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EVALUATION OF MICROBIAL CONSORTIA ON SYSTEMIC RESISTANCE AGAINST CHICKPEA WILT

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Abstract

The role of *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* (alone and in combination) in inducing systemic resistance in chickpea exposed to *Fusarium oxysporum ciceri*s was investigated**.** Chickpea seed (var. Radhey) treatment with *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* alone and in combination on the germination, wilt incidence, plant growth promotion, yield, production of chlorophyll, protein, peroxidase, polyphenol oxidase and total phenol content was recorded. The results of *in vitro* studies revealed better performances of treatment T_6 (Seed treatment with 1% *Trichoderma harzianum* + 2% *Pseudomonas fluorescens* + 2% *Bacillus subtilis* in combination). This treatment significantly reduced wilt incidence and increased germination, root length, shoot length, and yield as compared to untreated control. Treatment T_6 also induced 1.4 per cent increase in peroxidase, 1.6 in polyphenol oxidase and 2.3 in total phenol in chickpea during pathogenesis by *F. oxysporum ciceris* f. sp. Similarly, 1.9% increase in chlorophyll and protein was recorded with the treatment T_6 as compared to control. Present investigations will be helpful in formulating novel bioformulations using fungal and bacterial bioagents to control wilt in chickpea.

Introduction

Chickpea (*Cicer arietinum* L.) is an important crop of Indian subcontinent. Various microbial diseases are responsible for its low productivity. *Fusarium oxysporum* f. sp. *ciceris* a potential fungal pathogen causes wilt in chickpea and remains in soil in the form of chlamydospores which serves as primary inoculum. Resistant varieties have been proved to be an effective method to manage the disease but good resistant cultivars are not available to combat with the pathogen. The preference for biological control method is justified also by the undesirable side effects of pesticides. Various workers stated that use of biocontrol agents (BCAs) is an effective method to manage soil borne diseases of crops without resistant sources (Moradi *et al.* 2012, Mukherjee *et al.* 2012, Saxena *et al*. 2015). Earlier workers reported that *Pseudomonas* and *Trichoderma* spp. play an important role in increasing phenol (Ramamoorthy *et al.* 2002, Saravanakumar *et al*. 2007) peroxidases, polyphenoloxidases and phenylalanine ammonia-lyase (PAL) (Chen *et al*. 2000) in different crops. BCAs like *Trichoderma, Bacillus* and *Pseudomonas* are known as effective antagonists against many soil borne pathogens (Saxena *et al.* 2016). For induced systemic resistance, strains of fungal and bacterial antagonists as *Trichoderma, Bacillus* and *Pseudomonas* spp. appear to be promising. However, for biological management of chickpea wilt investigations on induced systemic resistance (ISR) is lacking. Therefore, the present study was undertaken to focus on the efficacy of *T. harzianum*, *B. subtilis* and *P. fluorescens* against chickpea wilt pathogen through induced growth and resistance.

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Materials and Methods

The pathogen *Fusarium oxysporum* f. sp. *ciceris* isolated on PDA medium from infected roots collected from different places of central Uttar Pradesh, India. *Fusarium* cultures were identified on the basis of their morphology as described by Booth (1971). Serial dilution technique (Johnson and Curl 1972) was adapted for isolation of *T. harzianum, Bacillus subtilis* and *Pseudomonas fluorescens* from rhizospheric soil collected from chickpea ecosystem (U.P., India). Purified cultures of *T. harzianum, B. subtilis* and *P. fluorescens* were maintained on potato dextrose agar (PDA) and nutrient agar (NA). For reconfirmation, purified cultures of fungal and bacterial bio-agents were sent to Indian Type Culture Collection (ITCC), New Delhi, India. Based on the identification report the fungal and bacterial bio-agents were identified as *T. harzianum* (ITCC 9864) *Bacillus subtilis* (ITCC B0047) and *Pseudomonas fluorescens* (ITCC B0034).

To assess the *in vitro* effect of *T. harzianum, B. subtilis* and *P. fluorescens* against *F. oxysporum* f. sp. *ciceris* a laboratory bioassay using dual culture technique (Morton and Stroube 1955) was conducted. Five mm disc from the growing colony of the pathogen was inoculated first with the help of sterilized inoculation needle and one day later, 5 mm mycelial disc from fungal antagonist was inoculated. Bacterial antagonists were streaked on one side of the Petri plates containing pathogen. Petriplates without antagonists served as control. Observations were recorded up to 72 hrs and per cent growth inhibition was calculated.

Compatibility among *T. harzianum, B. subtilis* and *P. fluorescens* was tested through dual culture inoculation technique to deduce whether they are compatible with each other or not (Siddiqui and Shaukat 2003). For this overnight cultures of *P. fluorescens* and *B. subtilis* were streaked in triplicates on PDA Petri plates at one side. At another side 5 mm disc of *T. harzianum* was placed and incubated at 25±1°C. Studies were undertaken to investigate the effect of talc-based formulation of *T. harzianum, B. subtilis* and *P. fluorescens,* as seed treatment (alone and in combination) for disease resistance, plant growth and defence response against *F. oxysporum* f. sp. *ciceris* in glasshouse conditions. Pathogen inoculum was prepared on sand-maize meal medium (50 g + 1.5 g + 10 ml water) (Miller 1946). Treated chickpea seeds (var. Radhey) were sown in soil infested with *F. oxysporum* f. sp. *ciceris (*@ 2% (w/w) filled in plastic pots of 8 cm dia. (Trivedi *et al*. 2013). Pots sown with untreated seeds were kept as control. For each treatment four pots were maintained. This experiment was repeated twice under glass house conditions with following treatments: T_1 - Chickpea seed treated with 1% *T. harzianum* (2×10^8cfu/g) @ 4g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_2 - Chickpea seed treated 2% *B. subtilis* (1×10^8cfu/g) @5g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_3 - Chickpea seed treated with 2% *P. fluorescens* (1×10^8cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_4 - Chickpea seed treated with *T. harzianum* (1%) and *P. fluorescens* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceri* @ 2% w/w, T_5 - Chickpea seed treated with *T. harzianum* (1%) and *B. subtilis* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_6 - Seed treatment with *T. harzianum* (1%) + *P. fluorescens* (2%) + *B. subtilis* (2%) and soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_7 - Seeds without prior treatment of bio-control agents + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w. Effect of these bioagents as seed treatment on root length, shoot length, vigour index, yield/treatment was also estimated. For assessment of defense-related enzymes and proteins chickpea leaves from each treatment were taken after one week of disease initiation for estimation of chlorophyll, peroxidase, phenol, polyphenoloxidase and protein content. One g sample from each treatment was weighed and mixed with 10 ml phosphate buffer (50 m mol, pH 7.0). The mixture was grinded in ice chilled mortar-pestle and centrifuged at 12,000 g for 20 min at 4ºC. The supernatant was used to assess chlorophyll, enzymes and protein content. For all graphical representations Micro Soft excel program was used. (Muthukumar *et al.* 2011)

To access chlorophyll content in each treatment 100 mg leaf sample was added with 80% acetone and grinded using mortar and pestle. The leaf homogenate was filter through filter paper.

Peroxidase activity was determined with dehydrogenation of guaiacol as a substrate. Polyphenoloxidase activity was calculated using method described by Zauberman *et al.* (1991). Extraction of protein was done using method described by Goggin *et al.* 2011. Protein content in all samples was calculated by the method described by Lowry (Lowry *et al*. 1951). Statistical analyses of laboratory and pot experiments were done by the method of CRBD prescribed by Goon *et al.* (1931).

Results and Discussion

Antagonistic potential of *Trichoderma harzianum*, (ITCC 9864), *Bacillus subtilis* (ITCCB0047) and *Pseudomonas fluorescens* (ITCC B0034) through dual culture indicated that colony growth of the pathogen after 72 hrs was 15.7 - 25.5 mm as compared to control. Colony growth of test pathogens was appressed and after coming in contact, the antagonists grew and sporulated over the pathogen colony due to their prolific growth habit and mycoparasitic character (Fig. 1). The results of *in vitro* studies revealed that *T. harzianum*, *B. subtilis* and *P. fluorescens* caused 49.0, 60.0, and 68.6 per cent reduction in mycelial growth of the pathogen, respectively over control (Table 1). Compatibility among *T. harzianum*, *B. subtilis* and *P. fluorescens* was

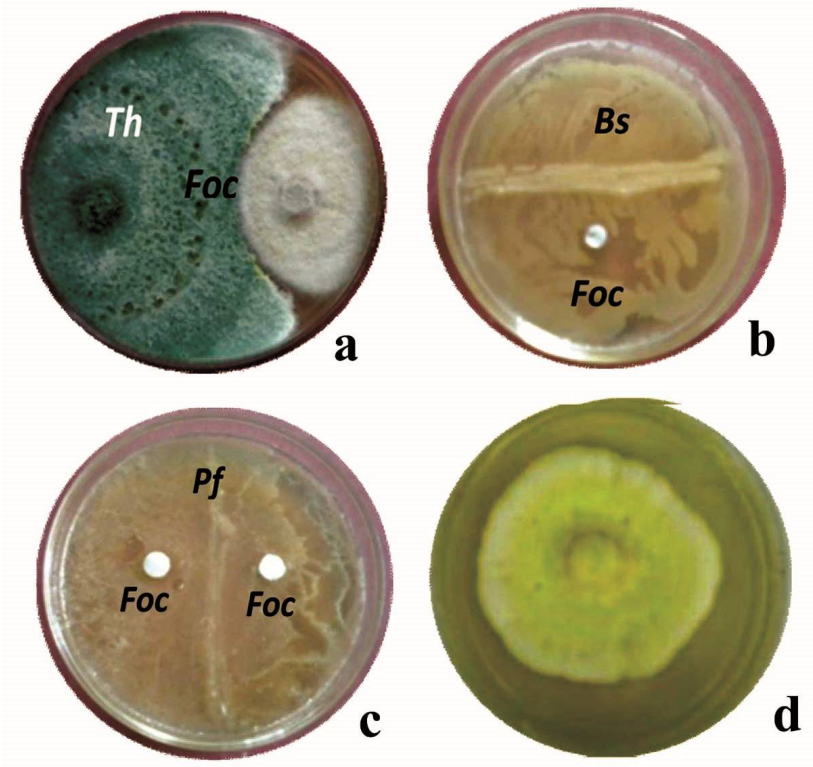

Fig. 1. Antagonism of *T. harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp. *ciceris.* (a) *Fusarium oxysporum* f. sp. *ciceris* + *T. harzianum*, (b) *Fusarium oxysporum* f. sp. *ciceris* + *B. subtilis*, (c) *Fusarium oxysporum* f. sp. *ciceris* + *P. fluorescens* and (d) *Fusarium oxysporum* f. sp. *ciceris* (control).

Table 1. Growth inhibition in *F. oxysporum* f. sp. *ciceris* in presence of bio-agents.

Treatments	Radial growth of pathogen (mm)	% inhibition in growth
Foc + Th	25.5	49.0
Foc + Bs	20.0	60.0
Foc + Pf	15.7	68.6
Control	50.0	-
SE(d)	1.2	-
CD = 0.05p	0.54	-

Foc = *Fusarium oxysporum* f. sp. *ciceris*, Th = *T. harzianum*, Bs = *Bacillus subtilis*, Pf = *Pseudomonas fluorescens.*

tested on the basis of inhibition zone. Studies revealed that no such zone was formed between fungal and bacterial bio-agents indicating that these were compatible with each other (Fig. 2). Jain *et al.* (2011) studied compatibility among *P. fluorescens, B. subtilis* and *T. harzianum* and found that all the three microbes were compatible with one another. Belkar and Gade (2012) studied compatibility of *P. fluorescens* with beneficial microbes. Among 15 isolates, 6 were most compatible with *T. harzianum* while 8 were the best compatible with *B. subtilis.* The results of *in vitro* studies revealed the supremacy of treatment T_6 (Seed treatment with 1% *T. harzianum* + 2%

Fig. 2. Compatibility among *T. harzianum, Bacillus subtilis* and *Pseudomonas fluorescens*.

P. fluorescens + 2% *B. subtilis* in combination). This treatment significantly reduced wilt incidence as compared to control. Among various treatments tested as seed treatment T_6 significantly reduced the wilt incidence as 60% followed by T_4 (seed treatment with 1% *T. harzianum* + 2% *Pseudomonas fluorescens*) and T_5 (Seed treatment with 1% *T. harzianum* + 2% *B. subtilis* in combination) with 46.8 and 45.6%, respectively reduction over control. Seedling treatments with 2% *B. subtilis*, 1% *T. harzianum* and 2% P. *fluorescens* (alone) also showed significantly better results over control. Untreated control T_7 showed the maximum of 50% wilt incidence. With regard to germination and seedling growth parameters, the treatment T_6 showed the highest germination percentage (80.0), shoot length (5.33 cm), root length (3.91 cm), vigour index (739.20) and yield (150.33 g/treatment). This was followed by T_4, T_5, T_3, T_1 and T_2. The least values of germination, growth parameters and yield were observed in untreated control T_7. (Table 2, Fig. 3) Niranjana *et al.* (2009) tested *Trichoderma viride* and *Pseudomonas fluorescens* as talc- wheat and found that seed dressing with such bioagents effectively reduced disease

incidence in wheat. The results demonstrated that *T. harzianum* with *P. fluorescens* and *B. subtilis* in consortia increased the growth and controlled the severity of wilt in chickpea plants. Thaware *et al.* (2017) made *in vitro* studies on the efficacy of *T. harzianum*, *P. fluorescens* and *B. subtilis* against *Fusarium oxysporum* f. sp. *ciceris* and observed that fungal and bacterial bio-agents

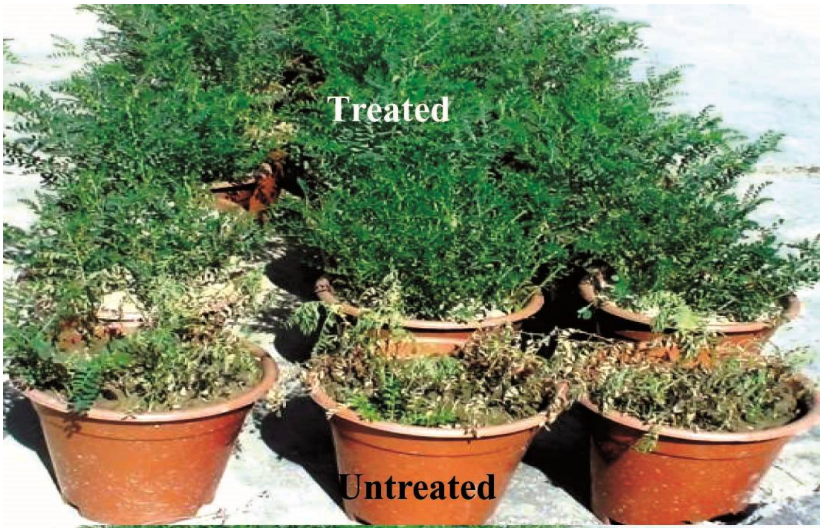

Fig. 3 Effects of chickpea seed treatment with *T. harzianum*, *B. subtilis* and *P. fluorescens* (alone and in combination) on wilt incidence in chickpea.

Table 2. Effects of seed treatment with *T. harzianum*, *B. subtilis*, and *P. fluorescens* on plant growth promotion and wilt incidence.

Treatments	Germination* (%)	Root* length (cm)	Shoot* length (cm)	Wilt incidence (%)	Disease control (%)	Yield* (g/treatment)	Vigour index
T_1	60.0	3.21	4.18	33.3	33.4	116.56	443.40
T_2	66.6	3.20	4.12	30.3	39.4	108.60	487.50
T_3	69.7	3.16	3.98	29.5	41.0	120.43	497.65
T_4	73.3	3.24	4.39	26.6	46.8	135.64	559.27
T_5	70.1	3.19	4.21	27.2	45.6	122.34	518.74
T_6	80.0	3.91	5.33	20.0	60.0	150.33	739.20
T_7	53.3	3.04	3.23	50.0	-	90.61	334.19
SE(d)	1.44	1.1	1.3	1.6	-	1.64	1.6
CD = 0.05	0.66	0.50	0.63	0.75		3.52	3.5

*Mean of three replications. T_1 = Chickpea seed treated with 1% *T. harzianum* (2×10^8cfu/g) @4g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_2 = Chickpea seed treated 2% *B. subtilis* (1×10^8cfu/g) @5g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_3 = Chickpea seed treated with 2% *P. fluorescens* (1×10^8cfu/g) @5g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_4 = Chickpea seed treated with *T. harzianum* (1%) and *P. fluorescens* (2%) + soil inoculated with *F. oxysporum* f.sp. *ciceri* @ 2% w/w, T_5 = Chickpea seed treated with *T. harzianum* (1%) and *B. subtilis* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_6 = Seed treatment with *T. harzianum* (1%) + *P. fluorescens* (2%) + *B. subtilis* (2%) and soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w and T_7 = Seeds without prior treatment of bio-control agents + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w.

Table 3. Effects of seed treatment with microbial bio-agents on peroxidase and polyphenol oxidase and phenol content in chickpea.

Treatments	Peroxidase activity A_{430}	Polyphenoloxidase activity A_{410}	Phenol content (mg catechol/g)
T_1	0.3	0.119	29.85
T_2	0.34	0.12	32.65
T_3	0.356	0.125	36.2
T_4	0.405	0.14	46.1
T_5	0.389	0.131	42.5
T_6	0.43	0.155	49.55
T_7 (Control)	0.291	0.092	21.3

T_1 = Chickpea seed treated with 1% *T. harzianum* (2×10^8cfu/g) @4g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_2 = Chickpea seed treated 2% *B. subtilis* (1×10^8cfu/g) @ 5g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_3 = Chickpea seed treated with 2% *P. fluorescens* (1×10^8 cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_4 = Chickpea seed treated with *T. harzianum* (1%) and *P. fluorescens* (2%) + soil inoculated with *F. oxysporum* f.sp. *ciceri* @ 2% w/w, T_5 = Chickpea seed treated with *T. harzianum* (1%) and *B. subtilis* (2%) + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_6 = Seed treatment with *T. harzianum* (1%) + *P. fluorescens* (2%) + *B. subtilis* (2%) and soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_7 = Seeds without prior treatment of bio-control agents + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w.

Table 4. Effects of seed treatment with microbial bio-agents on chlorophyll content in chickpea.

Treatments	Chlorophyll content (mg/g)		
	Chl. a	Chl. b	Total chlorophyll
T_1	1.611	0.641	2.252
T_2	1.623	0.654	2.277
T_3	2.245	0.826	3.071
T_4	2.332	1.052	3.384
T_5	1.835	0.934	2.769
T_6	2.368	1.024	3.392
T_7 (Control)	1.291	0.483	1.774

T_1 = Chickpea seed treated with 1% *T. harzianum* (2×10^8 cfu/g) @ 4 g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_2 = Chickpea seed treated 2% *B. subtilis* (1×10^8 cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_3 = Chickpea seed treated with 2% *P. fluorescens* (1×10^8 cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_4 = Chickpea seed treated with *T. harzianum* (1%) and *P. fluorescens* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceri* @ 2% w/w, T_5 = Chickpea seed treated with *T. harzianum* (1%) and *B. subtilis* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_6 = Seed treatment with *T. harzianum* (1%) + *P. fluorescens* (2%) + *B. subtilis* (2%) and soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_7 = Seeds without prior treatment of bio-control agents + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w.

exhibited significant inhibition in mycelial growth of the pathogen. Zaim *et al.* (2018) investigated efficacy based formulation against several crop diseases. Similarly, Duffy *et al.* (1996) tested *Pseudomonas fluorescens* and *Trichoderma koningii* based formulation against take-all disease in of *B. subtlis* and *T. harzianum* separately and in combination against *Fusarium oxysporum* f. sp.

*ciceri*s and reported that seed treatment with both antagonists effectively reduced 93.67% wilt incidence as compared to control. Combined treatment also induced plant height, root length, shoot length and fresh and dry weights of the same. Peroxidase (PO) and polyphenoloxidase (PPO) activities were observed significantly higher in treatments where fungal and bacterial bioagents are in combinations as compared to untreated control. Maximum activity was recorded in treatment T_6, T_4. Table 3 clearly indicated that seed treatment with 1% *T. harzianum* + 2% *P. fluorescens* + 2% *Bacillus subtilis* induced maximum PO and PPO activity as 0.430 and 0.155,

Table 5. Estimation of protein content in chickpea plants treated with microbial bio-agents.

Treatments	Treatment details	Protein concentration (mg/ml)
T_1	1% *T. harzianum*	0.177
T_2	2% *B. subtilis*	0.151
T_3	2% *P. fluorescens*	0.201
T_4	1% *T. harzianum* + 2% *P. fluorescens*	0.226
T_5	1% *T. harzianum* + 2% *B. subtilis*	0.198
T_6	1% *T. harzianum* + 2% *P. fluorescens* + 2% *B. subtilis*	0.278
T_7	Control	0.142

T_1 = Chickpea seed treated with 1% *T. harzianum* (2×10^8 cfu/g) @ 4 g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_2 = Chickpea seed treated 2% *B. subtilis* (1×10^8 cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f.sp. *ciceris* @ 2% w/w, T_3 = Chickpea seed treated with 2% *P. fluorescens* (1×10^8 cfu/g) @ 5 g/kg seed + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_4 = Chickpea seed treated with *T. harzianum* (1%) and *P. fluorescens* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceri* @ 2% w/w, T_5 = Chickpea seed treated with *T. harzianum* (1%) and *B. subtilis* (2%) + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_6 = Seed treatment with *T. harzianum* (1%) + *P. fluorescens* (2%) + *B. subtilis* (2%) and soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w, T_7 = Seeds without prior treatment of bio-control agents + soil inoculated with *F. oxysporum* f. sp. *ciceris* @ 2% w/w.

respectively as compared to other treatments. Treatment T_7 showed the least enzyme activity as 0.291 and .092 indicating that bio-agents consortia have strong ability to induce defense response in treated plants (Figs 4, 5). Observations on phenol content also indicated that maximum phenol content was in chickpea plants treated with consortium of three microbes. Treatment T_6 showed maximum phenol content as 49.55 mg catechol/g followed by T_4 with 46.1 mg catechol/g. Least phenolic content as 21.3 mg catechol/g was observed in untreated control. Chlorophyll content was also observed higher in plants treated with T_6 as 3.392 mg/g followed by T_4 with 3.384 mg/g. Minimum chlorophyll content 1.774 mg/g was recorded in T_6 (Table 4). Observations on total protein content in treated chickpea plants revealed that seed treatment with T_6 was the best yielded protein as 0.278 mg/ml in pot trials. Next in order of superiority was treatment T_4 with 0.226 mg/ml protein. The minimum values of protein content as 0.142 mg/ml was observed in inoculated control with pathogen alone (Table 5). The present investigations indicated that chickpea plants pretreated with *T. harzianum, P. fluorescens* and *B. subtilis* in combination exhibited increased level of enzymes and phenol against wilt. The results indicated that seed treatment with such bioagents in combination induced 1.0 - 1.4-folds increase in PO while, 1.2 - 1.6-folds in PPO. In case of total phenol 1.4 - 2.3-folds increase was observed in chickpea during pathogenesis by *Fusarium oxysporum* f. sp. *ciceris.* Similarly, 1.0 - 1.9-folds increase in protein and 1.2 - 1.9-folds increase in total chlorophyll content were recorded in treated plants as compared to control. Combined treatment of fungal and bacterial antagonists was found to be

more effective than either bacteria or fungus alone. Interestingly, it was also observed that enzyme activities and phenol content were higher in plants treated with mixture of *T. harzianum* and *P. fluorescens* as compared to *T. harzianum* and *B. subtilis*. This is because bacilli typically have longer generation time and less PGPR activities than *Pseudomonas* spp. Increased level of chlorophyll and protein is concerned with the plant growth promotion activity induced by bio-control agents while peroxidase, polyphenol oxidase and phenol are known defence enzymes responsible for antagonism against various pathogens. The results indicated that formulation of such bio-agents in combination induced higher levels of defence enzymes in chickpea during pathogenesis by *Fusarium oxysporum* f. sp. *ciceris*. Jain et *al*. (2011) mentioned that microbial consortium comprising of *Bacillus subtilis, Pseudomanas aeruginosa* and *Trichoderma harzianum* increased resistance against infection by *Sclerotinia sclerotiorum* in pea. Thus, it is concluded that application of *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* in combination promote plant growth and induce chlorophyll, phenol, protein and activities of defense related enzymes which in turn induce biochemical defense mechanism in chickpea against wilt.

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