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MANAGEMENT OF COLLAR ROT OF CHICKPEA (*CICER ARIETINUM*) BY *TRICHODERMA HARZIANUM* AND PLANT GROWTH PROMOTING RHIZOBACTERIA

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Abstract: Collar rot (*Sclerotium rolfsii*) of chickpea (*Cicer arietinum*) is one of the devastating soil-borne diseases of fungal origin, due to which 10–30% yield loss is recorded annually according to severity of the disease. Management of collar rot of chickpea is not feasible in the absence of effective soil fungicides. However, *Trichoderma harzianum* and plant growth promoting rhizobacteria (PGPR) have shown high efficacy against this disease *in vitro* as well as in the field. We used *T. harzianum* (10^4, 10^6 and 10^8 spore/ml) and two PGPRs (*Pseudomonas fluorescens* strain 4 and *P. aeruginosa*) as foliar spray with the fresh and heat inactivated microorganisms. Foliar application of *T. harzianum* (10^8 spore/ml) and *P. fluorescens* strain 4 (10^8 cfu/ml) showed maximum efficacy in reducing plant mortality as compared to the control. Foliar application of fresh-and heat-inactivated (121°C for 10 min) *P. fluorescens* strain 4, and *T. harzianum* reduced 15–25% plant mortality but *P. aeruginosa* showed very little disease control of 10–15%. However, regarding plant growth promotion, it was observed that fresh-and heat-inactivated *P. fluorescens* strain 4 showed maximum efficacy followed by fresh and heat inactivated *P. aeruginosa* and *T. harzianum* as compared to the control. The disease-controlling efficacy was also associated with the increase in phenolic acid synthesis in chickpea plants. The control of chickpea collar rot by biocontrol agents is safe and ecologically sound and appears to be a healthy approach to the disease control.

Key words: *Sclerotium rolfsii*, *Trichoderma harzianum*, plant growth promoting rhizobacteria, disease management

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INTRODUCTION

Every year, pests detrimental to agriculture, forestry, and public health cause losses in million of dollars. Several strategies were used for the management of the crop pests. Among these strategies physical, chemical, biological and resistance breeding are the most common. In chemical strategy, pesticides are used with a broad range or spectrum of activity and controlling several pests. Increasing awareness of public concern regarding a continued use of agro-chemicals that are damaging to biotic and abiotic environment are driving the search of more environmentally safe methods that will contribute to the goal of sustainability in agriculture (Herman *et al*. 2004). There are a number of disadvantages in using such chemical pesticides because of their broad spectrum of activity and these pesticides may destroy non-target organisms such as beneficial insects and parasites of destructive pests (Haas *et al.* 2000).

However phytopathogens are the major constraint in crop production. The control of soilborne diseases such as collar rot of chickpea (*Sclerotium rolfsii*) is not feasible because of un-availability of soil fungicides and much higher cost required for controlling the disease (Maurya *et al*. 2007). However, the use of biocontrol agents like *Trichoderma* and soilborne, non-pathogenic bacteria with the ability to antagonize fungal phytopathogens and thus prevent plant disease represent a realistic alternative to chemical fungicides. *Trichoderma harzianum* is an effective biocontrol agent which mycoparasitized on *Sclerotium rolfsii, Rhizoctonia solani* and *Pythium aphanidermatum* (Elad *et al.* 1980, 1983; Sivan *et al.* 1984; Benhamou and Chet 1996; Chet 1987, 1990). The process involved in mycoparasitism may consist of sensing the host, followed by directed growth, contact, recognition, attachment, penetration, and exit (Chet *et al*. 1981; Benhamou *et al*. 1999; Davanlou *et al*. 1999).

Plant Growth Promoting Rhizobacteria (PGPR) have shown an effective management against disease as reported by Kerry (2000). Interest in use of biocontrol agents is continuing, both within India and internationally. After passed down through generations and even today much of the knowledge is left within the families of traditional practices like farmyard manure and compost flourish day by day to increase demand of organic food for safety of health. Plant growth promoting rhizobacteria (PGPRs) have ability to colonize the roots of plants and produce antifungal metabolites represent a real alternative for the replacement of chemical fungicides (Burelle *et al*. 2006). Several reports indicate that when these bacteria are applied as foliar spray, they induce resistance in plants. There are reports which also indicate that heat inactivated PGPRs also have the ability to check various bacterial diseases to a limited extent. The approaches adopted in this experiment may be the development of one of novel techniques for enhancement of biocontrol efficacy of various biocontrol agents (Sarma *et al*. 2002; Burelle *et al*. 2006).

Keeping these in view an experiment was conducted to see by foliar application of fresh-and heat-inactivated *T. harzianum* and plant growth promoting rhizobacteria (Pf4 and Pag) and see the status of phenolic acids induction in plants and management of collar rot (*S. rolfsii*) of chickpea (*Cicer arietinum*).

MATERIALS AND METHODS

The Host

C. arietinum cv Avrodhi a widely acceptable cultivar of chickpea which has wilt resistance and widely grown in India, was selected for these experiments. Chickpea

is an annual plant with plant height ranging from 30 to 70 cm. The foliage is covered with glandular hairs, which secretes highly acidic exudates, and is considered important in conferring tolerance to insect pests, such as the pod borer. Leaves are compound, arranged in the alternate manner and generally perallel with 11 to 13 leaflets. Flowers are axillary, solitary, or in inflorescence of two or three white, pink, purplish, or blue in color (Anonymous 1984). In the experiment, seeds were grown in earthen pots (15 cm dia.) containing sterilized soil. Twenty days old plants were selected for conducting the study.

Biocontrol agents

Trichoderma sp., was isolated from the native soil of Agricultural farm of the Banaras Hindu University, Varanasi and identified as *T. harzianum*. The fungus grows toward hyphae of other fungi, coils around them, and attaches to host mycelium (Herman *et al*. 2004). Conidiophores are erect and produce side branches bearing whorls of short phialides. Branches are not swollen at the apex and bear terminal conidial heads. Conidium one-celled, ovoid, and produced successively from tips of phailides collected into small wet masses. Individual cells range from 25–70 µm in length and 2.5–3.5 µm in diam. Colonia are grow quickly producing white, yellow, or green cushions of sporulating branches.

Plant growth promoting rhizobacteria (PGPRs: *Pseudomonas fluorescens* strain 4 and *P. aeruginosa*) used in this experiment was obtained from EID Parry, India.

Isolation and purification of *S. rolfsii*

S. rolfsii was isolated by picking up sclerotia produced on the infected chickpea plant as well as other host plants in the Agricultural Research farm of Banaras Hindu University. Surface sterilization of sclerotia was done by dipping them in 0.1% $HgCl_2$ for five-ten seconds followed by 3–4 subsequent washing with sterilized distilled water. The sclerotia were then placed in a Petri dish containing PDA and incubated at 27 ± 2°C for germination. The cultures were purified and maintained on PDA.

Foliar spray of chickpea with fresh and heat-inactivated *T. harzianum* and plant growth promoting rhizobacteria of chickpea

Twenty-day old chickpea (cv Avrodhi) plants were used for this experiment. Foliar spray was done using three concentrations (10^8 cfu/ml) of fresh and heat-inactivated *T. harzianum* and plant growth promoting rhizobacteria (*P. fluorescens* strain 4 and *P. aeruginosa*) at the concentration of 10^8 cfu/ml. Untreated plants were maintained as control. Biochemical estimation (phenolic acids) of leaves was done after spraying with biocontrol agents. Sampling was done two times: 48 h after spraying with bioagents and 48 h after sclerotial germination. Plant mortality rate was used as a criterion to evaluate efficacy of the biocontrol agent. All the experiments were repeated twice during the cropping season of the year 2005–2006.

Extraction of phenolic acids from *S. rolfsii* from inoculated and non-inoculated plant leaves

Samples of leaves from twenty-days old plants were taken for the estimation of phenolics 48 h after the treatments and from the control. After 96 h, 100 sclerotia of *S. rolfsi*i were mixed with 100 g of soil. After inoculation of soil, samples of leaves

were taken after 48 h when the fungus colonized the collar region of the host. Harvested samples were initially crushed with a pestle in a mortar and finely crushed samples were suspended in 5–10 ml of ethanol-water (80:20; v/v). These samples were collected in screw-capped tubes and the suspension was subjected to ultrasonication (Branson Sonifier, USA) for 15 min at 4°C followed by centrifugation at 7500 rpm for 15 min. The clear greenish supernatant was subjected to charcoal treatment to remove pigments from each sample and was then transferred to glass tubes. The residue was re-extracted twice and supernatant was removed prior to evaporation under vacuum (Buchi Rotavapor Re Type). Dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing and filtered through membrane filter (pore size 0.45µm, Millipore) before HPLC analysis.

The reagents

Methanol and distilled water (HPLC-gradient grade) were supplied by Merck (Germany) and standard phenolic acids viz., tannic acid, gallic: (3,4,5-trihydroxybenzoic), vanillic (4-hydroxy-3-methoxybenzoic) cinnamic acid: caffeic (3,4-dihydroxycinnamic), o-coumaric (4-hydroxycinnamic), ferulic (4-hydroxy-3 methoxycinnamic) and salicylic acids were supplied from Merck, Himedia and Sigma, respectively.

High Performance Liquid Chromatographic (HPLC) analysis

High performance liquid chromatography of fractionated material was performed as described earlier using HPLC system (Shimadzu Corporation, Japan) equipped with two Shimadzu LC-10 AT*VP* reciprocating pumps, a variable UV-VIS detector (Shimadzu SPD-10 A*VP*), integrator and CLASS-VP software for data recording and processing (Shimadzu). Reversed phase chromatographic analysis was carried out in isocratic conditions using C-18 reversed phase HPLC column {(250 x 4.6 mm i. d., particle size 5 µm) Luna 5µ C-18 (2), Phenomenex, USA at 25°C. Running conditions included mobile phase methanol-0.4 % acetic acid (66:34, v/v), flow rate 1.0 ml/min, injection volume 5 µl and detection at 290 nm. Fractionated material (1mg/ml) and phenolic acids dissolved in HPLC grade methanol were injected thrice in the sample loop and the means of the peak areas of individual compounds were taken for quantification. Tannic, caffeic, vanillic, ferulic, cinnamic and salicylic acids were used as internal and external standards. Phenolic compounds present in the sample were identified by comparing retention time (Rt) of standards tannic (Rt. 2.94 min), gallic (Rt. 3.10 min), ferulic (Rt. 4.02 min) and cinnamic acids (Rt. 6.67 min). These phenolic acids were identified by co-injection of internal and external standards for their confirmation. The amount of individual compounds were calculated by comparing peak areas of reference compounds with those in the samples tested under similar elution conditions.

RESULTS

HPLC analysis of chickpea treated plant leaves 48 hrs after foliar application (10^8 cfu/ml) of fresh-and heat-inactivated *T. harzianum* and plant growth promoting rhizobacteria (Pf4 and Pag) indicated that four phenolic acids (gallic, tannic, ferulic, cinnamic acid) were continuously present in both treated and untreated plant leaves but their amount was variable. Among all the phenolic acids, gallic acid was at maximum followed by tannic, ferulic and cinnamic acids. HPLC analysis indicated that

foliar application of bioagents induced of phenolic acid production as compared to the control. In treated plant leaves, gallic acid (18.86 and 17.7 µg/g) was detected at maximum in case of Pf4 and *T. harzianum,* respectively followed by other treatments. Tannic, ferulic and cinnamic acids were also detected at maximum in treated plants leaves as compared to control. HPLC analysis of second sampling 48 h after sclerotia germinated plant leaves also showed the presence of phenolic acids but their amount was dependent on pathogenic interaction. After sclerotial germination gallic acid and tannic acid were drastically increased but other phenolic acids, viz., ferulic and cinnamic acid were also higher as compared to untreated plant leaves. (Table 1).

Table 1. Effect of foliar application of biocontrol agent (10^8 cfu/ml) of fresh-and heat-inactivated *T. harzianum* and plant growth promoting rhizobacteria (Pf4 and Pag) on production of phenolic acid in chickpea

Treatments	Phenolic acids (µg/g fresh weight)			
	TA	GA	FA	CA
	Ist sampling after 48 h of foliar treatment of bioagents			
Control	10.65 ± 1.35	12.65 ± 1.35	0.228 ± 0.16	0.150 ± 0.048
Th	14.26 ± 1.32	17.6 ± 1.32	0.366 ± 0.12	0.251 ± 0.074
HITh	13.13 ± 1.92	16.12 ± 1.92	0.236 ± 0.16	0.437 ± 0.072
Pf4	14.63 ± 1.74	18.63 ± 1.74	0.347 ± 0.019	0.640 ± 0.043
HIPf4	12.52 ± 1.62	15.52 ± 1.62	0.231 ± 0.075	0.175 ± 0.046
Pag	13.35 ± 1.75	14.35 ± 1.75	0.314 ± 0.032	0.177 ± 0.048
HIPag	11.75 ± 1.56	12.75 ± 1.56	0.128 ± 0.06	0.670 ± 0.012
CD: 6.21				
	IInd sampling after 48 h of sclerotial germination of *S. rolfsii*			
Control	14.65 ± 1.46	18.65 ± 1.46	0.042 ± 0.086	0.105 ± 0.047
Th	22.06 ± 1.45	26.6 ± 1.45	0.866 ± 0.089	0.285 ± 0.049
HITh	18.12 ± 1.35	24.12 ± 1.35	0.336 ± 0.045	0.437 ± 0.075
Pf4	24.63 ± 1.54	27.63 ± 1.54	0.947 ± 0.043	1.660 ± 0.046
HIPf4	21.52 ± 1.42	24.52 ± 1.42	0.331 ± 0.064	0.165 ± 0.042
Pag	18.35 ± 1.62	25.35 ± 1.62	0.514 ± 0.085	0.527 ± 0.023
HIPag	15.75 ± 1.72	23.75 ± 1.72	0.452 ± 0.19	0.400 ± 0.052
CD: 10.18				

Th: *T. harzianum*, HITh: Heat-inactivated *T. harzianum*, Pf4: *P. fluorescens* strain 4, HIPf4: Heat inactivated *P. fluorescens* strain 4, Pag : *P. aeruginosa,* HIPag : Heat inactivated *P. aeruginosa*, UDL: Under detection limit, ± standard error

Foliar application of *T. harzianum* and *P. fluorescens* showed good results in controlling the disease and reduced 30–40% of plant mortality followed by *P. aeruginosa* (20–25%) at 10^8 cfu/ml as compared to control in pots as well as in the field. In plant growth promotion, *P. fluorescens* and *P. aeruginosa* used at same dose showed similar efficacy and enhanced 12–15% plant growth as compared to control. Foliar sprays of fresh *P. fluorescens*, *P. aeruginosa* and *T. harzianum*, were less effective at 10^6 and 10^8 cfu/ml as compared to control. Disease controlling efficacy was observed at maximum in

case of *T. harzianum* and *P. fluorescens* at 10^8 cfu/ml followed by 10^6, 10^4 cfu/ml. Plant growth promotion studies, also showed variable results. Foliar spray with heat-inactivated *T. harzianum* and *P. fluorescens* strain 4, showed similar efficacy in controlling the disease and disease inhibition varied up to 15–25% under glasshouse treatment at various concentrations of cfu/ml of the microbes, fresh and heat-inactivated *P. aeruginosa* caused very little disease protection (10–15%) (Fig. 1).

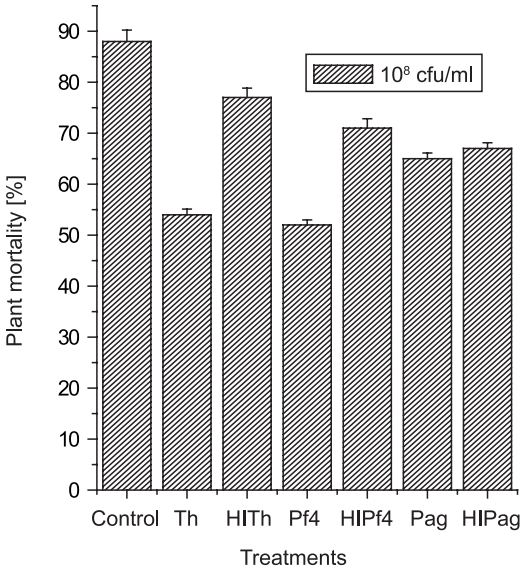

Fig. 1. Effect of foliar sprays of live and heat-killed *Trichoderma* and PGPRs on plant mortality of chickpea, A. foliar spray, Th: *T. harzianum*, HITh: Heat-inactivated *T. harzianum*, Pf4: *P. fluorescens* strain 4, HIPf4: Heat inactivated *P. fluorescens* strain 4, Pag: *P. aeruginosa*, HIPag: Heat inactivated *P. aeruginosa*

DISCUSSION

The results clearly indicated that the studied biological control agents provided chickpea protection against collar rot and caused plant growth promotion of chickpea. Several reports shows that soil and foliar application of plant growth promoting rhizobacteria and *T. harzianum* reduces the population of soilborne phytopathogens, especially *Rhizoctonia solani*, *S. sclerotiorum* and *S. rolfsii* (Elad *et al.* 1980; Sivan *et al.* 1984, Hoitink and Boehm 1999; Sivasithamparam and Ghisalberti 1998). Sarma *et al.* 2002 reported that soil application of rhizobacteria significantly reduced the population of *S. rolfsii* and inhibited 40% of plant mortality of chickpea as compared to control. Systemic effect induced by *T. harzianum* and PGPRs in plants against the disease were expressed more consistently in case of soil or seed treatment than in the foliage of plants. *T. hamatum* 382 (T382) was identified as one of the highly efficient biocontrol agents having ability to induce resistance and suppression of foliar diseases (Han *et al*. 2000; Harman *et al*. 2004; Krause *et al*. 2001; Krause *et al*. 2003). Isolates of several other *Trichoderma* spp. have also been described as being able to reduce the severity of

foliar diseases, presumably by inducing systemic resistance in plants (McBeath and Kirk 2000; Yedidia *et al*. 2003).

The investigation of biological control agents proved the ability to manage collar rot disease of chickpea. *Trichoderma* and PGPRs have been widely used in managing *S. rolfsii* as well as several other soilborne phytopathogens. Thus, the application of *Trichoderma* and *Pseudomonas* species not only control the disease but also enhance plant growth and are ecologically safe and can be regarded as healthy approach for controlling collar rot (*S. rolfsii*) of chickpea.

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POLISH SUMMARY

ZWALCZANIE ZGNILIZNY SZYJKI KORZENIOWEJ CIECIERZYCY POSPOLITEJ PRZEZ *TRICHODERMA HARZIANUM* I BAKTERIE RIZOSFEROWE STYMULUJĄCE WZROST ROŚLIN

Zgnilizna szyjki korzeniowej (*Sclerotium rolfsii*) ciecierzycy pospolitej (*Cicer arietinum*) jest jedną z groźnych chorób przenoszących się poprzez glebę, która powoduje straty plonu wynoszące 10 do 30%, w zależności od jej nasilenia. Choroby tej nie można zwalczyć przy braku fungicydów przeznaczonych do doglebowego stosowania. W przeprowadzonych badaniach wykazano jednak, że zużycie do opryskiwania liści roślin grzyba *Trichoderma harzianum* lub dwóch gatunków bakterii rizosferowych stymulujących wzrost roślin, a mianowicie *Pseudomonas fluorescens* szczep 4 lub *P. aeruginosa* wykazuje maksymalną skuteczność ograniczania śmiertelności roślin w porównaniu do roślin z nietraktowanej kombinacji kontrolnej. Zastosowanie do opryskiwania liści żywych lub inaktywowanych wysoką temperaturą (121°C w ciagu 10 min.) bakterii *P. fluorescens* szczep 4 albo grzyba *T. harzianum*, zmniejszyło śmiertelność roślin o 15–25%, ale bakterie *P. aeruginosa* ograniczały chorobę w niewielkim stopniu. Biorąc pod uwagę stymulację wzrostu roślin zaobserwowano, że żywe oraz inaktywowane termicznie bakterie *P. fluorescens* szczep 4, wykazywały najwyższą skuteczność w porównianiu do żywych i inaktywowanych termicznie bakterii *P. aeruginosa* oraz grzyba *T. harzianum*. Skuteczność zwalczania choroby była także związana ze wzrostem syntezy w roślinach ciecierzycy kwasu fenolowego.