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Influence of *Trichoderma harzianum* on Wilt Disease in Chickpea Seedlings caused by *Fusarium species*

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In this study, potential and effective strain of *Trichoderma* (*T. harzianum* Th. Azad) has been investigated and their effect of pre sowing seed treatment on germination, seedling establishment, seedling dry weight and vigour in chickpea genotype (Radhey) was observed. The different pre sowing seed treatments showed different responses against all eight tested quality parameters. Chickpea seeds were treated with *Trichoderm*a bioformulation and metabolite preparation. As a result, the percentage of seed germination was found to be higher in *Trichoderma harzianum* (Th. azad) metabolite treated seeds as compared to the *Trichoderma* bioformulation. Various attributes with their observations include seed germination (61% and 55%), root length (110.4 and 106.112 cm) shoot length (15.47and 15.2 cm) seedling length (25.48 and 24.94 cm), dry weight (.80 and .77 cm), vigour index I (1554.2 and 1371.7) and vigour index II (48.0 and 42.35). Protein content was also high in metabolite treated seeds (1.10 and 1.12) which suggests that metabolite treatment induces some Pr protein encoding genes. Among all treatments, control showed the poorest performance for all eight tested attributes.

Keywords: Germination, *Trichoderma*, vigour, chickpea.

Plant dieses play an important role in the destruction of crops.Phytopathogens cause important loses. Phytopathogenic fungi such as *Fusarium, Alterneria, Colletitrichum, Rhizoctonia, Sclerotium* etc. has spread during the last few years. *Trichoderma* species are the most commonly used biological control agents that are efficently used for the control of phytopathogens. The species of *Trichoderma* have attracted attention because of their effectiveness against various plant pathogens (Harman *et al*., 2004). They have shown impressive results against many phytopathogenic fungi (Papavizas, 1985 & *Samuels 1998*) including *M. phaseolina* (Aly *et al*., 2007). The mycoparasitic mechanism employed by *Trichoderma* includes following steps: Recognition of the host, attack and subsequent killing of the pathogens. During mycoparasitic action CWDEs enzyme and metabolites play a crucial role, they hydrolyze the cell wall of pathogen and ultimately released the contents of the organism (Kubick *et al* 2001). The antifungal action of *Trichoderma* includes a great variety of lytic enzymes such as chitinase, proteases, xylanase and glucanase (Lorito 1998, Lorito *et al* 1994a 1996a), which play a key role in bicontrol activity of *Trichoderma* (Kubicke *et al* 2001).

Chickpea (*Cicer arietinum* L.*)* remarkably predominates among other pulse crops in terms of both area and production and the crop is widely growing in‘ India as well as other tropical, sub-tropical and temperate regions of the world. Its seeds contain high amounts of protein (25.3-28.9%), even after dehulling (Hulse, 1991). Chickpea seeds are eaten fresh as green vegetables, parched, fried, roasted, and boiled; as snack food,

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sweet and condiments; seeds are ground and the flour can be used as soup, dhal, and to make bread; prepared with pepper, salt and lemon it is served as a side dish. Among various factors attributed to the low productivity of chickpea, such as susceptibility to wilt diseases is the most important. Chickpea is also affected by biotic and abiotic constraints and seed treatment is an important aspect to obtain higher germination and good quality seedlings. Wilt (*Fusarium oxysporum* f. sp. *ciceri* (Pad.) Snyd and Hans is one of the serious diseases of chickpea causing heavy loss upto 10-100% depending on fungal inoculum and environmental conditions). Chemical control of the disease is less effective against *Fusarium oxysporum* f. sp. *ciceri* as it survives in the soil for a longer period of time due to the presence of chlamydospores. Therefore, cultivation of resistant varieties is an economical approach available for the disease when only a few resistant varieties are available (Pande *et al*., 2004). As such, in the present context, biological control of dry root rot of chickpea offers great promise.

In the present study Trichoderma formulation and metabolite preparation obtained from *Trichoderma* species was employed to study their antagonistic effect on *Fusarium* wilt and their plant growth promotion activity.

MATERIALS AND METHODS

Isolation and Characterization of Fungal Culture

Trichoderma species was isolated from rhizospheric soil of field farm of C. S. Azad University of Agriculture & Technology, Kanpur, using serial dilution plate technique describe by Jonhson and curl (1976). Single spore cultures of *T. harzianum* (*Th* Azad) and *F. oxysporum* were sub-cultured on potato dextrose agar (PDA; 39 g/l, Himedia, Mumbai, India) in a temperature-controlled growth cabinet at 25±2°C for 7 days. It was morphologically identified by slide mounting and molecular identification characterized by ITS marker

Metabolite extraction

For metabolite extraction Trichoderma isolate was inoculated in 100 ml Potato Dextrose Broth in 250 ml conical flask and incubated at 28 ± 2°C for 14 days. After incubation period completes the content of the flask were filtered through the Whatman filter paper. Obtained filtrate is considered as the source of metabolites and is used for the chickpea seed treatment.

Trichoderma bioformulation preparation

Grains are cheap, easily available and act as best nutritive media for the mass multiplication of many micro-organisms. Bajra (*Pennisetum typhoides*) grains should be completely soaked in 2% sucrose solution in water for 6 h. After draining out the excess water, the soaked 250 g seeds of bajra should be filled in autoclavable poly-propylene (PP) bags of 30 × 20 cm2. The PP bags should be plugged with non-absorbent cotton followed by autoclaving at 15 lbs pressure for 30 min. After autoclaving, the bags should be left for cooling overnight. The next day, the bags should be individually inoculated by using 5 ml stock solution (10^6 to 10^8 CFU/ml) of starter culture grown for 100 days, with syringe. Before inoculation, the place from where the inoculation is to be made should be marked out with a small circle with the help of marker pen. Punctured place of injection of the PP bag must be sealed with cellophane tape. The bags should be incubated at 25 ± 2°C for 15 days in a temperature controlled room. After 15 days of incubation, the contents of the bags should be taken out and kept in hot air oven for drying overnight at 35°C. During the 15 days of incubation visual check every day is essential to ensure detection and elimination of contaminated PP bag(s). Formulation thus prepared should be ground to fine powder, while ensuring that during the process temperature does not go beyond at 35°C. The powdered formulation thus obtained should be mixed with pre-sterilized talc in 1:9 (*Trichoderma* spore:talc) ratio. Three samples should be taken from each, but lot during production and tested using a standardized method to determine the viability of the active ingredient expressed as colony forming units (CFU). The product thus prepared is ready for packaging at this stage. For storage, the finished product should be stored in vacuum filled plastic bags, covered by paper cartons of different sizes (250, 500 and 1000 g). These packets should then be kept in sealed cartons for transportation purpose

Seed Treatment

Wilt infected seed of chickpea (Radhey) was obtained from seed processing plant of C.S. Azad University of Agriculture & Technology,

Kanpur. One hundred seeds were counted and weighted to apply the recommended dose of *Trichoderma* bioformulations and metabolite preparation. All the treatments were tested as dry seed treatment method (Nene and Thapaliyal, 1977). The treated seeds were subjected to assess the germination and vigour as per the procedure recommended by ISTA at laboratory of Department of Seeds Science & Technology, C.S. Azad University of Agriculture and Technology, Kanpur. Seed germination was recorded 5 days after treating in all the experiments. Seed germination was recorded on the basis of number of the seed germinated out of total germination. Seedling length of the seed was recorded 10 days after treatments in all the experiments. Shoot and root lengths of the seeds were recorded on the basis of randomly selected ten plants per treatment in lab experiment (Dubey, *et al.*, 2011). The dry weight of the seedling were measured on the basis of randomly selected 10 germinated seeds per experiment were placed in hot air oven at 60º C for 36 hours. Two recommended methods *viz.*, germination per cent x seedling length for vigour index I (Abdul Baki and Anderson, 1973) and germination x dry weight for vigour index-II was adopted during the course of investigation.

Total protein extraction and quantification

Total protein was extracted using method developed by Goggin *et al.*, (2011). Briefly, 2.5 g of leaves were ground to a powder in liquid nitrogen, then placed in a centrifuge tube with two volumes of extraction buffer containing Tris-HCl 0.05 M, pH 8, 0.02% SDS, 30.3% urea, 1% 2-mercaptoethanol. After 20 min incubation on ice with gentle rocking, the tubes were centrifuged at 12000 g for 10 min. For purification, extracted proteins were precipitated in chilled methanol (-80°C) and incubated overnight at -80°C, then centrifuged for 30 min at 10000 g. The pellet was allowed to air-dry, re-suspended in minimal IEF buffer which contained 8 M urea, 2% [w/v] CHAPS, 60 mM DTT, 2% [v/v] IPG buffer, for 10 min with gentle rocking, and centrifuged at 12000 g for 30 min again. Protein concentration of samples were determined using Bradford assay (Bradford,1976) and lowry assay (***Lowry*** 1951). BSA was used as a standard.

RESULTS AND DISCUSSION

Isolate was identified up to species level based on phenotypic characters like colony colour and growth; size and shape of conidiophore, phialides and conidia using the available literature (Bisset 1991a,1991b and Samuels *et al.*1998) and confirmed molecular identification by ITS marker, sequences deposited to NCBI GenBank (Acc. No.-KC800922) and re-confirmation by ITCC, New Delhi (Acc. No.-ITCC-6796). Finally, potential and effective bio-agent *T. harzianum* (*Th* Azad/6796) was submitted to microbial data bank at NBAIM, Mau (Acc.No.-F-03109).

In the current study, it was observed that both the seed treatments were significantly superior over control (untreated seeds). Among the eight seed quality parameters *viz*. germination, shoot length, root length, seedling length, seedling dry weight, vigour index I and vigour index II (Table-1). Treatment T1- crude metabolite extract (*Trichoderma harzianum* (Th. azad) was found to be significantly superior and effective with 61%

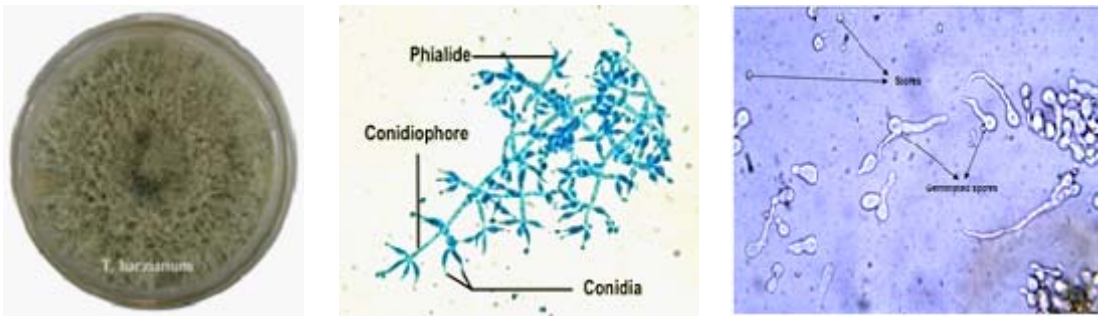

Fig. 1. Light micrographs of *T. harzianum* (*Th* Azad/6796) (A) Growth on PDA Media (B) Microscopic observation at 40x(C) Spore germination

germination of chickpea variety Radhey from control followed by T2 (*Trichoderma* bioformulatio 5%), similarly the beneficial impact of seed treatment was also recorded for root length, shoot length, seedling length and dry weight vigour index-I and vigour index-II similarly T1 treatment (crude metabolite extrat*)* excelled overall significant superior performance by contributing root length (110.4), shoot length (15.47), seedling length (25.48), dry weight (.80), vigour index I (1554.2), vigour index- II (48) respectively sfollowed by T2 treatment. Germination and seedling length along with seedling dry weight are important attributes, which determine the quality of seed of any seed

Table 1. Effect of different seed treatments on quality of chickpea seeds

Treatments	Germination %	Root length	Shoot length	Plant height	Dry weight	Vigour index-I	Vigour index-II
Crude secondary metabolite extract	61	110.4	15.47	25.48	0.80	1554.2	48.0
Trichoderma formulation	55	106.1	15.2	24.94	0.77	1371.7	42.35
Control	44	94.1	13.9	21.61	0.73	950.0	32.12

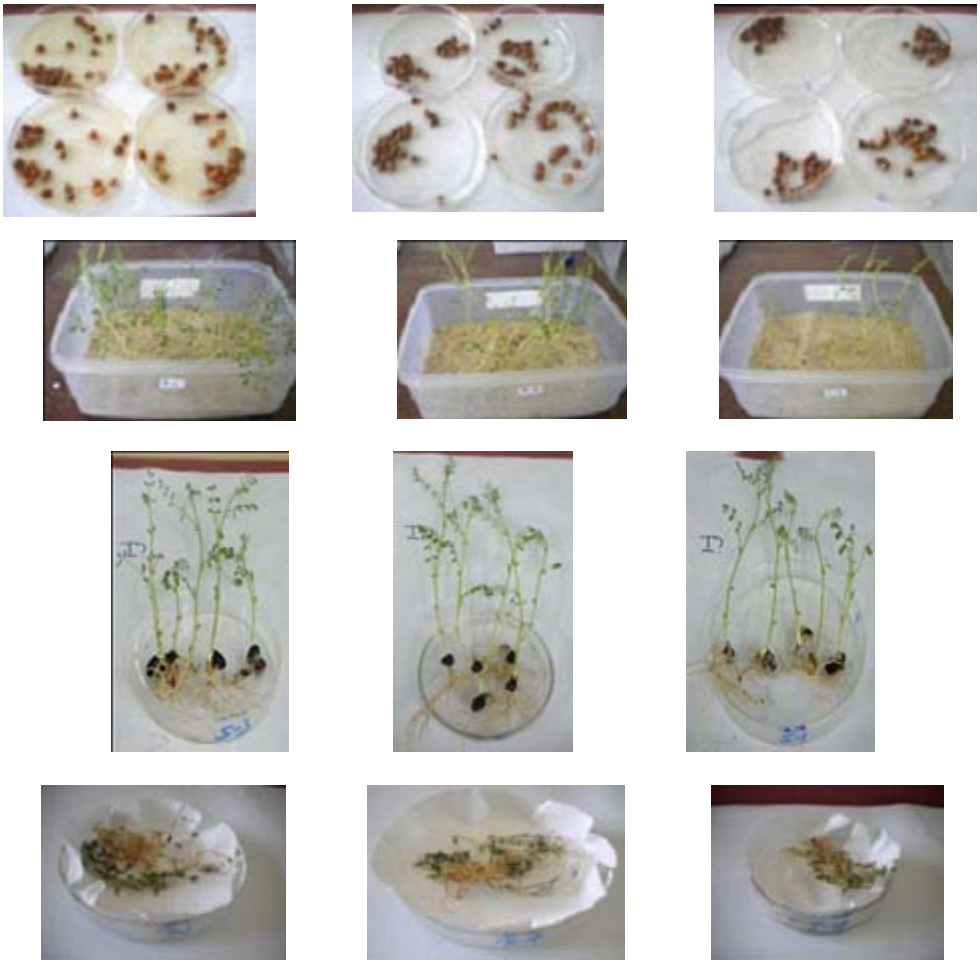

Fig. 2. Chick pea seed treatment process (A) showing the seed treatment process of Fig1b: Uprooted *cicer aritenum* plants *Cicer aritenum* seeds and their growth in crystal sand (B) Uprooted *cicer aritenum* plants and (C) Dried *Cicer aritenum* plants

lot. Besides these quality seed parameters seed vigour index also plays very crucial role in predicting the fate of any seed lot under biotic and abiotic stress conditions. Shukla, 2008 Cokkizgin and Cokkizgn, 2010 also reported germination per cent, plumule length, radical length and vigour index in case of lentil and *Terminalia arjuna* showed better performance for all these attribute with chemical treatments. The data given (Table 1) is also revealed that over all superior performance was contributed by T1 treatment (crude metabolite extract*)* achieving the highest vigour index-I as well as vigour index-II. The impact of control treatment (T3) for germination, root length, seedling length and for dry weight was poor. Dube, *et al*., 2011 and Shahid *et al*. 2011 also suggested

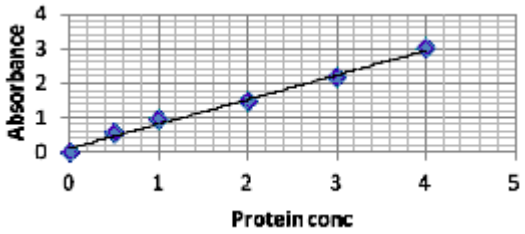

Fig. 3. BSA standard curve for Bradford

Table 2. Protein concentration of chickpea seedling

Treatment	Protein Conc.
T1	1.10
T2	1.03
T3	.82

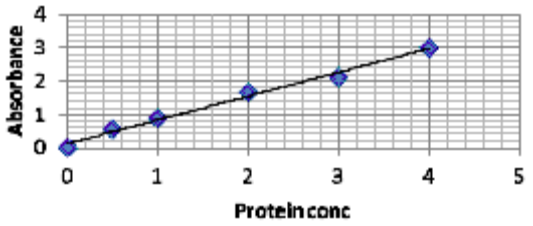

Fig. 4. BSA standard curve for Lowry

Table 3. Protein concentration of chickpea seedling

Treatment	Protein Conc.
T1	1.12
T2	1.06
T3	.96

the effect of soil application and seed treatment with *Trichoderma* species on seed germination shoot, root length dry root in case of chickpea. The above result concluded that out of three treatment including control T1 treatment (*metabolite extract*) are better seed treatments to enhance quality seed parameters in chickpea seed (Radhey), which can finally be converted into superior yield even in adverse conditions. Shahid *et al* 2015 showed that application of *Trichoderma* mycolytic enzymes results in better germination and grwth in chickpea seedlings.

Protein estimation by Bradford and Lowry method

Amount of protein extracted from chick pea seedling was determined by Bradford and Lowry's method and the results of the same can be seen in Table 2 &3 below. In *Trichoderma* metabolite treated seeds higher amount of protein content was found

In this study, effect of metabolite and bioformulation of *T. harzianum* (Th. Azad) was investigated on germination, seedling establishment, seedling dry weight and vigour in chickpea genotype (Radhey). The different pre sowing seed treatments showed different responses against all the eight tested quality parameters. Chickpea seeds were treated with *Trichoderm*a metabolite and bioformulation. As a result, the percentage of seed germination was found to be higher in *metabolite treated* seeds followed by bioformulation treated seeds. Various attributes with their observations include seed germination (61% and 55%), root length (110.4 and 106.112 cm) shoot length (15.47and 15.2 cm) seedling length (25.48 and 24.94 cm), dry weight (.80 and .77 cm), vigour index I (1554.2 and 1371.7) and vigour index II (48.0 and 42.35). Among all treatments, control showed the poorest performance for all eight seed quality attributes.

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