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MICROBIAL CONTROL OF *FUSARIUM WILT* OF CHICKPEA CAUSED BY *FUSARIUM OXYSPORUM* F. SP. *CICERIS*

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Abstract

Use of biocontrol agent not only provide effective disease control, but it is safe and environmental friendly and being widely used against plant pathogens including *Fusarium* spp. Antagonistic fungi inhibit plant pathogens either directly through mycoparasitism, or indirectly through antibiosis, promoting plant growth and enhanced plant defensive mechanism. In present study, different antagonistic fungi *viz*., *Trichoderma pseudokoningii*, *T. polysporum*, *T. harzianum*, *Paecilomyces lilacinus*, *P. variotii* and *Gliocladium virens* were tested against *Fusarium oxysporum* f. sp. *ciceris* under *in vitro* and *in vivo* conditions. In dual assay, all the antagonistic fungi inhibited the growth of test pathogen. However, *P. lilacinus* produced the least inhibition of test pathogen while, *G. virens* produced maximum inhibition. Variations observed in the interaction between the used antagonists and *F. oxysporum.* The use of antagonistic fungi remains highly effective to enhance seed germination and plant growth, to reduce plant mortality and root infection as well as application of antagonists remarkably enhanced the grain yield in treated chickpea plants.

Key words: *Trichoderma* spp., *Gliocladium virens*, *Paecilomyces* spp., chickpea, *Fusarium* wilt.

Introduction

The chickpea wilt is an economically important disease caused by *F. oxysporum* f. sp. *ciceris* that is widely distributed in all chickpea growing regions of the world (Nene & Reddy, 1987; Haware, 1990; Jalali & Chand, 1992). Average annual yield losses of 10-15% are the consistent feature of this disease (Trapero-Casas & Jimenez-Diaz, 1985; Campbell & Madden, 1990; Jalali & Chand, 1992), but under severe conditions, losses may reach up to 100%, drought conditions with high temperature favor the disease (Haware & Nene, 1980; Navas-Cortes *et al*., 2000).

Fusarium oxysporum f. sp. *ciceris* is a soil borne pathogen that can survive in the soil for 3-6 years without any host (Ayyub *et al.*, 2003; Haware *et al*., 1996). Chemicals are widely used for control of this disease (Jimenez-Diaz & Trapero-Casas, 1985; Dwivedi & Updhyay, 1988; Subhani *et al*., 2011; Kamdi *et al*., 2012), but it is not an eco-friendly and economical option. Beside other disadvantages, injudicious use of chemicals harms beneficial soil microorganisms. However, some chemicals such as Carbendazim and Thiphanate-methyl are very effective in controlling this disease (Maitlo *et al*., 2014b). Use of resistant variety may be the best option, but in our previous screening study out of thirty one locally available cultivars, no one was completely immune (Maitlo *et al*., 2014a). Integrated management strategies, including the selection of variety, early sowing, bio-control treatment, minimum use of fungicides for reducing pathogen inoculum and amendments of antagonists may reduce Fusarium wilt epidemic (Navas-Cortes *et al*., 1998; Landa *et al*., 2004, 2006).

Numerous antagonistic microorganisms such as *Penicillium*, *Trichoderma*, *Bacillus*, *Aspergillus*, *Streptomyces* and *Pseudomonas* are recognized as potential biocontrol agents (Tilston *et al*., 2002; Fuchs & Larbi, 2004; Dubey *et al*., 2007). Fusarium wilt of chickpea is effectively reduced by specific strains of *Bacillus subtilis* and *Pseudomonas* (Anjaiah *et al*., 2003; Jamali *et al*., 2004; Saikia *et al*., 2005). Combined or alone application of *Bacillus subtilis* and *Pseudomonas fluorescens*, with a nonpathogenic strain of *F. oxysporum* reduce this disease and increases the yield (Landa *et al*., 2004). Some isolates of *P. fluorescens* and chemical inducers such as salicylic acid systemically induced resistance against Fusarium wilt of chickpea. Alone application of *P. fluorescens* reduced the wilt disease by 26-50% as compared to control. While, reduction was more pronounced and reaches 52- 64% when chemical inducers (salicylic acid) were applied with *P. fluorescens* as soil treatment (Saikia *et al*., 2003). Antagonistic fungi usually colonize plant roots prior to start of plant growth and protection against infections. Antagonists show a different type of interactions with pathogens and hosts such as mycoparasitism, competition for nutrients, antibiosis and induction of plant defensive mechanisms (Hoitink & Boehm, 1999; Benitez *et al*., 2004). In present study, comparative efficacy and mechanism of different antagonistic fungi against *F. oxysporum* f. sp. *ciceris* causing chickpea wilt is studied.

Materials and Methods

Source of test pathogen and antagonistic fungi: *F. oxysporum* f. sp. *ciceris* was isolated in the previous study (Maitlo *et al*., 2014b) from wilted chickpea plants, collected from a chickpea field of Pulses Research Station, Agriculture Research Institute Tandojam, Sindh Province, Pakistan. The culture of bio-control agent's *viz*., *Paecilomyces lilacinus, P. variotii, Trichoderma pseudokoningii, T. harzianum, T. polysporum* and *Gliocladium virens* were obtained from the Culture Collection Center, Department of Agriculture and

Agribusiness, University of Karachi, Karachi. Cultures were multiplied and maintained on PDA medium for further use.

***In-vitro* evaluation of antagonistic fungi:** Above-mentioned bio-control agents screened for their antagonistic activity against *F. oxysporum* by dual culture assay. Eight-millimeter diameter discs of antagonistic fungus and test pathogen placed on the solidified PDA medium at opposite sides to each other. Petri plates inoculated separately with *F. oxysporum* served as control. These plates incubated at 25±1℃ and after each 24 hours, the colony diameter of the test pathogen and the antagonistic fungus recorded by drawing a straight line. The percentage inhibition of radial growth (PIRG) was determined with the help of a following formula (Jinantana & Sariah, 1997; Nashwa *et al*., 2008):

$$PIRG = (R1 - R2)/ R1 \times 100$$

where;
R1: Radial growth of test pathogen in control plate (without antagonistic fungus);
R2: Radial growth of test pathogen in the treated plate (with antagonistic fungus).

The interactions between the antagonists and test fungus were noted as described by Yaqub and Shahzad (2005).

A. Colonies of antagonist and test fungus meet each other; test fungus overgrew the antagonist colony.
B. Antagonist inhibited the growth of test fungus by coiling around the hyphae of test fungus.
C. Colonies of test fungus and antagonist meet each other, no more growth of either was observed.
D. Colonies of antagonist and test fungus intermingled.

Effect of antagonistic fungi on plant growth and disease development

Pot experiment: Surface sterilized seeds of chickpea variety 'Rabbat' with 5% bleach and sown into earthen pots of 20 cm diameter @10 seeds/pot having 2-kg sandy clay loam soil sterilized by steam. Before sowing, the soil was artificially infested with *Foc* inoculum @ 10^4 conidia $gram^{-1}$ of soil (Bhatti & Kraft, 1992). The soil also amended with the culture of antagonistic fungi with three concentrations, i.e. 10^3, 10^4 and 10^5 cfu $gram^{-1}$ of soil. The experiment was conducted with three replicates using Randomized Complete Block Design (RCBD). Plants irrigated with sterilized water, when required. After forty-five days of sowing, plants uprooted carefully and roots washed with tap water. Data regarding seed germination, plant mortality, root infection, root weight and length, shoot weight and shoot length was recorded. The root infection calculated by the following formula:

$$\text{Root infection (\%)} = \frac{\text{Total number of root pieces colonized by the pathogen}}{\text{The total number of root pieces studied}} \times 100$$

Field experiment: All biocontrol agents also evaluated under field conditions against Fusarium wilt. The experiment designed with four replications as Randomized Complete Block Design (RCBD). The total size of a plot was 5×3 $m^{2,}$ there were10 rows with 30 cm row-to-row distance. Each antagonist was multiplied on sand maize meal water medium (90 g sand, 10 g maize meal, and 20 ml distilled water). The chickpea seeds treated with a bio-control solution containing 10^6 conidia ml^{-1} (Nene *et al*., 1981; Dubey *et al*., 2007). The field irrigated time to time to maintain 50% water holding capacity. The data on plant length (root and shoot) and plant weight (root and shoot), disease incidence, root infection, and grain yield was recorded at the time of harvesting.

Results

Influence of bio-control agents against *F. oxysporum* f. sp. *ciceris*: Mycelial growth of test pathogen inhibited by *Trichoderma polysporum, T. pseudokoningii, T. harzianum, Paecilomyces variotii* and *Gliocladium virens* as compared to the control in dual culture assay. However, *P. lilacinus* produced the least inhibition of test pathogen. Type of inhibition/interaction between antagonists and pathogen has given in the table 1. *Gliocladium virens* produced maximum inhibition (38.00%) followed by *P. varioti* (35.41%), *T. harzianum* (15.88%), *T. pseudokoningii* (14.70%) and *T. polysporum* (11.76%). The *T. pseudokoningii, T. polysporum* and *T. harzianum* displayed D-type interaction, whereas, *P. lilacinus, P. variotii* and *G. virens* displayed C-type interaction with pathogen (Table 1).

Effect of bio-control agents on plant growth and disease development

Pot experiment: The application of all antagonists significantly enhanced the chickpea seed germination as compared to control (Fig. 1a & b). *Trichoderma harzianum* appeared as the highly effective antagonist that provided maximum seed germination at higher used concentration (10^{-5} cfu $gram^{-1}$ of soil). The high and medium concentration (10^{-5} and 10^{-4} cfu $gram^{-1}$ of soil) followed by lower concentration (10^{-3} cfu $gram^{-1}$ of soil) of *T. polysporum, T. pseudokoningii* and *G. virens* were also found equally effective for enhancing the seed germination (Fig. 1b). However, *P. lilacinus* appeared least effective among the used antagonist at its all used concentrations.

Similarly, the application of antagonists significantly decreased the plant mortality as compared to control (Fig. 2). Minimum plant mortality observed in plants grown in soil amended with the medium and higher doses of *T. harzianum, T. polysporum, T. pseudokoningii* and *G. virens*. The maximum plant mortality of 60% observed in untreated plants and second highest in plants treated with *P. lilacinus*.

The applications of antagonistic fungi effectively inhibited the activities of the inoculated pathogen and significantly reduce the infection. The highest root infection was found in inoculated-untreated plants (84%) followed by plants treated with *P. lilacinus*. The low, medium and higher dose of *P. lilacinus* produced 23, 39 and 72% root infection, respectively (Fig. 3).

Table 1. Effect of different antagonistic agents on mycelial growth of *F. oxysporum*. Each number represents the mean of six replications.

Bio-control agents	Incubation days	Colony diameter (mm)		Inhibition (%)	Type of interaction
		Pathogen	Bio-control agent		
Trichoderma harzianum	3	28	61	15.88	D
Trichoderma polysporum	3	30	60	11.76	D
Trichoderma Pseudokoningii	3	29	61	14.70	D
Gliocladium virens	5	30	60	38.00	C
Paecilomyces variotii	5	31	59	35.41	C
Paecilomyces lilacinus	7	60	30	00.19	C

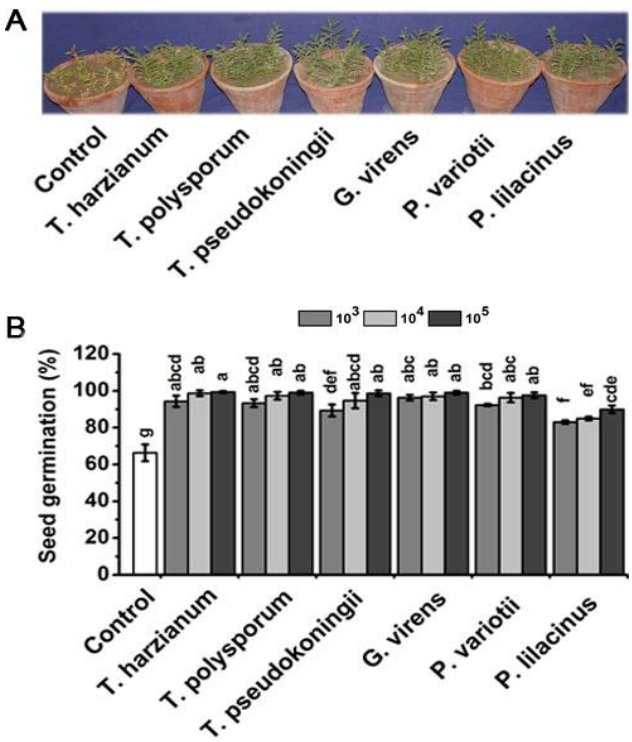


Fig. 1. Effect of antagonistic agents on germination of chickpea seeds inoculated with *F. oxysporum*. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$) Tukey-Kramer HSD test. The photographs were taken on day 45.

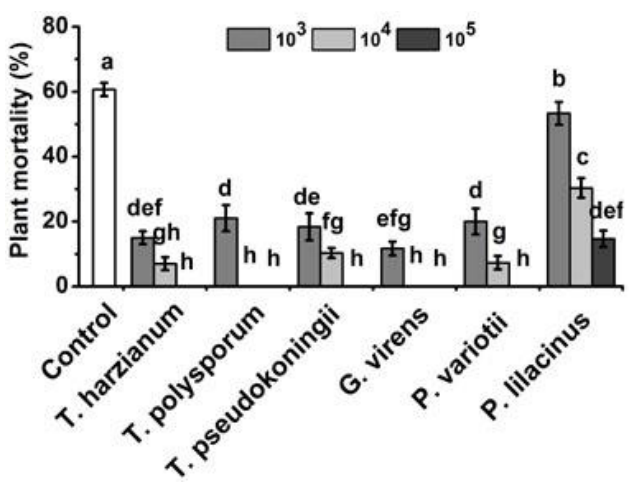


Fig. 2. Effect of antagonistic agents on mortality of chickpea plants inoculated with *F. oxysporum*. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

Bio-control agents not only reduced the infection initiated by the inoculated pathogen, but also enhanced plant growth. As significantly maximum, shoot length and weight, root length and weight found in plants treated with higher and medium doses of *Trichoderma* spp., *G. virens* and *P. variotii* followed by their lower doses. Among biocontrol agents, the application of *P. lilacinus* showed less plant growth (Fig. 4a & 4b).

Field experiment: Disease incidence significantly reduced in treated plants with bio-control agents as compared to the control (untreated) plants. Among different bio-control agents, the significantly minimum disease incidence observed in plants treated with *T. polysporum* and *G. virens* (5%), *P. variotii* (6%), *T. pseudokoningii* (7%) and *T. harzianum* (9%). The maximum disease incidence was recorded in un-treated plants (80%) followed by the plants treated with *P. lilacinus* (36%) (Fig. 5). The plants treated with *T. polysporum* showed maximum shoot length (33.3 cm), followed by *G. virens* (31.3 cm) and *T. pseudokoningii* (31 cm). The significantly lowest shoot length was recorded in un-treated plants (15.4 cm) followed by plants treated with *P. lilacinus* (21.2cm) (Fig. 6a). The un-treated plants produced the minimum shoot weight of (3.4 gm) followed by plants treated with *P. lilacinus* (7.8 gm) (Fig. 6b). Almost similar trends also observed in terms of root weight and length. The highest root length (15.8 & 15.7 cm) and root weight (3.65 & 3.67 gm) was recorded in plants either treated with *G. virens* and *T. polysporum*, followed by *T. pseudokoningii*, (15.2 cm & 3.25 gm), *T. harzianum* (14.3 cm & 3.15 gm) and *P. variotii* (14.3 cm & 3.12 gm). Among bio-control agents, *P. lilacinus* produced lowest root length and weight (11.2 cm and 2.25 gm) as compared to the rest of bio-control agents (Fig. 6c & d).

In field experiment, the applications of these bio-control agents significantly reduced the root infection as compared to control. *G. virens* and *T. polysporum*, appeared as the most effective bio-control agents which produced significantly lowest root infection (20%). The next most effective bio-control agent was *T. pseudokoningii*, in which treated plants showed 26% root infection, followed by *T. harzianum* and *P. variotii* produced 33% root infection. While the *P. lilacinus* appeared to be a less effective bio-control agent, which showed 55% root infection. Although, this was much lower than those recorded in untreated plants 82% (Fig. 7).

Grain production significantly increased in plants treated with antagonistic fungi as compared to the un-treated plants. Remarkably maximum grain production noted in plants treated with *G. virens*, *T. polysporum* and *T. harzianum* followed by *P. variotii* and *T. pseudokoningii*. Whereas the lowest grain yield was found in untreated plants followed by plants treated with *P. lilacinus* (Fig. 8).

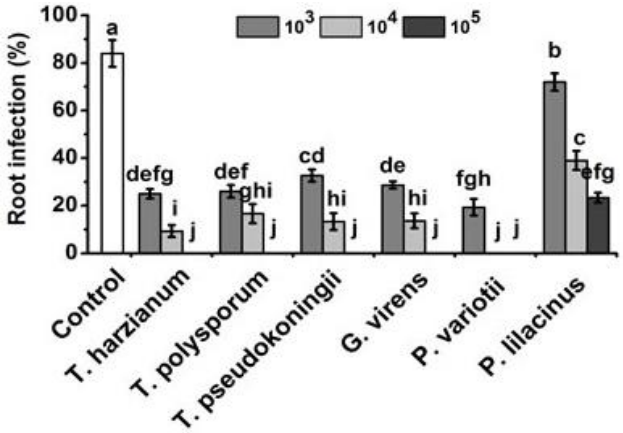
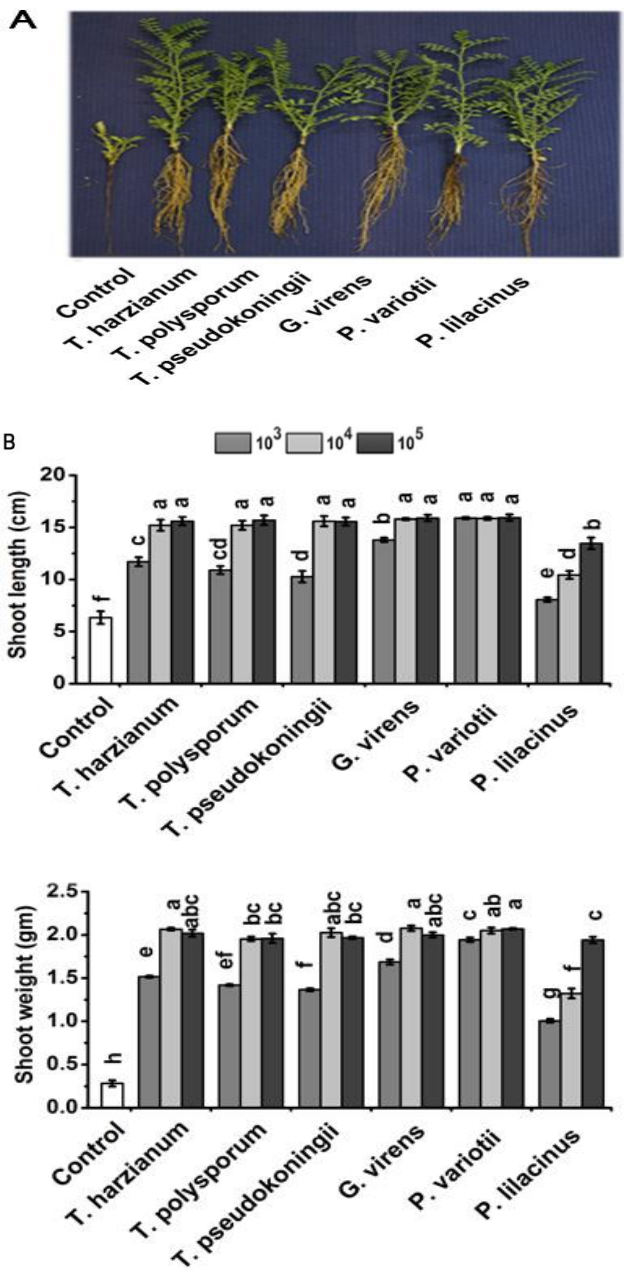


Fig. 3. Effect of antagonistic agents on root infection of chickpea plants inoculated with *F. oxysporum*. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).



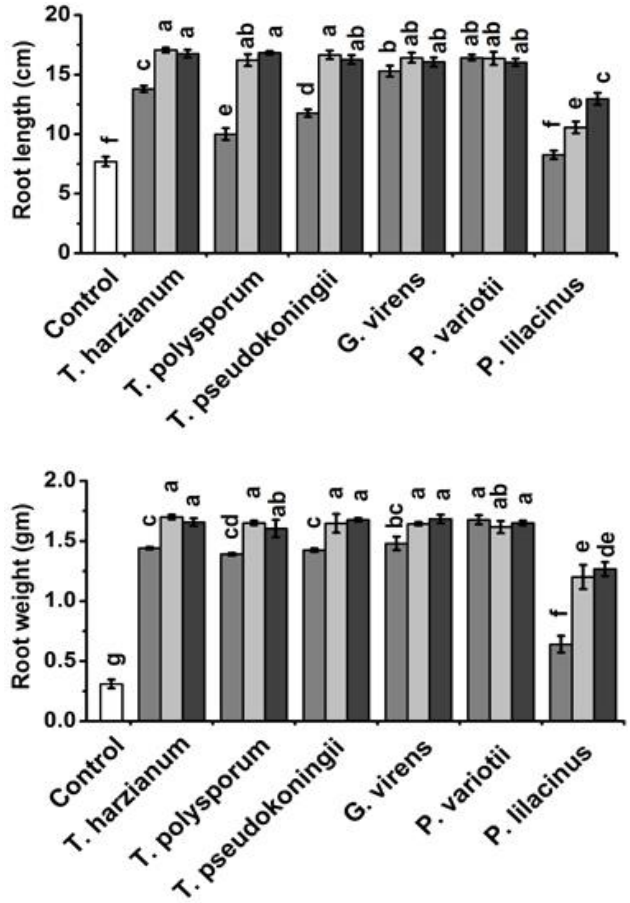


Fig. 4. Effect of antagonistic agents on growth of chickpea plants inoculated with *F. oxysporum*. (A) The plants grown in soil containing different concentrations (10^3, 10^4 and 10^5) of antagonistic agents. (B) The shoot length and weight, root length and weight of chickpea plants were measured at 45 dpi with all concentrations of bio-control agents. The experiments were repeated triplicate with the concentrations of bio-control agents with the same results. Ten plants were used for each treatment in each experiment. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

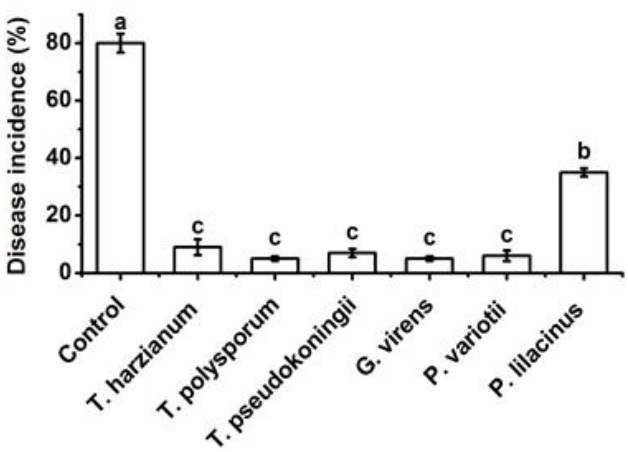


Fig. 5. Effect of antagonistic agents on incidence of chickpea plants inoculated with *F. oxysporum* in infested field. The disease incidence percentage was recorded during 2012 in chickpea infested field. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

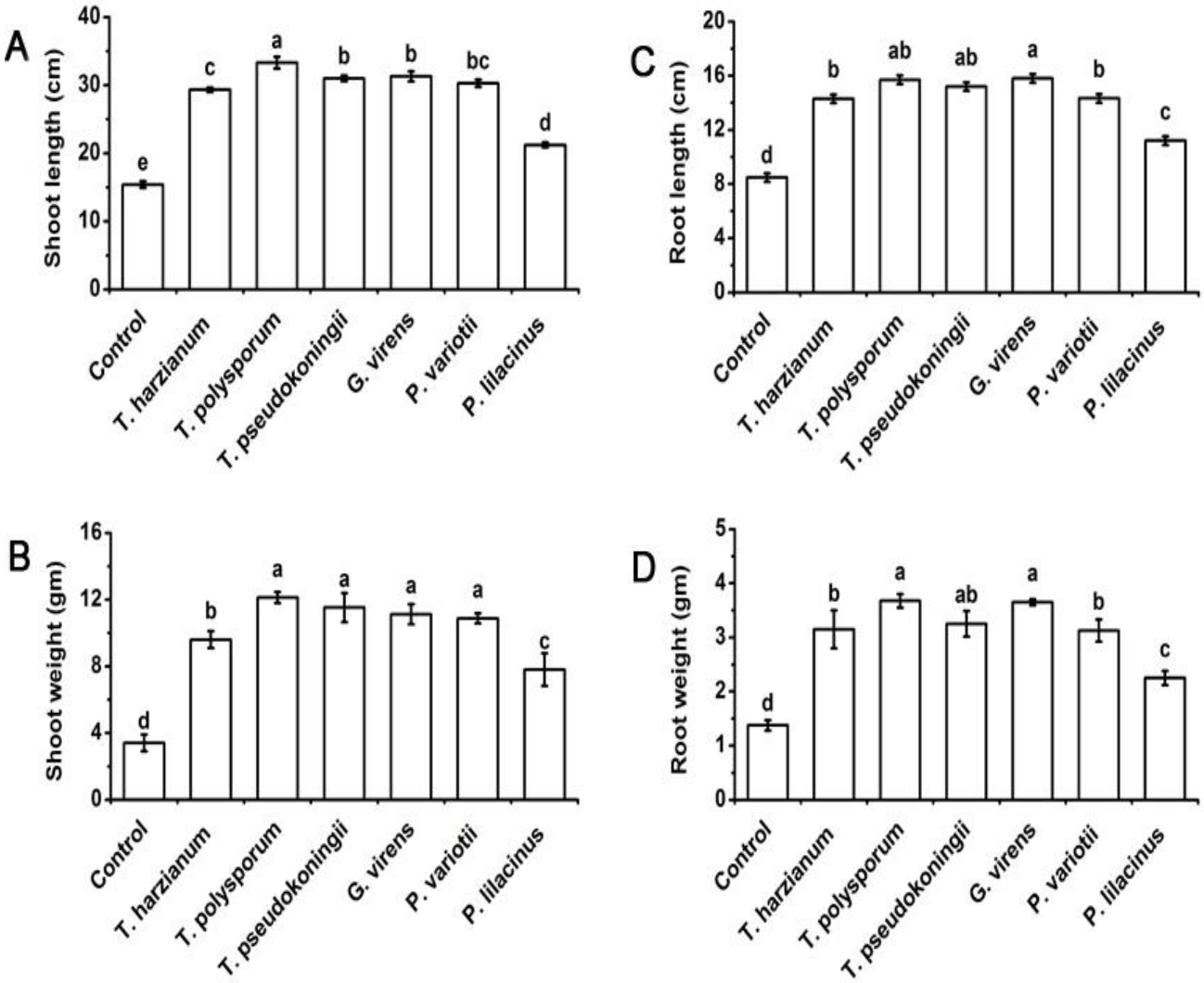


Fig. 6. Effect of antagonistic agents on the growth of chickpea plants inoculated with *F. oxysporum* in infested field. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

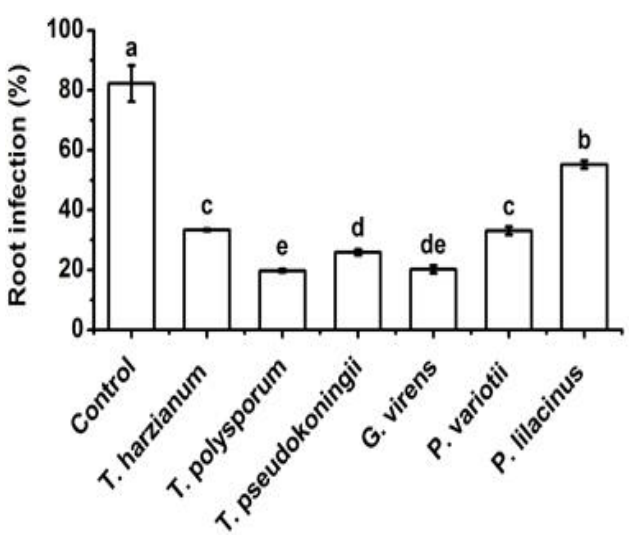


Fig. 7. Effect of antagonistic agents on root infection of chickpea plants inoculated with *F. oxysporum* in infested field. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

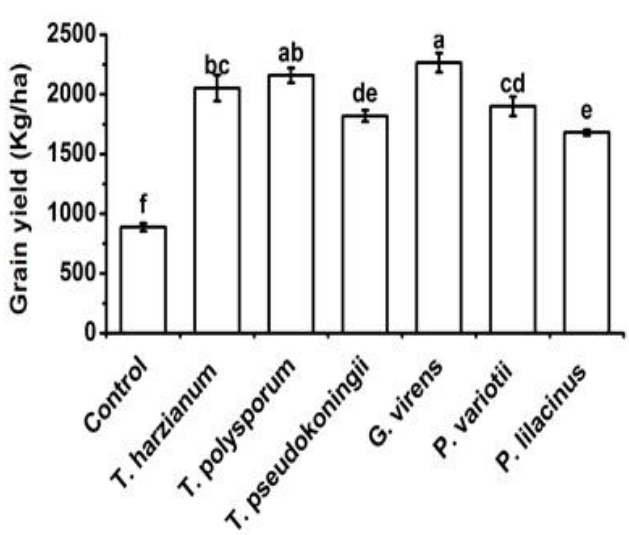


Fig. 8. Effect of antagonistic agents on grain yield of chickpea plants inoculated with *F. oxysporum* in infested field. Bars indicate the standard deviation. Mean with different letters at the upper side of the columns indicate significant differences ($p<0.05$, Tukey-Kramer HSD test).

Discussion

Fusarium oxysporum f. sp. *ciceris* cause destructive wilt disease in chickpea plants. A robust control method is required that manage this soil borne vascular wilt pathogen. Soil borne diseases caused by fungi are difficult to control, especially when the pathogen form resistant structures such as chlamydospores or sclerotia surviving many years in the soil even in the absence of a host crop. Species belong to *Fusarium* are one of similar examples, its chlamydospore surviving 3-6 years in the soil (Haware *et al*., 1996; Ayyub *et al*., 2003). The use of biocontrol agents not only provides effective disease control, but it is safe and environmental friendly; therefore, being widely used against plant pathogens including *Fusarium* spp. There are many reports which revealed the potential of antagonistic fungi against *F. oxysporum* (Poddar *et al*., 2004; Sheikh *et al*., 2006; Zote *et al*., 2007; Dubey *et al*., 2007; Dawar *et al*., 2008; Mukhtar, 2008; Tsai *et al*., 2008; Christopher *et al*., 2010; Akrami *et al*., 2012; Edward *et al*., 2013).

Biocontrol agents isolated from plants and rhizospheric soil usually screened first *In vitro* to determine their antagonistic potential. However, the results obtained *In vitro* frequently do not correlate with those obtained *In vivo*. In present study, *G. virens*, *P. variotii*, *T. harzianum*, *T. pseudokoningii* and *T. polysporum* successfully inhibited the mycelial growth of the *F. oxysporum* f. sp. *ciceris* in dual culture assay. *T. harzianum* found to produce maximum inhibition of mycelial growth of *F. oxysproum* isolated from banana and chickpea plants (Thangavelu *et al*., 2004; Khan *et al*., 2012).

Colonization of plant roots through antagonistic fungi implies the ability to stimulate plant growth and to suppress plant pathogens. Several molecular studies revealed that *Trichoderma* metabolites or roots colonization by *Trichoderma* alters the proteome and transcriptome of plants (Marra *et al*., 2006; Alfano *et al*., 2007; Shoresh & Harman, 2008). The application of biocontrol agents also found effective in promoting the seed germination as well increased vegetative plant growth and decreased wilt incidence. Antagonistic fungi reduced infection and root colonization by pathogen (Thangavelu *et al*., 2004). The reduction in incidence and infection of Fusarium wilt with the application of antagonistic agents has been reported in many crops including *Gliocladium* spp. in eggplant (Watanabe, 1994), *T. harzianum, G. virens, P. lilacinus, Bacillus subtilis* and *Streptomyces* sp. in okra and soybean (Ehteshamul-Haque *et al*., 1990), *Trichoderma viride, T. viriens* and *T. harzianum* in chickpea plants (Dubey *et al*., 2007; Nikam *et al*., 2007; Subhani *et al*., 2013).

This study depicts that all bio-control agents significantly reduced the pathogen infection and disease development with the enhancement of germination, growth of plant and grain production as compared to control plants. *G. virens* and *T. polysporum* were most effective antagonistic fungi followed by *T. pseudokoningii* and *T. harzianum*; whereas, *P. lilacinus* was the least effective antagonistic fungus. Enhancement of plant growth with antagonists is in agreement with (Paulitz *et al*., 1985; Chang *et al*., 1986; Ahmed & Baker, 1987; Kumar *et al*., 2007; Yaqub & Shahzad, 2011) that support the results of the present investigation. Plant growth promoting abilities of *Trichoderma* and *Gliocladium* are well documented (Chet *et al*., 1997; Harman & Bjorkman, 1998). Application of *Trichoderma* may serve as potential biofertilizer for improvement in yield and quality of produce (Molla *et al*., 2012).

Conclusion

The antagonistic fungal biocontrol agents appeared highly effective in controlling chickpea wilt and increasing seed germination, plant growth and yield. Results emphasize their vital importance to control threatening plant pathogens that may serve as alternate of toxic chemical fungicides commonly in use for controlling devastating plant diseases.

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