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Seed Biopriming and *Trichoderma* enriched FYM Based Soil Application in Management of Chickpea (*Cicer arietinum* L.) wilt complex

Pratik Jaisani, H.N. Prajapati, D.L. Yadav and R.N. Pandey

Centre for Transfer of Mass Production Technology of Bioagent: *Trichoderma* spp.
Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand- 388 110, India.

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Chickpea or Gram (*Cicer arietinum* L. Family: Leguminosae) is the premier legume grown in the Indian subcontinent, covering a very large area under cultivation as a rabi crop. Chickpea crop cultivation is prone to various diseases chiefly wilt complex due to *Fusarium oxysporum* f. sp. *ciceri* and *Sclerotium rolfsii* which is mainly soil and seed borne in nature. *In vitro* application of *T. viride, T. harzianum* and *T. virens* conidial suspension to chickpea seeds gave significant results with high plumule length upto 13.3, 12.7 and 12.4 cm and radical length upto 6.4, 5.8 and 5.3 cm, respectively. The seedling emergence and vigour index were significantly high in all treatments compared to pathogen check. Among the treatments, seed biopriming application of *T. viride* along with soil application of FYM enriched *T. viride* @ 100g/m² resulted in better seedling emergence (97.0%) and highest vigour index (3496.0), followed by seed biopriming application of *T. viride* alone with seedling emergence (96.0%) and high vigour index (3235.2).

Keywords: Chickpea, *Trichoderma* spp., biopriming, *Fusarium oxysporum* f. sp. *ciceri, Sclerotium rolfsii* and Management.

Chickpea is the world's third most important pulse crop after bean and pea, with India accounting for approximately 75% of the world chickpea production (FAO, 1993). Chickpea is grown in tropical, sub-tropical and temperate regions. Kabuli type is grown in temperate regions while the deshi type chickpea is grown in the semi-arid tropics. Chickpea is valued for its nutritive seeds with high protein content of 25.3- 28.9 % after dehulling (Hulse,1991). Its cultivation in marginal lands is often limited with wilt, collar and stem rot caused by *Fusarium oxysporum* f. sp. *ciceri* and *Sclerotium rolfsii,* respectively. *F. oxysporum* f.sp. *ciceris* (Padwick) Matuo and Sauto (FOC) is aimportant seed and soil-borne pathogen causing huge losses to chickpea production world-wide causing 10-90 percent losses (Nene *et al*. 1996; Singh and Dahiya, 1973 and Jalali and Chand, 1992).

S. rolfsii has got wide host range; is a destructive soil borne pathogen, which attacks over 500 plant species including chickpea and limiting seed yield production (Durrell, 1968; Barnett and Binder, 1973; Elad*et al.*, 1980 and Mukherjee and Raghu, 1997). The disease has been reported to cause seedling mortality in chickpea up to 90 % in favorable conditions (Dubey, 1982).

Various species of *Trichoderma* have been studied for their biocontrol ability to manage disease complex caused by *Fusarium* spp. and S. *rolfsii* (Lewis andPapavizas, 1980; Papavizas, 1985;

* To whom all correspondence should be addressed.

Lewis *et al.,* 1995; Selvarajan and Jeyarajan, 1996). The present study was undertaken to evaluate the biocontrol ability of *Trichoderma* as seed bioprimingand soil application of *T. harzianum, T. viride, T.virens* enriched FYM efficacy on incidence of wilt and collar rot of chickpea under green house conditions.

MATERIALS AND METHODS

Collection and maintenance of bioagents and the pathogens

Fresh culture of bioagents *viz*. *T. harzianum, T. viride* and *T.virens* and the pathogens *viz. Sclerotium rolfsii* and *F. oxysporum* f.sp. *ciceri* maintained on PDA slants at 28±1°C in Department of Plant Pathology, B.A. College of Agriculture, A.A.U were used for studies.

Effect of biopriming of chickpea seeds on emergence, plumule and radical length *in vitro*

One hundred seeds of Chickpea variety GG-2 were taken in Petriplates and treated with slurry of *T. viride, T. harzianum* and *T. virens* having 2 x 10^8 conidia/ml for 10 hours and incubated at 28±1°C in BOD incubator. The slurry was prepared by mixing 50 gram of solid talc based formulation (2 x10^8 cfu/g) dissolved in 250 ml of distilled water. After 10 hours of treatment, the seeds were checked for germination, seedling emergence, plumule length and radicle length etc.

Mass production of *S. rolfsii and F. oxysporum* f.sp. *ciceri* in sand maize medium

F. oxysporum f.sp. *ciceri* and *Sclerotium rolfsii* were separately multiplied by inoculating twenty five fungal disks of 5 mm size from 7 days old culture in pre-sterilized plastic trays containing sterilized sand maize medium. The medium was prepared in proportion of powdered Maize 40g, sand 160g and distilled water 50 ml and sterilized at 121°C for 15 min at 15 lb pressure. The inoculated trays were covered with surface sterilized polythene sheets. The culture was allowed to grow for two weeks till fine mycelial growth was observed on the medium.

Mass production of *T. viride, T. harzianum,* and *T. virens* in liquid broth

Mother culture of *T. viride, T. harzianum* and *T.virens* was multiplied in Potato Dextrose Broth (PDB) at pH 7.0. Conidial as well as mycelial suspension of 100 ml of *T. viride, T. harzianum* and *T. virens* were poured in 250 ml of PDB subjected to sterile 500 ml Borosil conical flasks. The flasks containing bioagents in medium were incubated at 28 ± 2° C in BOD incubator. After 10 days of incubation, the mycelial mat was harvested by decanting the spent broth. The mycelial mats were blot dried and the spores per gram of mycelial mat were determined by making a spore suspension in sterile distilled water. Mass production of *T. viride, T. harzianum* and *T.virens* were carried out in 20 litres vessel of laboratory scale Cleaver Scientific fermentor with pre-standardized parameters *viz*. aeration @ 1vvm, agitation @ 140 RPM, pH @ 7.0 and Temperature control @ 28°C in PDB medium. The medium was inoculated with starter mother culture of the bioagents grown in rotary shaker for three days @ 5 percent in context to the volume of medium used in the process. The bioagent was allowed to grow in fermentor for 15 hours at above standardized parameters and later kept for 12 hours in pre-sterilized vessels in open aerated environment under aseptic conditions for sporulation in BOD incubator at 28 ± 2° C. Entire fermented biomass was mixed with 300 mesh talc powder in a blender of packing machine. Talc-based formulation of the bioagent was prepared by mixing the fermented *Trichoderma* biomass with double the quantity of talc and shade dried. Thereafter, 0.1% carboxy methyl cellulose powder was added as sticker to enhance shelf life of *Trichoderma*. To determine the number of colony forming units (cfu) of the bioagents in the formulation, 1g of evenly mixed *Trichoderma* homogenized in sterile water. Serial dilutions (1:10; 1:100 and 1:1000) were prepared. Aliquots of the each dilution was evenly spread on *Trichoderma* selective medium (TSM) suggested by Elad and Chet, 1983.

Mass production of *T. viride, T. harzianum* and *T. virens* on sorghum grains for FYM enrichment through soil application

Sorghum grains were selected as solid substrate due to cheap cost and suitability for growing the *Trichoderma*. One hundred fifty grams of robust healthy sorghum grains were taken in a 500 ml flasks. The grains were rinsed by rotating in distilled water thrice and soaked for overnight. The grains in the flasks were autoclaved at 121°C and 15 lb pressure for 20 min. The grains were inoculated with 1 ml of conidial suspension containing 2 x 10^8 spores/ml of *T. viride, T. harzianum and T. virens*

separately. The inoculated seeds were shaken well to evenly disperse the inoculum of *Trichoderma*. The inoculated flasks were incubated at 28 ± 2° C under 12/12 h cycles of light and darkness for 14 days. During the incubation, the grains in the flasks were shaken gently once in a day to prevent aggregation. The entire mass was harvested after 14 days of incubation when the substrate was completely overgrown with light green fungal mycelial growth. The entire grains showing the fungal growth were gently half-crushed with sterilized glass rod in each flask and 250 ml of distilled water was added to the multiplied mass with crushed grains. The mixture prepared contained $6x10^8$ cfu/g of spore count. Thus prepared mixture of seed and *Trichoderma* was used as source of inoculum for FYM enrichment. The prepared mixture at rate of 1 gram was suspended in 9 ml of sterile distilled water and the cfu was assess of the spores using haemocytometer.

Preparation of Talc based formulation for seed biopriming application

Talcum powder formulation of the *Trichoderma* was prepared by blending fermented mycelial biomass of *Trichoderma* in sterilized talcum powder in the ratio of 1:2.5 (w/w) grade. Prior to mixing of mycelial biomass with talcum powder, 0.1 percent of carboxy methyl cellulose was added as a sticker to 1 litre of broth and mixed well. Talcum powder was autoclaved at 121° C for 30 min on aluminium trays. Then the mycelial biomass was mixed thoroughly with the sterilized talcum powder under aseptic conditions in a biosafety cabinet (Labcon Co., Germany). Formulations of each i.e. *T. viride, T. harzianum* and *T. virens* thus prepared were spread on sterile filter paper towel in a hood of biosafety cabinet and large clumps were broken and mixed thoroughly to evenly distribute the inoculum in the talc powder. The formulations were allowed to dry for few hours under cool shade dry condition. Completely dried formulations were mixed in a blender for 10 min, packed and sealed in airtight polypropylene bags and stored at cool place for further study. One gram of each sample of formulations were suspended in 9 ml of sterile ultra pure water obtained from Millipore RO system. Serial dilutions of the suspension were evenly distributed on PDA plates and incubated at 28±2° C for 5 days. The number of spores/gram of mycelium incorporated was determined. In both the formulations a conidial with a concentration of 2×10^8 spores/ml was prepared in a aqueous solution of for laboratory and green house studies.

Source of chickpea seeds

Seeds of Chickpea cultivar Gujarat Gram-2(GG-2) obtained from Agricultural Research Station, Derol, Anand Agricultural University was taken for laboratory and green house studies.

Seed biopriming (Biological Seed treatment)

One kilogram Seeds of Chickpea cultivar GG-2 were taken in sterilized conical flasks and treated with slurry of *T. viride, T. harzianum* and *T. virens* with spore load of 2×10^8 conidia/ml, made by mixing 50 gram of solid talc based formulation (2×10^8 cfu/g) dissolved in 250 ml of distilled water treated for 10 hours at room temperature. After 10 hours of treatment the seeds were air-dried and then were subjected for inoculation of *F. oxysporum* f. sp. *ciceri* and *Sclerotium rolfsii* separately. The parameters measured were disease complex incidence, seed germination, seedling vigour and emergence. An untreated control was also maintained.

Enrichment of FYM with *T. viride, T. harzianum* and *T.virens* for soil application

Ten gram of formulation of each of *Trichoderma* i.e. *T. viride, T. harzianum* and *T.virens* containing 2×10^8cfu/g spore load were multiplied on sorghum grains was mixed with 1 kg of FYM to obtain 1:100 proportion. The moisture level of the organic source was maintained at 40 percent and was incubated at 28±2°C for 21 days. It was mixed evenly at three days interval and supplied with adequate moisture. After entire process, one gram of FYM along with overgrowth of mycelium was suspended in 9 ml of sterile distilled water to assess the concentration of the spores. The FYM enriched with *Trichoderma* i.e. *T. viride, T. harzianum* and *T.virens* was added to soil @ 100 g/m^2 in each pot one week before sowing.

Efficacy of *T. viride, T. harzianum* and *T.virens* against *F. oxysporum* f.sp.*ciceri and Sclerotium rolfsii* under greenhouse conditions

The bioprimed seeds were sown in five plastic pots of 20 cm diameter containing a mixture of soil and sand in a ratio of 2:1 (v/v). There were five replications per treatment, and five pots per

replication. The experiment was conducted in a completely randomized design (CRD) with three replications. The emerging seedlings were challenge with inoculation of*F. oxysporum* f.sp. *ciceri* and *Sclerotium rolfsii*by sick soil method with the mycelia grown on sand maize medium. Pots were maintained under greenhouse conditions (90–95% RH, 22–25°C temperature) and observed for disease development. The seedlings were observed and rated for disease incidence when they showed any one of the typical Fusarial and Sclerotial infection like drooping of leaves; necrotic lesions at base, lodging-off and wilted plants. Seed germination was recorded 15 days after sowing. Plant samples (5 plants from each treatment) were drawn after 4 weeks of sowing. Shoot and root length of each plant was measured. The vigour index was calculated by multiplying the sum of root and shoot length with germination percent. Observations on wilt and collar rot incidence were recorded at 30 days after sowing. Seeds sown in infested soils without pre application of bioagents in pot served as control. Statistical significance was analyzed by analysis of variance.

RESULTS AND DISCUSSION

Among the treatment Seed biopriming application of *T. viride* along with soil application of *T. viride* enriched FYM @ 100g/m^2 resulted in higher seedling emergence of 97.0% and highest vigour index of 3496.0, followed by seed biopriming application of *T. viride* alone with seedling emergence of 96.0% and high vigour index of 3235.2 (Table 1). Seed biopriming application of *T. harzianum* along with FYM enriched *T. harzianum* @ 100g/m^2 resulted in good seedling emergence of 94.0% and vigour index of 3245.7, followed by seed biopriming application of *T. harzianum* alone with seedling emergence of 93.0% and vigour index of 2904.6. The seedling emergence and vigour index were significantly higher in all the treatments and were at par among each other in comparison to pathogen check. Rajput *et al.* (2010) reported *T. harzianum* to be most effective under biological seed treatment on chickpea inhibiting *F. oxysporum* f.sp. *ciceri* upto 63.23 percent and *Sclerotium rolfsii* upto 86.00 percent with maximum seedling vigour index of 1866.0 was observed in seed treatment with *T. viride.*

However, significant results were observed during *in vitro* application of *T. viride, T. harzianum* and *T. virens* with high plumule length upto 13.3, 12.7 and 12.4 cm and radicle length upto 6.2, 5.6 and 5.3 cm respectively. Similar increase in seedling and plant growth of chickpea due to soil application of *T. harzianum* prior to sowing was reported by Sharma *et al.* (1999). Singh *et al.* (1997) observed that the growth of chickpea roots, shoots and leaves was enhanced in the presence of different fungal antagonists, with maximum growth in soil inoculated with *T harzianum.* Mechanisms like production of hormone-like metabolites and release of nutrients from soil or organic matter by soil application of *T. harzianum* one week before sowing resulted in significantly less collar and root rot complex incidence (4.9 and 1.2%) as compared to *Trichoderma* spp. *T. viride, T virens* and *T harzianum.* Seed biopriming alone also gave significantly less wilt and collar rot incidence (16.0%), (19.0%) and (24.0%) compared to pathogen check (83.0%). In earlier study reported

Table 1. Effect of biopriming on chickpea seeds *in vitro*

S. No.	Treatments	Plumule length (cm)	Radicle length(cm)
1	T_1:Seed biopriming for 10 hrs with suspension of solid talc based formulation (2x10^{8}cfu/g) of *Trichoderma viride* @ 50 g in 250 ml of water/kg of seed	13.3	6.2
2	T_2:Seed biopriming for 10 hrs with suspension of solid talc based formulation (2x10^{8}cfu/g) of *Trichoderma harzianum* @ 50 g in 250 ml of water/kg of seed.	12.7	5.6
3	T_3:Seed biopriming for 10 hrs with suspension of solid talc based formulation (2x10^{8}cfu/g) of *Trichoderma virens* @ 50 g in 250 ml of water/kg of seed.	12.4	5.3
4	T_4:Untreated check	9.6	3.9
	CD (P=0.05)	0.45	0.43

Table2. Management of wilt and collar rot of chickpea through seed biopriming and soil application of *Trichoderma* enriched FYM.

Treatment	Seedling emergence (%)	Shoot length (cm)	Root length (cm)	Vigour index (%)	Wilt and Collar rot complex incidence % at 30 days
T_1: Seed biopriming for 10 hrs with suspension of solid talc based formulation ($2x10^8$cfu/g) of *Trichoderma viride* @ 50 g in 250 ml of water/kg of seed	96* (9.81)**	21.3	12.4	3235.2	16
T_2: Seed biopriming for 10 hrs with suspension of solid talc based formulation ($2x10^8$cfu/g) of *Trichoderma harzianum* @ 50 g in 250 ml of water/kg of seed.	94 (9.71)	19.6	11.3	2904.6	17
T_3: Seed biopriming for 10 hrs with suspension of solid talc based formulation ($2x10^8$cfu/g) of *Trichoderma virens* @ 50 g in 250 ml of water/kg of seed.	91 (9.55)	20.7	12.1	2984.8	24
T_4: T_2+ soil application of *T. harzianum* enriched FYM (10g/ kg FYM) @ 100 g/ m^2 soil)	93 (9.59)	22.8	12.1	3245.7	19
T_5: (T_3+ soil application of *T. virens* enriched FYM (10g/ kg FYM) @ 100 g/ m^2 soil)	91 (9.50)	21.6	12.0	3057.6	24
T_6: T_1+ Soil application of *T. viride* enriched FYM (10g/ kg FYM) @ 100 g/ m^2 of soil)	97 (9.83)	24.2	12.6	3496.0	14
T_7: Untreated Check.	11 (3.42)	16.9	8.3	277.2	83
CD (P=0.5)	1.65	1.60	1.23	101.75	2.06

by Prasad and Rangeshwaran, 2000c also indicated that soil application of *T. harzianum* granules before sowing resulted in significantly less *rhizoctonia* root rot incidence in chickpea. Lewis *et al.* (1995) reported that amending soilless mix with pre-gelatinized starch-flour granules of *T. virens* and *T. hamatum* protected eggplant, pepper and zinnia seedlings from damping-off caused by *R. solani*. Lewis and Larkin (1997) obtained significant reduction in damping-off of eggplant caused by *R. solani* by application of *rice* flour based extruded granular formulation of *T. virens, T. hamatum, T: viride* and *T. harzianum*.Similar results were reported by other workers, where wilt *(F. oxysporum*f. sp. *ciceri)* and rootrots caused by *Macrophomina phaseolina* and *Fusarium solani* reduced due to soil application and seed application treatments with bioagents like *T. harzianum* and *T. viride* (Selvarajan and Jeyarajan, 1996; Gowrily *et al.,* 1995; and Okhavat and Karmpour, 1996). Coating chick pea seeds with biocontrol agents like *Bacillus subtilis,* G. *virens, T. harzianum* and *T. viride,* and carboxin (vitavax) significantly controlled *F. oxysporum* f. sp. *ciceri*

Fig. 1. Mass multiplied culture of *T. harzianum, T.viride* and *T.virens* in solid based sorghum medium

Fig. 2. Mass multiplied culture of *Trichoderma* spp. inMass multiplied culture of *F. oxysporum f.sp.* solid based FYM medium *ciceri and S. rolfsii* complex in sand maize medium

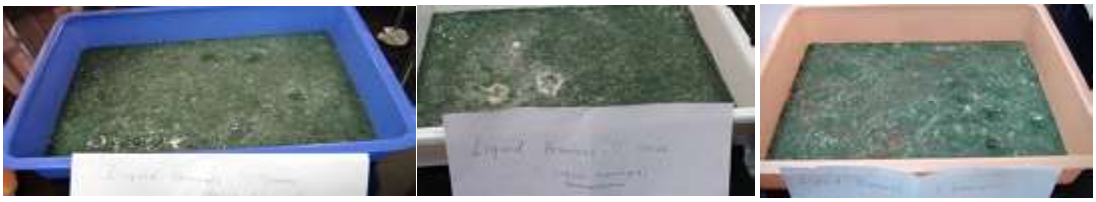

Fig. 3. Mass multiplied culture of *T.viride, T.virens* and *T. harzianum* in liquid based PD broth medium

Fig. 4. Non-bioprimed and *T.viride, T. harzianum* and *T.virens* bioprimed chickpea seeds *in vitro*

wilt by 30-45.8% (De *et al.,* 1996). The present study clearly indicates the positive effect of the three bioagents on seed and plant health. Seed biopriming with *T. viride* prior to sowing along with soil application of FYM primed *T. viride* was most effective in controlling wilt and collar rot of chickpea under green house as well as can be further applied in large scale for better crop management in field conditions.

Fig. 5. Efficacy of *Trichodermaviride*bioprimed along with *T. viride*enriched FYM in green house conditions

Fig. 6. Chickpea seedling infected by wilt and collar rot

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