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Management of *Fusarium* Wilt using mycolytic enzymes produced by *Trichoderma harzianum* (Th. Azad)

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The main aim of this study was to isolate the best chitinase and glucanase enzyme producing *Trichoderma* strain to manage the *Fusarium* wilt disease of *Cicer aritenum* under *in vitro* conditions. We also studied the effect of *Trichoderma* strains on the growth and development of *C. aritenum* plants. Seven strains of *Trichoderma* were screened against the *Fusarium* pathogen to isolate the best biocontrol agent causing maximum inhibition of *Fusarium* growth. *Trichoderma harzianum* (Th. Azad) was found to be the best strains among all the tested strains. *Trichoderma* treated plant exhibited the least disease incidence as compared to control plants. *Trichoderma* treated plant showed a significant stimulatory effect on all the tested eight parameters as compared to control.

Key words: *Trichoderma*, antagonistic activity, chitinase, glucanase, Biocontrol agent, phytopathogenic fungi.

INTRODUCTION

Plants are the major source of food, fibre, fodder, medicines and many other useful products (Naseby et al., 2000). Various insects, bacteria, virus, fungi and other pests attack plants at various stages of their development (Rifai, 1969; Elad, 1983). *Fusarium oxysporum* and *Sclerotinia sclerotiorum* are the major plant pathogens which cause rot, and wilt in plants. For the control of these phytopathogens different chemical fungicides are used (Papavizas, 1985). Extensive use of these chemical fungicides has lead to the development of fungicide resistant strains. Thus, there is a need for identifying alternative measures which can be efficiently used for the control of phytopathogens. *Fusarium* is an important disease which attacks chickpea, bean, wheat, barley and other grains worldwide, especially in humid and semi humid areas (Schroeder and Christensen, 1963; Howell et al., 2003; Haggag and Amin, 2001). The disease caused by this fungus is characterized by wilted plants, yellowed leaves and root rot, and minimal or absent crop yield (Nemec et al., 1976; Harman et al., 2000, 2006). In many regions of the world, chickpea (*Cicer arietinum* L.) is a popular vegetable and chief source of protein in the human diet. During chickpea cultivation problems have occurred that were connected to diseases which could reduce yield and crop quality. Chickpea is susceptible to *Fusarium* root rot strain (*Fusarium solani* (Mart.) Sacc. f.

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sp. eumartii (C. Carpenter) (Snyder and Hans) and *Fusarium* wilt strain (*F. oxysporum* Schlechtend.: Fr. f. sp. ciceris (Padwick) Matuo and K. Sato). Rhizosphere is the first line of protection for roots and rhizospheric micro-organisms producing HCN, siderophore or leading to antibiosis, competition, parasitism and cell lysis can ideally be used as biocontrol agents (Shahid et al., 2012a). As chitinase and β-1, 3-glucanase are two main hydrolytic enzymes associated with fungal cell wall lysis, the purpose of this study was to isolate the best chitinase and glucanase producing *Trichoderma* strain and to screen their antagonistic activity against *F. oxysporum* (Elad, 1999; Pandey et al., 2014 b). So, we can employ *Trichoderma* species for the control of *Fusarium* wilt (Shahid et al., 2012b).

MATERIALS AND METHODS

All the microbes used in this study were isolated from the soil of different locations of UP. All the microbes were purified using serial dilution plate method and preserved on PDA media at 4°C.

Screening of *Trichoderma* strains

All the isolated strains were screened against *Fusarium* by dual culture method to identify the potential and effective strains. Out of different strains of *Trichoderma* screened against *Fusarium* *Trichoderma harzianum* (Th. Azad) showed the maximum inhibition against *Fusarium* under *in vitro* conditions. β-1, 3-Glucanase and chitinase were assayed in culture filtrates and reducing sugar was evaluated by using dinitrosalicylic acid (DNS) solution. One unit of β-1, 3-glucanase/ chitinase enzyme was defined as the amount which liberates 1 U/ml of reducing sugar per hour. *Trichoderma harzianum* (Th. Azad) has earlier been proved successful in their ability to biocontrol diseases in a broad range of plant species (Lorito et al., 1994). Czapek Dox broth (pH 7) was inoculated with *T. harzianum* and incubated for ten days for glucanase enzyme production. For chitinase production chitinolytic media was inoculated with *T. harzianum* under aseptic conditions and incubated at 120 rpm, 28°C. The enzyme activity was measured after seven days of incubation period. It was found that *T. harzianum* produced 2.01 U/mg of glucanase enzyme and 6.2 mg/ ml of chitinase enzyme (Pandey et al., 2014 a and c). *Trichoderma* grown in PD broth at 28°C and 120 rpm for seven days were centrifuged at 6800 g for 12 min at 4°C. Pellet was collected and resuspended in distilled water to obtain a population density of 1×10^8 CFU/ml. 1% carboxy methyl cellulose (CMC) was mixed with the suspension to make slurry and were used to coat surface sterilized *C. aritinum* seeds. Seeds were allowed to dry overnight under sterile condition and CFU was counted by dilution plate method and found to be 3.6×10^8 CFU/seed (Mukesh Srivastav et al., 2014; Wells et al., 1972). Spore inoculum (10^5 conidia/ml) of the selected fungal strains was mixed with sterilized seeds. Sowing was done in pots filled with crystal sands. Three replicates of each treatment were designed: In treatment 1 only *C. aritenum* seeds (C) were used. In treatment second seeds were treated with only biocontrol agent (S) and in treatment third seeds were treated with bioagent as well as with pathogen (R). For each treatment four replicates were used. After 40 days the plants were uprooted and growth (root/shoot length, germination index, total weight, Dry wt of plant, total nitrogen and protein content) were recorded.

RESULTS AND DISCUSSION

In pot experiments, the germination of half of the seeds was inhibited by *F. oxysporum.* However, in the presence of bioagent all the seeds germinated successfully. Similarly, the germination index with *F. oxysporum* alone was only 25% (Table 1 and Figure 1a). The interaction with *T. herzianum* led to better increase in all the 9 attributes as compared to control. About 80% increase in the total weight was recorded when *T. herzianum* was inoculated in *F. oxysporum* infested seeds as compared to uninoculated pathogen. Protein and nitrogen content was also high in the R treatment as compared to S and C (Table 2 and Figures 1b and c). The extensive use of chitinase and glucanase producing microorganism as biological control agents against many fungal pathogens has been reported (Vipul et al., 2014, Vipul et al., 2015). Reports have indicated that application of different species of *Trichoderma* have been found (Vipul et al., 2015; Bell, 1982; Elad, 1999; Ramezani, 2009). Our work has demonstrated the ability of isolates (*T. herzianum*) to destroy the phytopathogens because of mycolytic enzymes production which were biologically active in soil conditions and showed excellent promise as biocontrol agent (Jayarajan and Ramakrishnan, 1991; Biswas and Das, 1999). Several workers (Jayalakshmi et al., 2009; Muhammad and Amusa, 2003; Bunker and Mathur, 2001; Shabir et al., 2012) have reported the inhibition of soil borne fungi, *F. oxysporum* f. sp. ciceri by *Trichoderma* species, due to production of extracellular cell wall degrading enzyme such as chitinase, β-1, 3-glucanase, β-1, 6-glucanase, protease, cellulease and lectin, which help *Trichoderma* in colonizing the host.

Trichoderma species are the most studied biocontrol agents that are used against a variety of fungal plant pathogens. *T. herzianum* is among the most potential species of *Trichoderma* that are commonly used for phytopathogen control. *Trichoderma* employs several mechanisms to combat the effect of phytopathogens. The various mechanisms employed by *Trichoderma* are, secretion of CWDEs, secondary metabolite production, mycoparasitisim, competetion for food and space and induction of host defence response.

Conflict of interests

The authors did not declare any conflict of interest.

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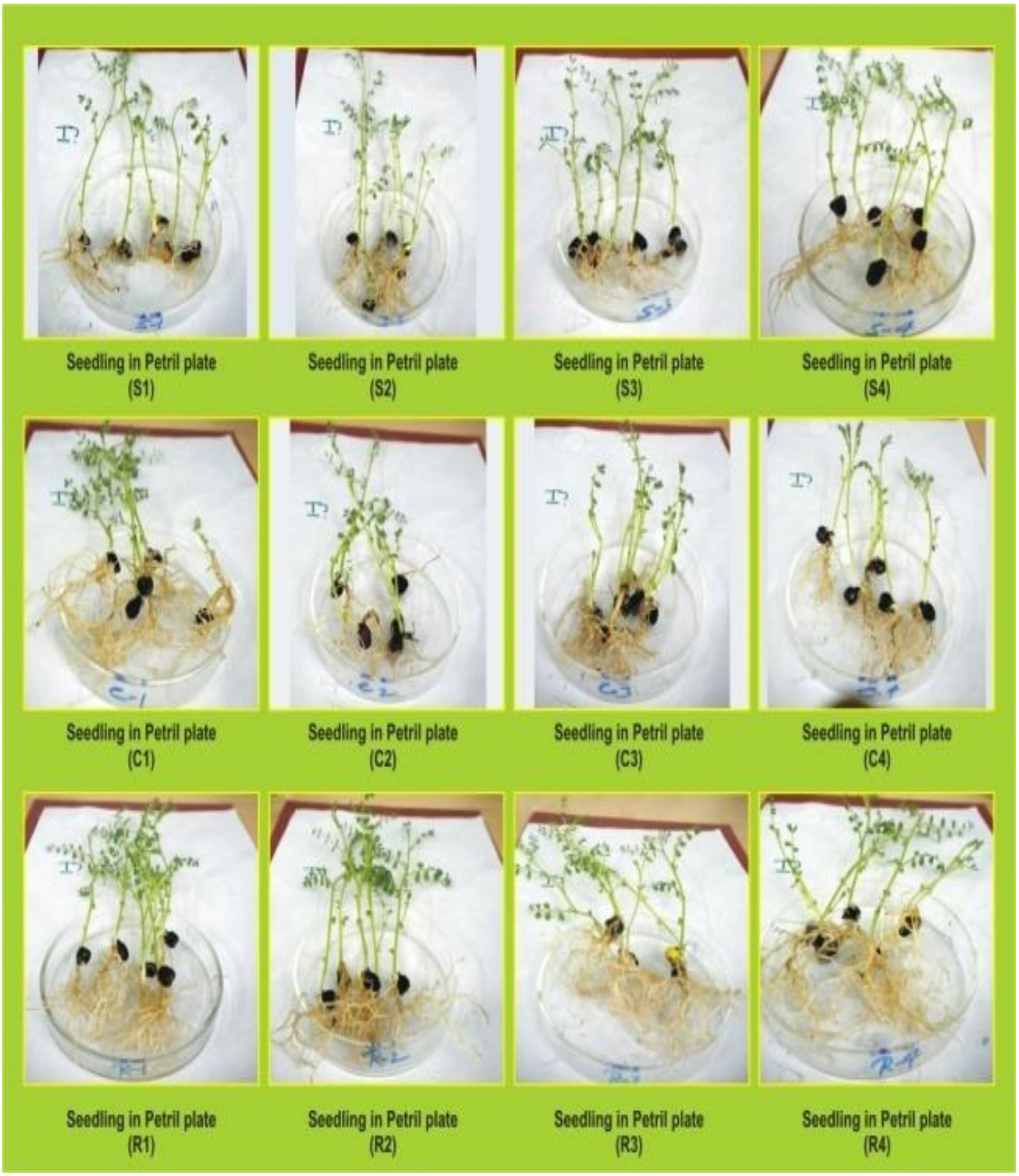
A

Figure 1A. Effects of *Trichoderma* application on all the three treatments **(C** only *Cicer aritenum* seeds, **S** seeds treated with only biocontrol agent and **R** seeds treated with bioagent as well as with pathogen) of *Cicer aritenum* seeds. For each treatment four replicates were used. **A:** Seed treatment process of *Cicer aritenum* seeds and their growth in crystal sand.



B

Figure 1B. Effects of *Trichoderma* application on all the three treatments **(C** only *Cicer aritenum* seeds, **S** seeds treated with only biocontrol agent and **R** seeds treated with bioagent as well as with pathogen) of *Cicer aritenum* seeds. For each treatment four replicates were used. Uprooted *cicer aritenum* plants.

C

Figure 1C. Effects of *Trichoderma* application on all the three treatments **(C** only *Cicer aritenum* seeds, **S** seeds treated with only biocontrol agent and **R** seeds treated with bioagent as well as with pathogen) of *Cicer aritenum* seeds. For each treatment four replicates were used. Dried *Cicer aritenum* plants.

Table 1. Effect of mycolytic enzymes produced by *Trichoderma* on the different growth parameters of all the three treatments (C only *Cicer aritenum* seeds, S seeds treated with only biocontrol agent and R seeds treated with bioagent as well as with pathogen) of *Cicer aritenum* seeds.

Treatment	Germination	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Dry Weight (g)	Vigour Index I (g)	Vigour Index II (g)
R1	20	11.3	14.8	21.0	0.36	420.0	7.2
R2	24	4.0	17.0	24.8	0.50	595.2	12.0
R3	17	7.6	18.4	29.2	0.46	496.4	7.82
R4	17	4.8	16.9	25.1	0.39	426.7	6.63
Average	**19.5**	**16.925**	**16.77**	**25.02**	**0.42**	**484.5**	**8.41**
S1	13	6.2	14.2	19.0	0.38	341.9	5.33
S2	18	7.8	13.2	18.3	0.28	288.0	4.32

Table 1. Contd

S3	13	10.8	13.1	16.7	0.30	221.0	4.68
S4	20	8.2	16.9	22.5	0.38	288.0	6.2
Average	**16**	**8.25**	**14.35**	**19.12**	**0.335**	**284.7**	**5.13**
C1	12	4.8	15.00	26.3	0.41	228.0	4.56
C2	10	5.1	12.0	16.0	0.24	183.0	2.8
C3	16	3.6	9.4	17.0	0.36	267.0	4.8
C4	14	5.6	9.6	14.4	0.31	315.0	5.32
Average	**13**	**4.77**	**11.5**	**18.42**	**0.33**	**248.3**	**4.37**

For each treatment four replicates were used.

Table 2. Biochemical effect of mycolytic enzymes produced by *Trichoderma* on all the three treatments (C only *Cicer aritenum* seeds, S seeds treated with only biocontrol agent and R seeds treated with bioagent as well as with pathogen) of *C. aritenum* seeds.

Treatment	Nitrogen (%)	Protein (%)
R1	0.370	2.31
R2	0.330	2.05
R3	0.300	2.18
R4	0.320	2.02
Average		**2.14**
S1	0.318	1.98
S2	0.310	1.93
S3	0.290	1.81
S4	0.300	1.87
Average		**1.89**
C1	0.274	1.71
C2	0.266	1.66
C3	0.280	1.75
C4	0.260	1.62
Average		**1.68**

For each treatment four replicates were used.

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