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Effect of combined application of *Trichoderma harzianum* and *Bacillus subtilis* against wilt disease complex of chickpea caused by *Fusarium oxysporum* and *Rhizoctonia solani*

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ABSTRACT

In this study, antagonistic effects of *Bacillus subtilis* and *Trichoderma harzianum* isolated from *Rhizosphere* of chickpea were evaluated against *Fusarium oxysporum* as potential biocontrol agents *in vitro* and *in vivo*. Fungal inhibition tests were performed using plate assay. Isolates were selected according to their high antagonistic efficiency in *in vitro* which was shown as inhibition zones in the dual-culture assay. *Bacillus subtilis* was isolated in cyst form and is transferred to inert carriers like peat, lignite ore or can be transferred to liquid medium. In lab conditions (controlled) seeds of chickpea are 20 treated directly with *Bacillus* sp. culture and dried for a while and treated with cultures (generally spores) of *Trichoderma* sp. These treated seeds are sown in earthern pots containing black soil having few spores of *Fusarium oxysporium* (MTCC, Chandigarh). Observations are made after every 2-4 days. Combined application of *Bacillus subtilis* and *Trichoderma harzianum* has synergistic effect on the growth of *Fusarium oxysporium* in *in-vitro* environment. Our results indicate that PGPR improve growth parameters in this plant and can help in the biocontrol of pathogen.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important food legume grown in the world. In 2004, global chickpea production was about 8.6 million metric tons, second only to dry beans among edible pulses (Smith and Jimmerson, 2005). It is grown in over 45 countries in all continents of the world. The crop provides a high quality protein to the people in developing countries. In the developed countries, consumers consider it as a health food. A member of the family Leguminosae, chickpea can fix nitrogen from the atmosphere. The growth habit is erect, with most of the pods formed in the top part of the plant. In recent years, world production of chickpea has ranged from 6.65 million MT up to 8.94 million MT. India produces a significant amount of the chickpeas, but consumes all of that locally. India usually accounts for around 70 per cent of production, with Pakistan, Turkey, Canada, Mexico, Iran, and Australia accounting for an additional 23 per cent.

Chickpea production is often successful in rotation with cereal grains such as durum wheat. Chickpea does not leave a lot of crop residue, so cereal crops with tall stubble grown before and after chickpea provide much-needed residue to protect the soil from erosion (Ingle *et al.,* 2008 and Zape *et al.,* 2009). Disease problems are the biggest production problems chickpea grower face in India. Some problems are seed-borne (such as ascochyta blight and sclerotinia stem rot), and can be minimized by the use of a proper seed treatment, and by using disease free seed. Other problems are common soil-borne diseases (root rot) with multiple host crops, which again can be minimized by the proper use of a seed treatment (Kandoliya and Vakharia, 2013 and Singh *et al.*, 2013). But once a crop has the disease, there is no fungicide treatment that can limit the damage of that disease (although some diseases can be prevented by at least one, if not multiple, in-crop fungicide treatments).

Fusarium wilt (*Fusarium oxysporium*, *Fusarium solani*) :

– Host crops – Chickpea, dry bean, sunflower, soybean, lentil, pea, canola, clover, flax.

– Biology – The fungi causes root rot and are soil borne. Root rotting fungi can attack any part of the root system and even the lower portion of the stem at the soil line. The fungus is seed-borne (Haware *et al.*, 1978) and soil-borne (Haware *et al.*, 1986). When young seedlings are infected with root rot, they usually die. Infected plants may appear yellowed, stunted and wilting took place. Finally yellowish patches can be observed in the field, damaging almost 90 per cent of the crops (Singh *et al.*, 1987).

Cultural practices adhering to proper rotations are one of the best preventative measures for all diseases. Damage due to *Fusarium* sps can be minimized by using disease free seed, treating seed, adhering to proper rotations, and not planting the new crop beside a field from last year that had the disease (minimizing infection by air-borne ascospores). Infected chickpea straw should be deep ploughed to hasten decomposition. Seed treatment Thiram (0.15%) + carbendazim (0.1%) proved to be very effective against *Fusarium oxysporum* f.sp. *ciceris* (Nikam *et al.*, 2007). Chlorothalonil is a preventative fungicide, and will not stop infections once they have started. It is effective for 10-14 days.

Biological methods of controlling the infections can be effective in preventing infections. *Bacillus species* and *Trichoderma* sps are the competent biofungicide that can be applied to chickpea crops either as preventive measure or as reactive measure or can be used as both (Shrivastava, 2013 and Kohire *et al.*, 2012).

Bacillus species (Fig. A) are gram negative (rods) soil bacteria that :

- Produce endospores
- Produce a wide range of antibiotics (>70) and sederophores (Davison, 1988).
  – Species known to produce antibiotics: *licheniformis, pumilus, circulans, cereus, Brevibacillus laterosporus, Paenibacillus polymyxa* – Shown to have antibacterial and antifungal activity against phytopathogens (Todorova and Kozhuharova, 2009)
- Produce compounds that stimulate plant defenses – Phenylalanine ammonia-lyase (PAL).

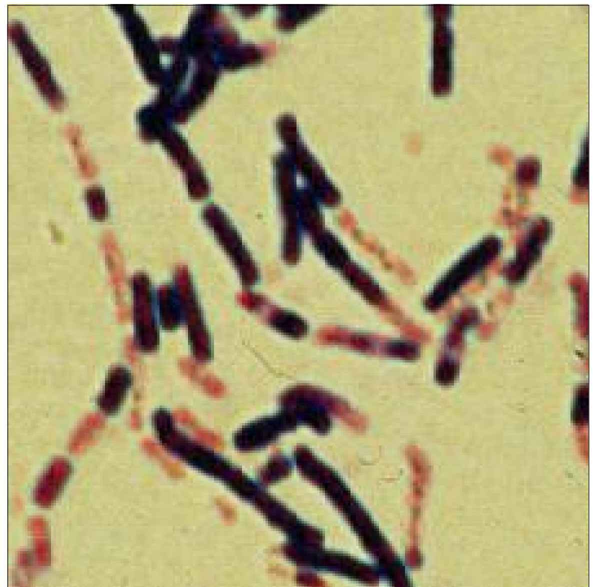

Fig. A : *Bacillus subtilis*

Bacillus subtilis inhibited fungi (*Phytophthora* spp., *Pythium* spp., *Fusarium* spp., *Sclerotium* spp., *Rhizoctonia* spp.) seed treatment : chickpea (48%), Cotton (33%), peanuts (37%) yield increases.

Trichoderma sps (Fig. B),

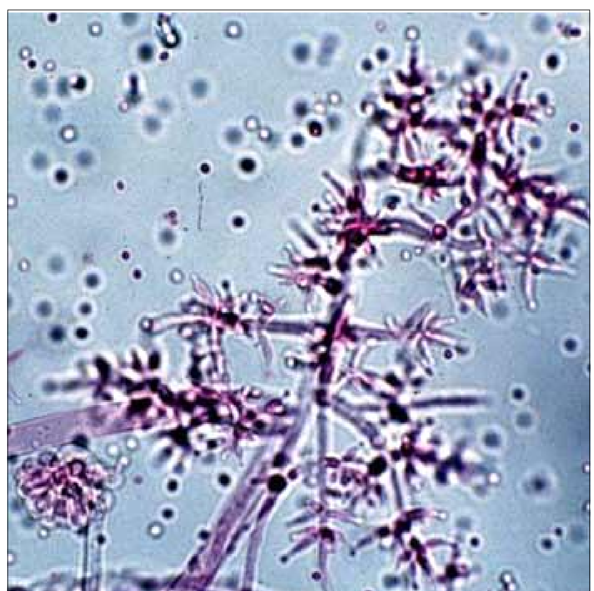

Fig. B : *Trichoderam harzianum*

- Species of *Trichoderma* sps. are common in soil (especially water-logged soil), dung and decaying plant materials,
- Fast-growing, white, green, or yellow sporulating filaments. Conidiophores produce side branches bearing whorls of short phialides. Single-celled conidia produced successively from the tips of the phialides and collect in small wet masses,
- Trichoderma species are strongly antagonistic to other fungi. Appears to kill other fungi with a toxin and extracellular enzymes having amylolytic, chitinolytic, pectinolytic, proteolytic, lipolytic and cellulolytic activities (Gachomo and Kotchoni, 2008).

Mode of action :

Diffusible inhibitors (toxins, antibiotics)
- Volatile inhibitors (alcohols, ketones, sesquiterpenes)
- Competition
- Mycoparasitism
  - Chemotaxis to target
  - Galactose residues (T) bind to lectins on host
  - Coiling
  - Extracellular enzymes (cellulases and chitinases)
  - Appresoria like structures and penetration (Fig. C).

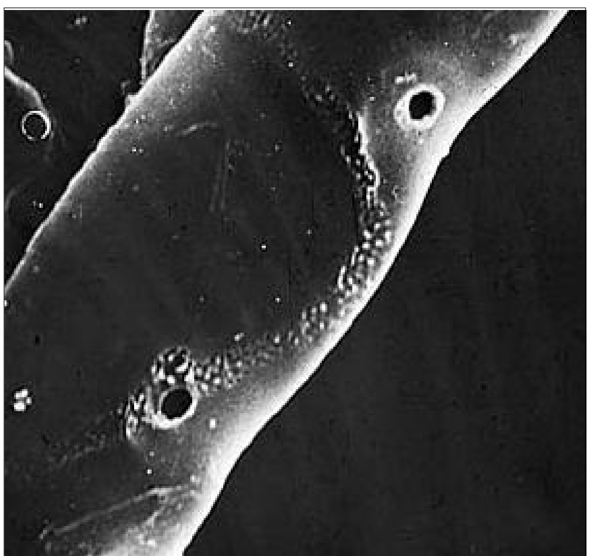

Fig. C : *Trichoderma harizianum* causes perforation in *Fusarium* spp.

MATERIAL AND METHODS

Production and formulation of *Bacillus subtilis* :

Bacillus subtilis strain CBE4 (MTCC, Chandigarh) is revived on nutrient agar media (NAM) incubated at 25 - 28°C for 48 hrs (Fig. D.)

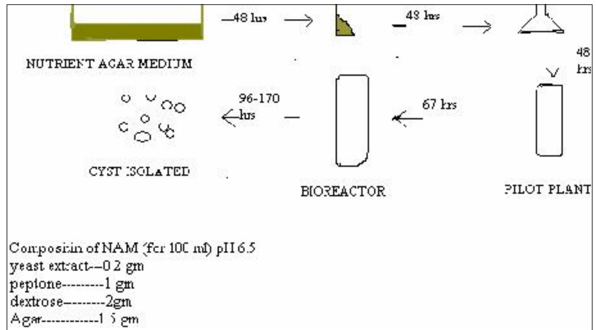

Fig. D : Production of Bacillus culture (endospores) on large scale

Bacillus subtilis is isolated in cyst form and is transferred to inert carriers like peat, lignite ore or can be transferred to liquid medium. Around 10-12 per cent of Bacillus culture (containing approximately 10^9 cfu) was mixed with 85- 90 per cent of talc. This formulation is inert and can be directly applied to the seed before sowing.

Production and formulation of *Trichoderma harzianum* :

Trichoderma harzianum (T-22, MTCC, Chandigarh, India) can be grown in either potato dextrose agar (pH 7.2) or in corn meal agar or in rice medium. Last 2 options are economical and produce more fungal spores, which can be directly added to the inert carrier medium. Cornmeal is blended in 800 ml distilled water and left in refrigerator at 4°C overnight. Next day cornmeal is heated at 60°C for 1 hour, make up the volume to 1L. Add agar and heat to dissolve. Then following procedure is performed (Picard *et al.*, 2000).

Inoculation of culture either takes place in square bottles (750 ml) containing PDA medium or in large plates containing cornmeal agar. Bottles are to be incubated at room temperature (> 25°C). After 8-10 days of profuse growth scum is harvested in aseptic condition. It should be noted that harvesting must be done in sporulating condition. The scum is then added in sterilized talc (100ml scum + 1kg Talc). Now this mixture is ready to apply to the seed or soil (Fig. E).

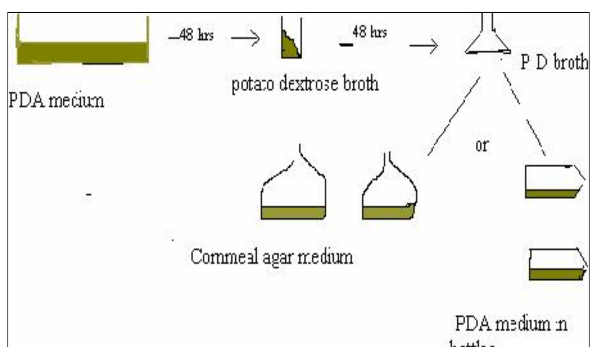

Fig. E : Production of *T. harzianum* (spores)

Treatment of chickpea seeds with *Bacillus subitilis* and *Trichoderma harzianum* :

In lab conditions (controlled) seeds of chickpea are treated directly with *Bacillus* sps. culture. These isolates have shown more effects in seed treatment. The effects of all treatments on shoot length, root length, dry weight of shoots and roots of chickpea plants were determined 50 days after inoculation by the pathogen.

In the field conditions, *Bacillus subtilis* is directly coated to the chickpea seeds at rate of 100 g of talc formulation for 15-20 kg of seeds. This can be achieved by making slurry of molasses or jaggery (*gud*) and adding culture to it. It should be mixed thoroughly and can be directly sprayed on the seeds. Seeds are then dried in shade for 10-15 min. Now *Trichoderma* sps. based formulation is applied on above treated seeds. 15-20 kg of seeds can be treated with 100-150 g of talc formulation dissolved in 20- 25 L of water.

RESULTS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented in Fig. 1 to 3.

Effects of bacterial antagonist on disease severity in controlled conditions :

The first symptom of disease was appeared 21 days after inoculation with *Fusarium oxysporium* (MTCC, Chandigarh). Observations are made after every 2-4 days. The effects of bacterial antagonist on the disease severity in both soil and seed treatments was studied. *B. subtilis* showed more effects, than other isolates. All bacterial isolates had significant effects on plant growth compared with the non-treated control in both methods. *B. subtilis* and *Trichoderma harzianum* significantly increased length of shoot and root of chickpea plants in soil and seed treatments. Moreover, these isolates increased dry weight of shoots and combined application of *Bacillus subtilis* and *Trichoderma harzianum* has synergistic effect in controling the growth of *Fusarium oxysporium* in *in-vitro* environment. *Trichoderma* sps. sowed more widely antagonistic effect on *Fusarium* sps. when grown separately with the pathogenic fungi. *Trichoderma* species also release certain growth regulating factors that increases the rate of seed germination (Windham *et al.*, 1985). On the other hand *Bacillus subtilis* sowed faster growth but proved weak antagonist as compared to *Trichoderma harzianum*. However, when both the species grown together proved to be efficient in controlling the growth of pathogenic fungi. In field conditions, *i.e.* in the natural environment, joint efforts of both the biopesticides show good results, which were notable.

Bacillus subtilis NB22 has a wide range of antifungal and antibacterial activities and can be used as a prominent agent to control various soil diseases in tomato (Phae *et al.*, 1992). The dual treatment by *Trichoderma* sps. and *B. subtilis* decreased the percentage of infection and increased survival rate than individual one. Moreover, the dual inoculation gave the highest records of growth parameters, seed yields and plant nutrient content than individual one (Morsy *et al.*, 2009).

Fig. 1 : *Trichoderma* sp.

Fig. 2 : *Fusarium oxysporium*

Fig. 3 : *Trichoderma* vs *Fusarium* sp.

Biological way of controlling pesticides (bacterial, fungal, protozoan, insects etc.) is very effective economically as well as environmentally. Though the mode of action of biopesticides is time consuming, but once acclimatized in the field environment, they execute very fast and have long-lasting effect in the field as compared to chemical pesticides (Chatar *et al.*, 2010 and Pandey *et al.*, 2012). Today, India and other developing countries need 2nd green revolution which can be achieved just by executing newly evolving technologies and not just discussing in papers. Similar work related to the present investigation was also carried out by Jha *et al.*, 2011; Katiyar, 2012 and Patil *et al.*, 2012.

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