies, in dual culture technique all tested antagonists inhibited the growth of *M. phaseolina.* Bacterial antagonists used in this study were found more effective and showed maximum inhibition in the growth of *M. phaseolina*. Bacteria produce different kinds of metabolites including antibiotics and toxins in the medium which inhibit the growth of pathogenic organisms (Laura *et al*., 1998).

In all tests (blotting paper, test tube and pot experiment) all antagonists when used for seed treatment did not show any detrimental effect on germination. Use of fungal antagonists enhanced the germination of sunflower seeds. Especially, treatment with *Trichoderma virid* and *Paecilomyces variotii* showed promising results. Other treatment showed non significant effect on germination. However seed treated with *M. phaseolina* reduced seed germination. *M. phaseolina* produce toxin known as phaseolinon which inhibit seed germination up to 50% (Bhattacharya *et al*., 1994).

Blotter and test tubes experiments were carried out to determine the direct effect of antagonists and pathogen on plant growth parameters. Radicle length, plumule length and vigour index was found better in seedlings which were treated with *T. viride* in blotter tests. Similarly, *T. viride* increased shoot length, root length and vigour index in test tube experiment. This is indicating that *Trichoderma* species produces plant growth promoting factors (Windham *et al*., 1986). Different species of *Trichoderma* gained considerable success against pathogenic fungi. *T. harzianum* protects the root system against *F. solani*, *R. solani* and *M. phasoelina* infection on a number of crops (Malik & Dawar, 2003).

Finally plastic pot experiment was performed to asses the effect of antagonists on *M. phaseolina* pathogenicity. In previous studies several microbial antagonists and biocontrol agents have shown promising results in the control of soil-borne pathogens (Ghaffar, 1992). In seed treatment, the antagonistic organisms readily multiply on seed surface, which in turn prevent the entry of the pathogen (Raguchander *et al*., 1998). Colonization percentage in all plant treated with antagonists remains lower than controls. Least colonizations percentage was observed in *P. variotii* and *T. viride* treated plants. *B. subtilis* also found effective to increase plant growth and decrease *M. phaseolina* infection. Seed treatment with *B. subtilis* have since been shown to control various diseases in a variety of crops, including diseases caused by *Rhizoctonia solani* Khün in wheat, brown spot of rice and damping off in tomato and sugarbeet (Merriman *et al*., 1974). The efficacy of antagonists in the control of *M. phaseolina* has been reported earlier in soybean (Vyas, 1994), sesamum (Sankar & Jeyarajan, 1996), pigeonpea (Lokesha & Benagi, 2007), eggplant (Ramezani, 2008), safflower (Sing *et al*., 2008) and sunflower (Zaki & Ghaffar, 1987; Shahnaz Dawar *et al*., 2008).

It is concluded that seed treatment with *Trichoderma viride, Rhizobium meliloti* and *Bacillus subtilis* as antagonists are effective for management of root rot fungus *M. phaseolina* on sunflower.

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