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Full Length Research Paper

Evaluation of biological control potential of locally isolated antagonist fungi against *Fusarium oxysporum* under *in vitro* and pot conditions

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***Fusarium oxysporum* f sp. *phaseoli* is responsible for wilt disease of *Phaseolus vulgaris* L., which results in extensive damage to the crop. Biological control of soil borne plant pathogens is a potential alternative to the use of environment harming chemical pesticides. Therefore, the study was undertaken to determine the potential of locally isolated antagonist fungi (*Aspergillus niger, Penicillium citrinum*, *Trichoderma harzianum,* and *Trichoderma viride*) to manage fusarium wilt of common bean. Under *in vitro* condition all antagonist species had inhibited the radial growth of pathogen; however in the case of *A. niger* this inhibition was insignificant. The maximum mycelial growth of all antagonists was recorded at 25°C and decreased above this temperature. Under pot conditions, all treatments were able to boost plant growth and provide significant reductions in disease levels. The highest plant growth and chlorophyll a+b content were observed in plants treated with *T. harzianum,* followed by *T. viride, P. citrinum* and *A. niger*. The effect of these treatments on fusarium wilt was found to be inversely proportional to the plant growth. Maximum control of wilt disease was observed in bean plants treated with *T. harzianum* (71.4%). Effectiveness of the other antagonists was recorded in the following order: *T. viride* (67.8%), *P. citrinum* (53.5%) and *A. niger* (35.7%).**

Key words: *Fusarium oxysporum* f sp. *phaseoli, Trichoderma* sp. biological control, fusarium wilt, *P. vulgaris*, antagonist fungi.

INTRODUCTION

Fusarium oxysporum, found in its many pathogenic forms, is the most damaging species of the genus where in plants are concerned. Recently a number of new disease reports on *Fusarium* have been submitted to the literature pool on agricultural research (Polizzi et al., 2010; Perveen and Bokhari, 2010; Felgueiras et al., 2010). Fusarium wilts caused by pathogenic forma specials of the soil inhabiting fungus, *F. oxysporum* can cause severe loses in a wide variety of crop plants (Larkin and Fravel, 1998). *F. oxysporum* f sp. *phaseoli* Kendr. and Snyder is responsible for wilt disease of *Phaseolus vulgaris* L. (common bean); this pathogen causes wilting and early death of the plants, which can cause extensive damage to the crop. Crop loss has been reported in South America and Africa (Abawi and Corrales, 1990).

P. vulgaris is a pulse legume grown in soils, ranging from light sand to heavy clay. It is the most important source of protein for low-income populations in Latin America and in Africa. Brazil is the largest producer and consumer of this legume worldwide and is widely grown in developed countries. It is considered to be the most

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Abbreviation: FOP, *Fusarium oxysporum* f sp. *Phaseoli;* **PDA,** potato dextrose agar.

important grain legume for human consumption which comprises 50% of the grain legumes consumed world-wide (Broughton et al., 2003; Graham et al., 2003).

Crop rotation, soil conditioning, use of resistant cultivars and fungicides are the common strategies used for the management of fungal diseases. The most effective method in preventing fusarium wilt is chemical fungicides; however the application of chemical fungicide has its shortcomings such as harming other living organisms and the reduction of useful soil micro-organisms (Lewis et al., 1996). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest management systems to reduce our dependence on chemical pesticides (Sutton, 1996). As with other vascular plant-diseases the sanitation measures are difficult to apply (Brayford, 1992). A promising strategy for the replacement of chemical pesticides has been the implementation of biological control. Research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). Interactions that leads to bio-control include antibiosis, competition, induction of host resistance, production of growth stimulating factors and predation (Cook and Baker, 1983). Many of these concepts have been extensively covered in recent reviews (Harman et al., 2004; Woo et al., 2006; Lorito et al., 2010; Druzhinina et al., 2011).

Several studies have demonstrated the reduced incidence of diseases in various crops (including beans) after supplementing the soils with fungal antagonists (Lewis et al., 1996; Bashar and Rai, 1994; Larkin and Fravel, 1998; Pieta et al., 2003; Pieta and Pastucha, 2004; Abd-El-Khair et al., 2011; Otadoh et al., 2011). The commercialization of biological control products has accelerated this approach (Fravel et al., 2003). *F. oxysporum* f sp. *phaseoli* can cause extensive damage to the crop, thus the need is to manage the disease. To reduce our dependence on chemical pesticides it is important to explore alternatives such as efficient bio-control agent. This study was undertaken to determine the potentiality of locally isolated antagonist fungi to manage fusarium wilt of common bean.

MATERIALS AND METHODS

Isolation and maintenance of *F. oxysporum* f sp. *phaseoli* and antagonist fungi

Isolate of *F. oxysporum* f sp. *phaseoli* (FOP) used in this study was isolated from naturally infected beans plants grown in field near Riyadh region, Saudi Arabia.

Antagonist fungi were isolated from soil samples of various farm fields of Riyadh region, Saudi Arabia. These fungi were isolated by soil plate methods, as described by Dhingra and Sinclair (1995) using potato dextrose agar (PDA) medium. *Trichoderma* spp. was isolated on selective media of Elad and Chet (1983). All species were maintained on PDA slants and were stored at 4°C till further use.

Identification of pathogen and antagonist fungi

Based on microscopic studies, the pathogen was identified as *F. oxysporum* f sp. *phaseoli* on the basis of presence, shape and size of macro- and micro-conidia (Leslie and Summerel, 2006). On the basis of cultural characters and microscopic observations, fungi isolated from various fields were identified as, *Aspergillus flavus, Aspergillus niger, Chetomium sp. Cladosporium cladosporioides, F. equiseti, Penicillium citrinum*, *Penicillium sp., Trichoderma harzianum,* and *Trichoderma viride.* Identification of these fungi was further confirmed by Indian Type Collection Center (ITCC), Indian Agriculture Research Institute (IARI), New Delhi, India.

Evaluation of antagonistic behavior of isolated fungi against *F. oxysporum* f sp. *phaseoli* by dual culture technique

Antagonistic behavior of fungi was evaluated against *F. oxysporum* f sp. *phaseoli* under *in vitro* by dual culture technique. Five millimeters mycelial disc of *F. oxysporum* and antagonist fungi namely *A. niger, P. citrinum*, *T. viride* and *T. harzianum* were cut with the help of reverse side of sterilized micropipette tips from the edge of 3 days old culture. One disc of each of antagonists were placed on the solidified PDA medium at one side of plates and one of *F. oxysporum* f sp. *phaseoli* at opposite to antagonist. Plates were incubated at 25 ± 2°C. The radial growth of test pathogens in treated and control plates were recorded after one week of incubation and the per cent inhibition of mycelial growth of the pathogens was calculated using following formula:

$I = (C-T/C) \times 100$ (Singh et al., 2002)

Where, I = Inhibition (%), C = Colony diameter in control plate and T = Colony diameter in treated plate.

Effect of temperature on mycelial growth of antagonist fungi

The effect of different temperature range (10, 25, 30, 40°C) on mycelial growth of antagonist fungi was evaluated by measuring radial growth of fungi on PDA. Five millimetre mycelial disc of *A. niger, P. citrinum*, *T. viride* and *T. harzianum* were obtained, as mentioned above, and placed on the solidified PDA medium. Radial mycelial growth was measured as mean of two perpendicular diameters, after one week and data were expressed as percent growth of fungi at each temperature.

Determination of percent seed germination

The seeds of *P. vulgaris* var. Strike were obtained from the local market of Riyadh and the experiment was conducted at Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.

Steam-sterilized sand was inoculated with the FOP spores (1.0×10^3 spores/g of soil mixture) before the seed were sown. Four surface sterilized (0.1% sodium hypochlorite) bean seeds were sown in each pot. Inoculums of A. *niger, P. citrinum*, *T. viride* and *T. harzianum* were prepared in the form of a conidial suspension (10^6 spores / ml) as described by Sivan et al. (1984). Bean seedlings were raised in a glass house containing one of the antagonist microorganism, seeds without any inoculation served as control. Plants were watered regularly. After 14 days of sowing, percent seed germination was determined.

Determination of *P. vulgaris* plant growth and inhibition of *F. oxysporum* under pot conditions

For pot trial, method described by Perveen et al. (2007) with slight

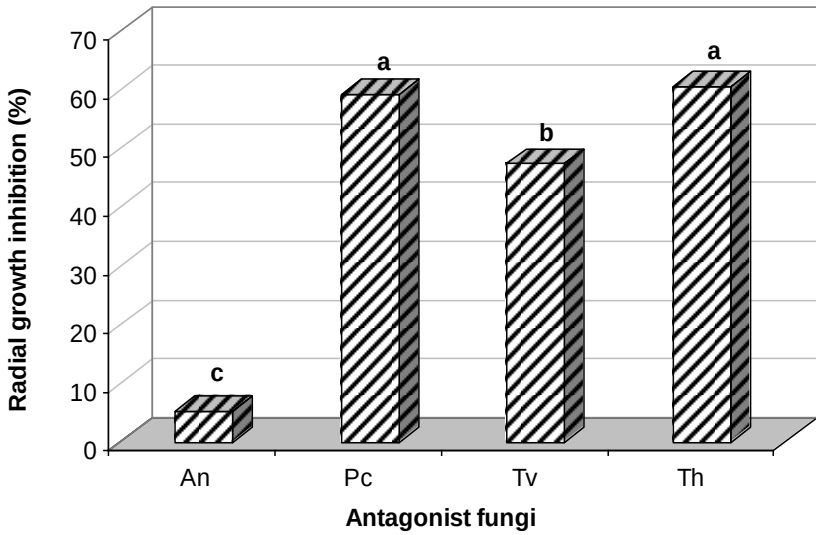

Figure 1. Radial growth inhibition of *F. oxysporum* f. sp. *phaseoli* by antagonist fungi under *in vitro* condition. Each value is an average of three replicates. Columns' showing different letters are significantly different (P≤ 0.05) according to Duncan's multiple range test. An = *A. niger,* Pc = *P. citrinum, Tv* = *T. viride,* Th = *T. harzianum.*

modification was used. Pots of 9 cm (diameter) were surface sterilized with 1% sodium hypochlorite and were filled with 100 g mixture of autoclaved peat moss soil and sand (5:1). Single sterilized *P. vulgaris* seed was sown in each pot.

F. oxysporum f sp. *phaseoli* culture grown on PDA were scraped with the help of sterilized spatula and were mixed with sterilized distilled water to get the final cfu of 1.0×10^6 spores/ml. 10 ml of FOP spore suspensions was added around the plant by removing soil. The spore suspension [3% (v/v) of *A. niger, P. citrinum*, *T. viride* and *T. harzianum*] were mixed with the pathogen infested soils, according to the following treatment scheme: FOP alone, FOP + *T. harzianum,* FOP + *P. citrinum,* FOP + *T. viride* and FOP + *A. niger*. Pots inoculated with only distilled water served as uninoculated control, whereas FOP alone inoculated pots served as inoculated control. Pots were arranged in glass house on a rack in randomized block design. Plants were irrigated with sterile water, as per requirement, were observed daily to record symptoms and growth for over one month. Plants were uprooted after one month to record the height, fresh weight and disease index (Perveen et al., 2007). Chlorophyll content of the third leaf from the apex was estimated according to the Lichtenthaler and Buschmann method (2001).

Statistical analysis

All experiments were performed in triplicate. Duncan Multiple Range Test was used to evaluate the significant differences between treatments (P ≤ 0.05). ANOVA analysis was done with the SPSS statistics software.

RESULTS

Under *in vitro* condition all tested antagonist fungi have inhibited the radial growth of *F. oxysporum* f sp. *phaseoli* at varying degrees (Figure 1). The highest inhibition of FOP was recorded in *T. harzianum* (59.8%) followed by *P. citrinum* (59%) and *T. viride* (47%), whereas *A. niger* (5%) had insignificant growth inhibition of pathogen (P ≤ 0.05).

Effect of temperature on the growth of antagonist fungi revealed that all antagonist fungi reached a peak in mycelial growth rate at 25°C (Figure 2). At 30°C, *P. citrinum*, *T. viride* and *T. harzianum* showed very slow growth and at 40°C the fungal growth was less than 20%. Whereas, at 30°C the growth of *A. niger* was 75%.

The *P. vulgaris* seeds treated with FOP and antagonist fungi in all treatments showed more than thirty three percent seed germination (Figure 3). Maximum seed germination was observed in seeds treated with *T. harzianum* and *P. citrinum* (90%), followed by *T. viride* and *A. niger* (58% and 33% respectively).

In the present study, all antagonists have significantly enhanced the height and fresh weight of bean seedlings as compared to the height and fresh weight of bean seedlings inoculated with FOP alone (Table 1). Maximum plant height was observed in plants treated with *T. harzianum* followed by *T. viride, P. citrinum* and *A. niger* (37.9, 34.2 and 15.8 cm, respectively). The FOP alone inoculated plants showed 94.3% reduction in the plant fresh weight as compared to uninoculated control plants. Whereas all antagonists were able to increase the plant fresh weight and showed significant reduction in disease levels. The least reduction in plant fresh weight was observed in plants treated *T. harzianum* (9.5%) followed by *T. viride* (13.3%), *P. citrinum* (23.2%) and *A. niger (*69.6%). Similarly, chlorophyll a+b content was found to be increased significantly in all treated plants as

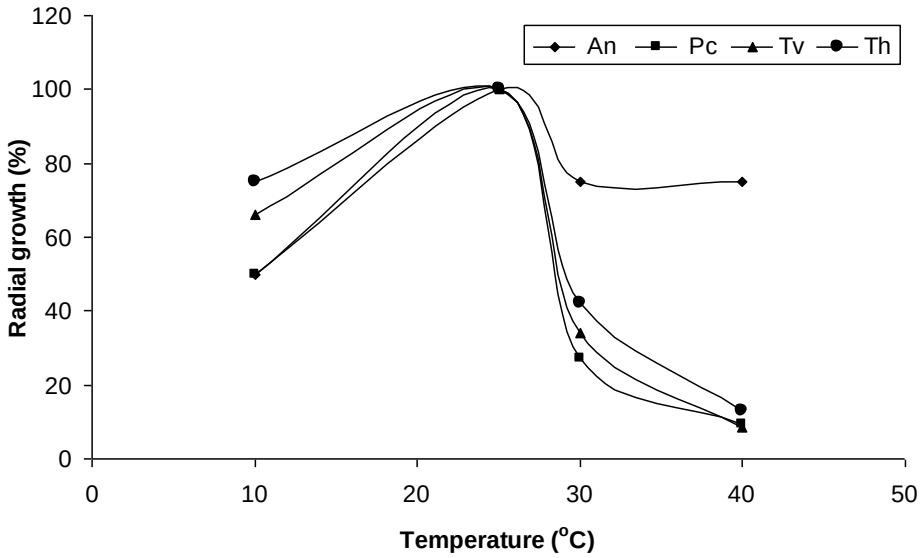

Figure 2. Effect of temperature on the mycelial growth of antagonist fungi. Each value is an average of three replicates. An = *A. niger*, Pc = *P. citrinum*, *Tv* = *T. viride,* Th = *T. harzianum.*

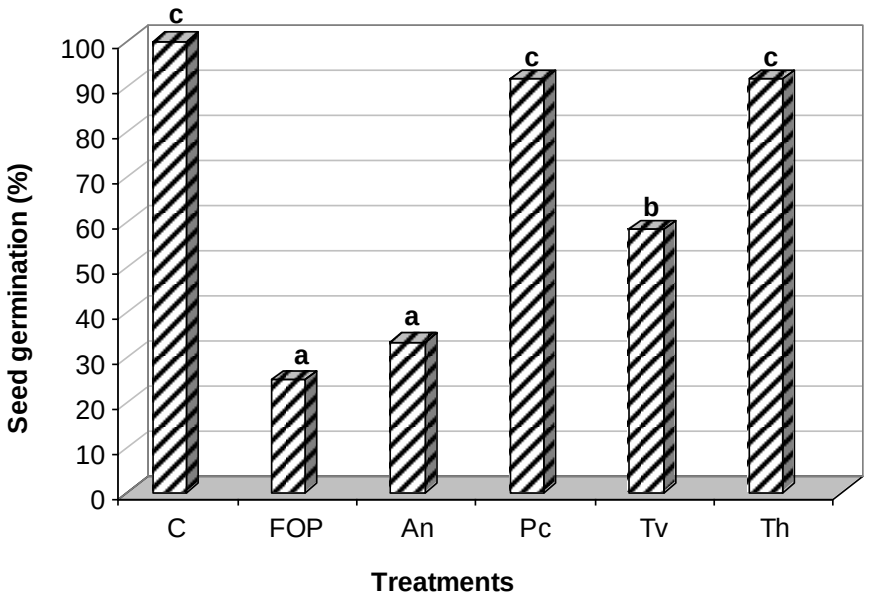

Figure 3. Effect of antagonist fungi on germination of seeds inoculated with *F. oxysporum* f. sp. *phaseoli.* Each value is an average of three replicates. Columns' showing different letters are significantly different (P≤ 0.05) according to Duncan's multiple range test. C = Uninoculated control, FOP = *F. oxysporum* f. sp. *phaseoli* only, An = *A. niger,* Pc = *P. citrinum, Tv* = *T.viride*, Th = *T. harzianum.*

compared to FOP alone inoculated plants (P ≤ 0.05).

The application of antagonists had lowered significantly the extent of wilt infection (disease index) by *F. oxysporum* f sp. *phaseoli* in comparison to FOP alone inoculated plants (Figure 4). The effect of these treatments on fusarium wilt was found to be inversely proportional to that on the plant growth. The maximum control of the wilt disease was observed in bean plants treated with *T. harzianum* (59.8%), effectiveness of the other antagonists was recorded in the following order:

Table 1. Effect of antagonist fungi on the growth of *P. vulgaris* plants inoculated with *F. oxysporum* f. sp. *phaseoli* under pot conditions[a].

Treatment	Plant height (cm)	Plant fresh weight (g)	Chl a+b (µg/mg)
C	41.8[e]	8.2[e]	3.08[a]
FOP	3.3[a]	0.47[a]	0.00[e]
FOP + An	15.8[b]	2.49[b]	1.37[d]
FOP+ Pc	34.2[c]	6.31[c]	1.97[c]
FOP +Tv	37.9[d]	7.11[d]	2.78[b]
FOP+ Th	38.9[d]	7.42[d]	3.03[a]

[a]Each value is an average of three replicates.Data followed by different letters in the column are significantly different (P≤ 0.05) according to Duncan's multiple range test .C = Uninoculated control, FOP= *F. oxysporum* f. sp. *phaseoli,* An = *A. niger,* Pc = *P. citrinum,* Tv = *T. viride,* Th = *T. harzianum,* Chl = Chlorophyll.

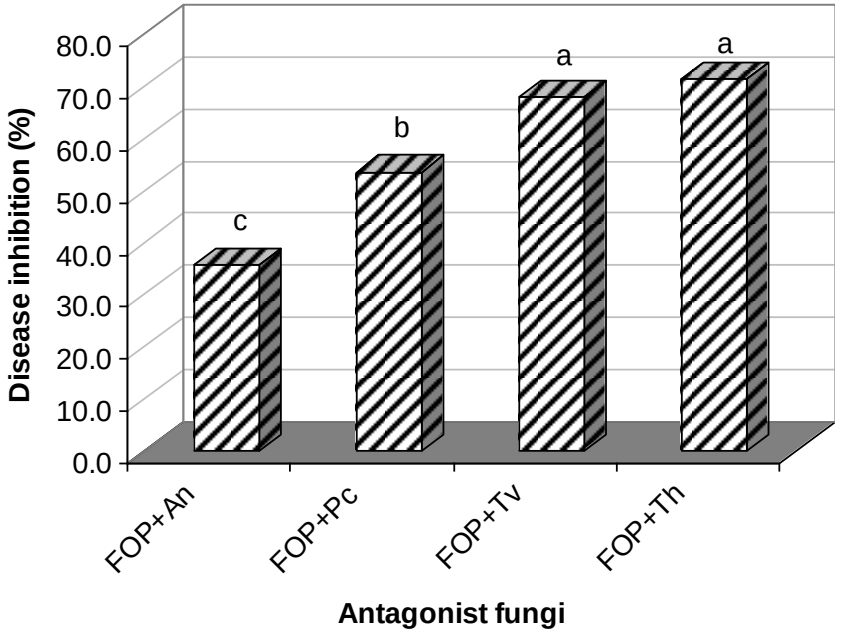

Figure 4. Fusarium wilt inhibition (%) by antagonist fungi under pot conditions. Each value is an average of three replicates. Columns' showing different letters are significantly different (P≤ 0.05) according to Duncan's multiple range test. An = *A. niger,* Pc = *P. citrinum, Tv* = *T.viride*, Th = *T. harzianum.*

T. viride (67.8%), *P. citrinum* (53.5%) and *A. niger* (35.7%). Some of the plants inoculated with FOP alone got succumbed to infection and cotyledons/roots of these plants were found to be severely infected with the pathogen (Figure 5).

DISCUSSION

Biological control of soil borne plant pathogens is a potential alternative to the use of chemical pesticides, which have already been proved to be harmful to the environment. There is a growing demand for sound, biologically-based pest management practices. Recent surveys of both conventional and organic growers indicated an interest in using biocontrol products (Rzewnicki, 2000; Van Arsdall and Frantz, 2001).

This study was undertaken to determine the potential of locally isolated antagonist fungi to act as biocontrol agent for the management of *F. oxysporum* f sp. *phaseoli* responsible for wilt disease of *P. vulgaris.* The results *in vitro* inhibition assay revealed that both species of *Trichoderma* and *P. citrinum* rapidly colonized the medium and were found to be effective in inhibiting growth of the FOP which may be due to fungistatic effect (Cook and Baker, 1983) or might be attributed to the

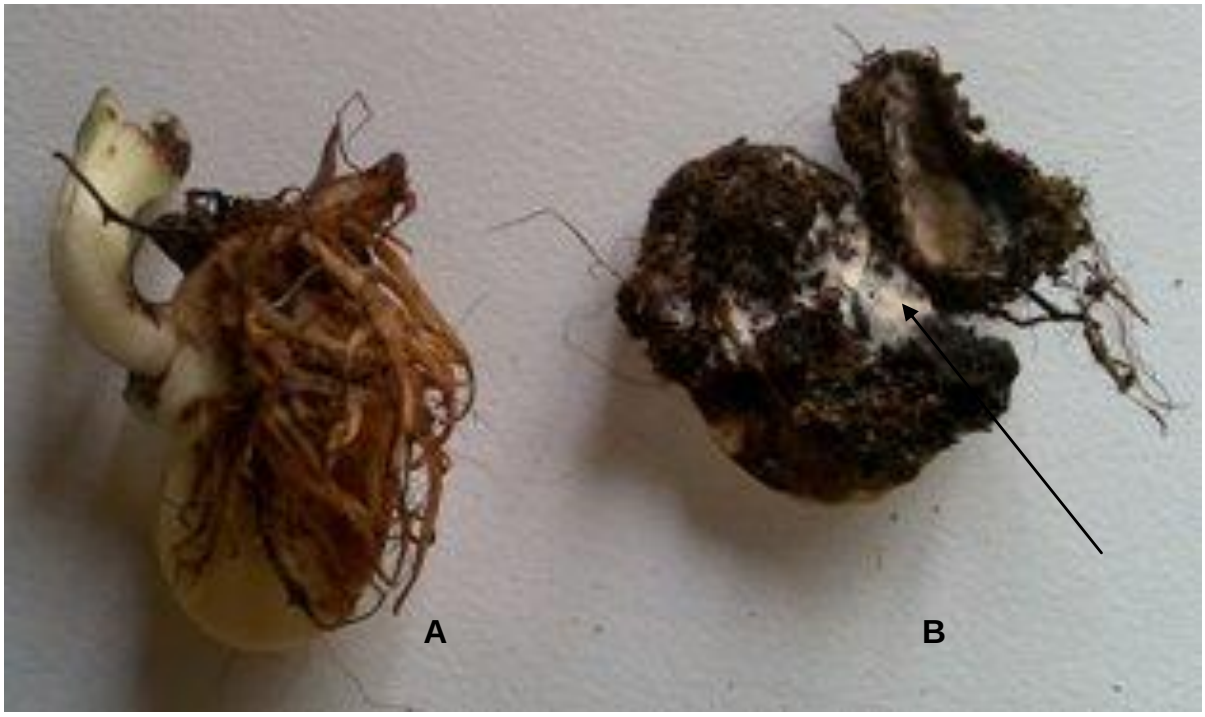

Figure 5. Cotyledons of plants uninoculated (A) and inoculated (B) with *F. oxysporum* f. sp. *phaseoli* (1.0×10^3 spores/g of soil mixture). Arrow indicates the mycelial growth *of F. oxysporum* f. sp. *phaseoli*.

secretion of antibiotics by the fungi or other inhibitory substances produced by the antagonists such as viridian, gliovirin, geodin, terricin, terric acid, aspergillic acid, dermadin etc. (Howell, 1998; Mondal et al., 2000; Vey et al., 2001; Landreau et al., 2002; Yan et al., 2006). The degree of effectiveness varies according to the nature, quality, and quantity of antibiotics/inhibitory substances secreted by the antagonists (Harman, 1998; Kubicek et al., 2001; Woo et al., 2006; Singh, 2006).

Temperature is a vital factor to manipulate the growth, sporulation and saprophytic ability as well as production of volatile and non-volatile metabolites, involved in nutrition, competition, mycoparasitism, and extra cellular cell wall degrading enzymes (Lorito et al., 1996; Harman and Kubicek, 1998; Kubicek et al., 2001; Woo et al., 2006). Therefore, effect of temperature on the growth of antagonist fungi was evaluated in order to determine the most suitable temperature for the growth of antagonist. In the present study, mycelial growth of *P. citrinum*, *T. viride* and *T. harzianum* was the highest at 25°C and it decreased above this temperature, whereas *A. niger* grew well at higher temperature. The optimum temperature for growth differs among the *Trichoderma* isolates; although most *Trichoderma* strains are mesophilic (Kredics et al., 2003; Hajieghrari et al., 2008). Similarly Pandey et al. (2001) observed that *Penicillium* and *Trichoderma* species prefer a mesophilic temperature range (15 to 35°C). *A. niger* showed good growth at higher temperature (41°C) and was categorized in xerophilic fungi (Cabrera et al., 2005). The results obtained from present study also support these observations.

In general all antagonist microorganisms tested has increased the percent seed germination. Pieta et al. (2003) observed that *T. harzianum*, *Trichoderma koningii* and *T. viride*, used as seed dressing, improved the seedling emergence and health of runner bean (*P. coccineus* cv. Eureka). Similarly, seeds of common bean were dressed, prior to sowing; with conidia of *T. harzianum* protected the germinating seedlings and plants against infection by soil borne pathogenic fungi, that is, *Fusarium* spp. and *R. solani* (Pieta and Pastucha, 2004).

Results of the effect of antagonist on the bean plant growth under pot condition revealed that seedlings grown in antagonist fungi treated soils had more plant height and fresh weight as compared to FOP alone inoculated plants (Table 1). Investigations suggest that the increased growth response caused by *Trichoderma* isolates may be through modification of the rooting system (Chao et al., 1986; Ahmad and Baker, 1987). *Trichoderma* species added to the soil or applied as seed treatments; grow readily along with the developing root system of the treated plant (Harman, 2006; Howell et al., 2000). It is well known that *Trichoderma* can parasitize fungal pathogens and produce antibiotics, besides the fungus have many positive effects on plants: increased growth and yield, increased nutrient uptake, increased

fertilizer utilization efficiency, increased percentage and rate of seed germination and induced systemic resistance to plant diseases (Harman et al., 2004; Harman, 2006). A study carried by Yedidia et al. (1999) reported that *T. harzianum* inoculation improved the uptake of nutrients by the plant at a very early growth stage. The plants treated with *P. citrinum* also showed positive plant growth response. Although in dual culture experiment *A. niger* had a non significant effect on inhibition of FOP growth, but under pot conditions its presence has enhanced significantly the percent seed germination as well as it has boosted the plant growth. The positive response of bean plants on the addition of *A. niger* and *P. citrinum* may be due to the fungistatic activity or the plant growth promoting activities in soil (Whipps and Mc Quilken, 1993; Bashar and Rai, 1994; Mondal et al., 2000; Singh et al., 2002; Yadav et al., 2011).

Results indicated that all antagonist species significantly reduced the disease incidence in pot conditions. These results agreed with Abou-Zeid et al. (2003), Pieta and Pastucha (2004), Abd- El-Khair et al. (2011) and Otadoh et al. (2011). They reported that *Trichoderma album, T. hamatum, T. harzianum*, *T. koningii, Trichoderma reesei* and *T. viride* protected the germinating bean seedlings against *Fusarium* spp. and *R. solani* infection. Some recent studies indicated that these fungi can induce systemic resistance in plants, thus increasing the plant defense response to diverse pathogen attack (Harman et al., 2004; Woo et al., 2006; Lorito et al., 2010).

One of the most important indicators of physiological activity is the rate of photosynthesis, which is related to the chlorophyll content of plants. In the present study the chlorophyll a+b content was found to be increased significantly in all treated plants as compared to FOP alone inoculated control. Previous reports suggested that applying biological control agents to infected plants increase mineral levels [(nitrogen (N), phosphorous (P), potassium (K) and magnesium (Mg)], chlorophyll biosynthesis and photosynthetic activity (Mahmoud et al., 2004; Henry et al., 2009; Morsy et al., 2009).

The present study demonstrated that *P. citrinum*, *T. viride* and *T. harzianum* have potential to be used as a biological control agent to protect bean plants from *F. oxysporum* f. sp. *phaseoli.* However, antagonist fungi with the highest level of bio-control *in vitro* may not perform as well *in vivo* since environmental conditions and competition with other microorganisms are much more restrictive. Therefore, the biocontrol potential of these antagonist fungi may be further evaluated in field condition.

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