In vitro Characterization of Trichoderma viride for Abiotic Stress Tolerance and Field Evaluation against Root Rot Disease in Vigna mungo L.

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Abstract

Soil-borne phytopathogenic fungi pose serious threats to yield of several crops. Biological control is an eco-friendly approach in the effective management of crop diseases. Trichoderma viride is an important soil-borne fungus, which play an important role in antagonism by secretion of different hydrolytic enzymes. Black gram is an important pulse crop world-wide and its yield is severely affected by Macrophomina root rot. Abiotic stresses greatly influence the performance of biocontrol agents. T. viride was evaluated for its in vitro abiotic stress tolerance ability and its field bioefficacy against root rot disease in blackgram. Growth of T. viride decreased with increasing in salinity, temperature and drought. T. viride effectively inhibited the growth of R. solani (45%) and M. phaseolina (40%) under in vitro conditions. T. viride was compatible with 0.25% mancozeb, 1.0% copper oxy chloride and metalaxyl. Among three doses, plants treated with 6 g.kg⁻¹ of T. viride showed highest yield of 1375 kg.ha⁻¹ and lowest root rot incidence of 14.77% which were statistically on par with 4 g.kg⁻¹ T. viride treated plants. To conclude, this study identified an abiotic stress tolerant T. viride for effective management of root rot disease and enhanced yield of Vigna mungo when applied as seed dresser at a concentration of 4 Kg⁻¹ under field conditions.

Introduction

Diseases caused by soil-borne phytopathogenic fungi pose serious threats to yield of several crops world-wide [1-2]. Biological control, the use of specific microorganisms that interfere with plant pathogens and pests, is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection [3-4]. Trichoderma spp. are fungi that are present in nearly all agricultural soils and in other environments such as decaying wood. Major mechanisms involved in the biocontrol activity of Trichoderma spp. are competition for space and nutrients, production of diffusible and/or volatile antibiotics and hydrolytic enzymes like chitinase and β-1,3-glucanase. These hydrolytic enzymes partially degrade the pathogen cell wall and leads to its parasitization [5]. This process (mycoparasitism) limits growth and activity of plant pathogenic fungi. Different species of Trichoderma have the potential to control soil-borne plant pathogens more effectively than chemicals [6] and they also exhibit plant-growth-promoting activity [7,9]. Use of these fungi is not as harmful to the environment as chemical pesticides. They are present in substantial quantity in nearly all agricultural soils and in other environments such as decaying wood and their use is only now being recognized world over as an alternative in plant disease control [4].

Blackgram (Vigna mungo L.) is one of the important pulse crops gaining importance all over the world in recent years. It is rich in proteins and contains amino acids in higher quantities than any other cereals and pulses. It is affected by number of diseases caused by fungi, bacteria and viruses. Among them the root rot caused by Macrophomina phaseolina is a major barricade that leads to severe crop loss. Biocontrol of black gram root rot disease by Trichoderma spp. has been an alternative to chemical control [10].

Like crops are affected by abiotic stresses, microbes are also known to be affected by these conditions. However, successful deployment of these organisms in stressed ecosystems depend on their ability to withstand and proliferate under adverse environments such as drought, high temperatures, salt stress, mineral deficiency, chemical and heavy metal toxicity which are major problems in rainfed agro-ecosystems. The principal stress factors in India are drought or soil moisture stress, which adversely affects nearly two third area of arid and semi arid eco systems. High temperatures, soil salinity/alkalinity, low pH and metal toxicity have a significant influence on the performance of agriculturally important microorganisms (AIMS). The selection and deployment of aims in stressed ecosystems therefore requires concerted research and technology development.

In view of this, we laid out an experiment carried out a study on abiotic stress tolerance of T. viride and its field bioefficacy in controlling root rot in black gram under typical rainfed conditions.

Material and Methods

Fungal culture

Trichoderma viride culture was procured from Centre for Plant Protection Studies, Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu, India and maintained by sub-culturing in Elad and Chat medium (g. Lit-1) (mycological peptone-5.0; dextrose-10.0; agar-agar-20; ph-5.2). It was commercialized under the trade name ‘Trikoraksha’ 1% WP.

Screening for abiotic stress tolerance of T. Viride

High temperature tolerance: A 5mm disc of T. Viride was inoculated on the potato dextrose agar (PDA) (peeled potatoes-200gm, dextrose-10gm, distilled water-1000 ml with pH-6.5) and incubated at

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30°C, 45°C and 50°C. The results were recorded based on growth and sporulation pattern compared to control plates which were incubated at 30°C.

**Salinity tolerance:** A 5 mm disc of *T. viride* was placed on 0.1, 0.5, 0.75, 1.25, 1.5, and 2.0 M NaCl amended in PDA medium and incubated at 30°C for 4 days. Percentage reduction of growth in salt amended media was calculated by using the formula (100 x A-B)/A), where A is radial growth in control plate in 'mm' of the isolate and B is radial growth in salt amended plate.

**Drought tolerance:** A 5 mm disc of *T. viride* was transferred to the PDA media containing 10%, 20%, 30%, 35% and 40% of polyethylene glycol (PEG 6000 Da) and incubated at 30°C for 4 days. Percentage reduction of growth in PEG amended media was calculated as described above.

**Agrochemicals**

Poison food method [11] was adopted to study the compatibility of *T. viride* with chemical fungicides. 0.1%, 0.25%, 0.5% & 1% solutions of mancozeb, carbendazim, copper oxy chloride and metalaxyl were prepared filter sterilized and incorporated into PDA medium. A 5 mm disc of actively growing *T. viride* was placed on the opposite side of the plate and incubated at 30°C for 5 days. Antagonistic activity was measured as zone inhibition and growth reduction.

**Screening for antagonistic activity of *T. viride***

To evaluate the *in vitro* antagonistic activity against selected phytopathogens viz., Botrytis ricini, Fusarium oxysporum, Macrophomina phaseolina, Sclerotium rolfsii and Pyricularia oryzae. A 5 mm disc of actively growing culture was placed on the periphery of the malt-dextrose agar (g. Lit -1) (peptone-2.0, malt extract-20.0, yeast extract-2.0, dextrose-5.0, agar-agar-20.0, ph-5.8) plate. Similarly test pathogen was plugged with cotton wool and wrapped in aluminium foil before autoclaving at 121°C for 1 h for two consecutive days. After cooling, the flasks were inoculated with a 2 cm mycelial plug cut from the periphery of mancozeb, carbendazim, copper oxy chloride and metalaxyl were prepared filter sterilized and incorporated into PDA medium. A 5 mm disc of actively growing culture was placed on the periphery of malt-dextrose agar and incubated at 30°C for 4 days. Results were recorded by the percent reduction of growth compared to fungicide free inoculated plates.

**M. phaseolina production and soil inoculation**

Rice grains were soaked in water for 30 min, air dried and filled in 500 ml Erlenmeyer flasks @ 50g/ flask. The mouth of each flask was plugged with cotton wool and wrapped in aluminium foil before autoclaving at 121°C for 1 h for two consecutive days. After cooling, the flasks were inoculated with a 2 cm mycelial plug cut from the periphery of a 7-day-old culture of *M. Phaselia*. The inoculated flasks were incubated at 28±2°C for 15 days for the rice seeds to be completely colonized by the pathogen. The inoculum was stored at 4°C before use in the field. One hundred colonized rice seed were placed on PDA and incubated at 28±2°C for five days and cultures were examined under microscope for the presence of pathogen. The number of seeds showing the mycelial presence of the pathogen and percentage recovery of *M. Phaselia* from the inoculant was calculated. Ten colonized rice seeds were introduced per planting hole to induce *M. Phaselia* infection in field.

**Field experimental design and seed treatment**

A field trial was conducted in randomized block design (5x5 m²) with one cultivar (*V. mungo* var. T9) in two different plots at Hayathnagar research farm (N 17.34 and E 78.59) of Central Research Institute for Dryland Agriculture, Hyderabad, India. The plot was contaminated with *M. phaseolina* as described, in which negative control of disease, three different dosages of *T. viride* (2.0, 4.0 and 6.0 g kg⁻¹ seed) as seed dresser were applied with 4 replications. Positive control of disease was maintained in healthy plot with 4 replications. Seeds were sown in plots with 45x15 cm spacing. Germination was recorded 10 days after sowing (DAS). The root rot incidence was recorded at 15 days interval till harvest. After maturity, dry weight of the plants and grain yield (kg/ha) were recorded.

**Assessment of phytotoxicity and disease incidence**

Observations on phytotoxicity were recorded on 1, 3, 7 and 14 days after seed treatment by recording the necrosis of leaf and stem region. The root rot incidence in black gram was assessed by morphological observation of the dead plant roots, followed by the isolation of pathogen under *in vitro* conditions. The percent of disease incidence was calculated based on the formula

\[
\text{Number of infected plants} \times 100
\]

\[
\text{Total number of plants}
\]

**Results**

**Abiotic stress tolerance**

High temperature tolerance pronounced effectively in the test strain. At 30°C 1.2×10⁷ cfu/g were observed whereas at 45°C cfu were 3.8×10⁷/g and decreased further at 50°C (10⁷ cfu/g) (Figure 1).

In case of salinity, growth decreased significantly with increase in salt concentration in the medium. When compared to control plate, 0.1 M salt added medium had 92.9% growth followed by 0.5 M (85.6%), 0.75 M (69.4%), 1.25 M (35.2%), 1.5 M (21.1%) growth was observed and at 2 M salt concentration growth was completely inhibited (Figure 2).

Drought tolerance of the test isolate appeared to be more or less similar to other stresses (Figure 3). At 10% of PEG concentration growth was higher with only 1% decrease than control. Whereas, at 20% PEG concentration, 94.3% growth was recorded which, gradually decreased thereafter to 79.5% in 25% PEG and 68% in 30% and in 35% PEG 63% growth was observed (Figure 3). Profuse sporulation was observed in presence of 10 and 25% PEG concentration whereas, 25 and 30% PEG concentration showed scanty sporulation and at 35% PEG concentration sporulation was completely absent.

![Figure 1: Temperature tolerance of *T. viride.*](image-url)

\[
\text{Growth} \times 10^7 \text{ CFU/mL}
\]

\[
\text{Temperature (°C)}
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<th>Temperature (°C)</th>
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The test *T. viride* strain was also more tolerant to chemical fungicides. Among the tested fungicides, 0.5% mancozeb completely inhibited the growth of *T. viride*, whereas, 100% and 56% growth was recorded with 0.1% and 0.25% mancozeb respectively compared to control. Test strain was more compatible with copper oxy chloride (COC) than any other chemicals tested. *T. viride* was not inhibited at 0.1-0.5% of COC tested whereas, at 1% of COC the growth was reduced to 45%. In case of metalaxyl, 47% and 13.5% growth was recorded at 0.5% and 1% concentration respectively (Table 1).

### In vitro antagonistic activity of *T. Viride*

The test isolate was also effective in inhibiting the growth of potential phytopathogenic fungi (Figure 4). *Botrytis cinerea* growth was inhibited by 27% compared to control whereas, 31% inhibition of *Fusarium oxysporum* f. Sp. *ciceri*, *Macrophomina phaseolina* (40%), *Rhizoctonia solani* (45%), *Sclerotium rolfsii* (36%) were recorded whereas, in case of *Pyricularia oryzae* only 12% growth was inhibited (Figure 4).

### Root rot disease control

*Vigna mungo* treated with *Trichoderma viride* was able to effectively combat root rot disease in the conducted field trial, denoting the importance of *T. viride* in disease management. Highest seed germination of 97.3% was observed when *T. viride* was used at the concentration of 4 g.Kg⁻¹, whereas at 6 g.Kg⁻¹ seed coating showed 97.1% germination. In case of plant dry mass, 6 g.Kg⁻¹ *T. viride* treated seeds showed highest dry mass of 18.88 gm followed by 4 g.Kg⁻¹ *T. viride* treated seeds. Highest disease incidence was observed in un-treated control plants with 39% root rot incidence whereas, lowest disease was observed where *T. viride* @ 6 g.Kg⁻¹ was applied. Highest yield of 1375 kg.Ha⁻¹ followed by 1370 kg. Ha⁻¹ was observed in the plots treated with 6 g.Kg⁻¹ and 4 g.Kg⁻¹ *Trichoderma* respectively. In case of plants sown in sick plot the yield was only 870 kg. Ha⁻¹ (Table 2) compared to the seeds sown in healthy plot (1140 kg. Ha⁻¹). None of the treatments showed any adverse effect on the plant in terms of necrosis on leaves and stem which are common symptoms of phytotoxicity. Hence, the products could be safely recommended as seed dressers.

### Discussion

The test *T. viride* isolate has the ability to withstand different abiotic stresses suggesting that the inoculant has better survival, efficacy, adaptability and thereby improving plant productivity under
rainfed conditions. Widden and Hsu [12] observed that the ability of different species of *Trichoderma* to colonize pine or maple litter differed with temperatures. The reason behind evaluation of abiotic stress tolerance in the current strain was that the stress tolerant strains can be efficiently deployed in extreme environments where they can show better rhizosphere competence and saprophytic competitive ability. Interestingly, some of the abiotic stress tolerant microbes also protected plants from abiotic stresses like drought [13], chilling injury [14], high temperature [15], and salinity [16]. A few recent reports demonstrated that these fungi alleviate abiotic stresses. Field data indicates that they may confer tolerance to drought stress at least in part through promotion of deeper root penetration into the soil profile [17]. In a recent report, *Thamatum* increased tolerance of cocoa plants to water deficit through increasing root growth that provided greater water resources to treated plants and delayed the onset of water deficit in these plants [18].

Results showed that *T. viride* could restrict the growth of potential phytopathogens in dual culture which prove its efficacy in management of crop diseases. The growth inhibition of tested pathogens may be due to antibiotic secretion of like trichodermin, derminad, trichovirdin and sesquiterpene heptical acid [19], nutrient impoverishment and ph alteration in the medium [20]. Hence, *T. viride* has a potential to develop as a biological agent to control the common post harvest diseases. The growth inhibition of postharvest fungi by dual culture in this study could be due to its fast growing nature, secretions of harmful extra-cellular compounds like antibiotics, cell wall degrading enzymes such as glucanases, endochitinases and chitinases and mycoparasitism [19,21,22].

There is scanty information about interactions between pesticides and those fungi which are antagonistic to various plant pathogens. Our experiments with routinely used chemical fungicides have confirmed the published reports and also have assessed the activity of different chemical fungicides on *T. viride*. This concluded that this bio-agent can be used in combination with COC, metaxyl and mancozeb at determined dosages. This study also suggests that, even in chemical fungicides contaminated fields the organism has a better survival for enhancing crop productivity. There have been few reports on the effect of some pesticides on naturally occurring strains of *T. viride*: for example, Pribela et al. [23] have reported that benomyl and dichlofluanid are highly active against this fungal biocontrol agent. Baicu [24] identified some pesticides which can be used along with *T. viride* in integrated control of plant pathogens.

Results obtained from this study showed that *Trichoderma* species could be used effectively to control the root rot disease. This ability of the *Trichoderma* as a biocontrol agent was also reported by Upadhyay and Mukhopadhyay [25]. They reported that *T. harzianum* isolate IMI 238493 lyses the mycelia and sclerotia of *Sclerotium rolfsii*. Inbar et al. [26] also observed hyphal interaction between the mycoparasite, *T. harzianum* and the soilborne pathogen, *Sclerotinia sclerotiorum*. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods [27]. *Trichoderma* spp. are now the most common fungal biological control agents that have been comprehensively researched and deployed throughout the world.

The inhibitory activity of *T. viride* against soil borne fungal pathogens found here were similar to the findings of [28,29]. The inhibitory effects observed here were mainly attributed to competition for space, nutrition between the pathogens and antagonists. Antagonists may also affect growth of pathogen either through antibiosis or mycoparasitism. Besides, they may also produce antifungal phenolic compounds [30].

Aggarwal et al. [31] reported isolates of *T. viride* improved growth of wheat crop such as shoot length, root length and 1000 grain weight. Singh and Singh [32] observed maximum disease reduction in pyrítica treatment followed by neem cake. Similar type of results were recorded by Basu and Maity [33] who reported that stem rot of potato was reduced by the amendments of NPK+FYM. The increase in yield in *Trichoderma* treated plots could be due to control of root rot and its plant growth promoting activities. Plant growth promoting activity of *T. harzianum* was well documented for its phosphate and micronutrient solubilization [34]. Highest yield was recorded in 6g.Kg⁻¹ *T. viride* treatment which was statistically (P<0.05) on par to 4g.Kg⁻¹ treatment. So, 4g.Kg⁻¹ *T. viride* as seed treatment in *Vigna mungo* is optimum dosage for attaining disease control and maximum yield.

**Conclusion**

This study has identified a potential strain of abiotic stress, chemical fungicides tolerant *Trichoderma viride* capable of effectively controlling root rot disease in *Vigna mungo* L. And attaining maximum yield under field conditions when applied as seed dresser at a concentration of 4g.Kg⁻¹. This research opens a new way in disease management of rainfed crops for enhancing crop productivity in rainfed agro-ecosystems for the benefit of small and marginal farmers.

**References**

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