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RESEARCH ARTICLE

Study on Isolation, Effectiveness and Formulation of Native Trichoderma spp. from Jhum, Virgin and Cultivated Land of Nagaland

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ABSTRACT

Ten Trichoderma isolates named as Trichoderma isolate1 to Trichoderma isolate 10, were isolated from different soil samples collected from virgin, Jhum, cultivated, and fallow land of Medziphema block of Nagaland state by serial dilution agar plate method on Trichoderma selective medium (TSM). All the Trichoderma isolates were tested against Fusarium solani and Sclerotium rolfsii by dual culture method. All the isolates were highly effective against both the pathogens with 83-93 percent and 72-89 per cent growth inhibition of Fusarium solani and Sclerotium rolfsii, respectively. Among the tested Trichoderma isolates, Trichoderma isolate1 was observed most effective; therefore, it was mass multiplied in Potato Dextrose Broth and formulated in talc powder. The colony forming units were counted thrice to determine the number of viable conidia in product; the first counting was done on the same day of formulation, the second counting was done one month, and the last was done six months after formulation. It was observed that the colony forming units decreased from 13 × 106 c.f.u. to 5.66 × 106 c.f.u. after six months of storage from the initial formulation which indicates that even after six months of storage, the Trichoderma isolate 1 formulated by using talc powder still contained good number of viable conidia. Thus, from the overall results obtained from the present work, it can be concluded that Trichoderma spp. as a ubiquitous fungus and an effective biological control agent can be one of the paramount components of integrated disease management.

Keywords: *Trichoderma; Jhum; Mass Multiplication, Talc Formulation; Nagaland.*

INTRODUCTION

The genus *Trichoderma* was identified by Persoon in 1794. The use of *Trichoderma* as an antagonist of plant pathogenic fungi and as an agent of biological control was discovered much later (Weindling, 1932 and 1934). *Trichoderma* is a saprophytic fungus found in soil, rhizosphere, and other habitats but has been used recently with greater emphasis as a biocontrol agent of plant diseases (Zhang *et al*., 2005). Through various research studies several species of *Trichoderma* are significantly inhibitory to oomycete and fungal plant pathogens like *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *F. culmorum* and *Geaumannomyces graminis* var. *tritici* under *in vitro*, greenhouse and field conditions (Chet and Baker, 1981; Whipps, 1987; Sivan and Chet, 1993; Inbar *et al*., 1994; Basim *et al*., 1999, Kucuk, 2000). *Trichoderma* species have been reported to produce various secondary metabolites, including **antibiotics that have direct antimicrobial roles. *Trichoderma* species have been known to induce systemic** resistance in plant (Harman *et al*., 2006).

Trichoderma as antagonist has several mechanisms of action (Chet, 1987 and Kucuk, 2000) like production of lytic enzymes (Haran *et al*., 1996; Kucuk, 2000), production of antifungal antibiotics (Dennis *et al*., 1971, Brewer *et al*., 1987 and Almassi *et al*., 1991), competition with pathogens (Whipps, 1987) and promotion of plant growth (Inbar *et al*., 1994). Production of various metabolites by *Trichoderma* depends on several ecological factors that explain the antagonists' varying performance in different agro-ecological situations (Rifai, 1969; Henis, 1984; Papavizas, 1985).

In India, hundreds of researchers engaged themselves in research with various species of *Trichoderma* intending to develop biocontrol products for plant disease management (Sharma *et al*., 2014). It is pertinent to say that *Trichoderma* species have immense potential for use in the plant disease management in states like Nagaland where traditional farming system is followed and farmers are generally reluctant to use synthetic plant

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protection chemicals and interested to use natural products only. However, *Trichoderma* spp. must be isolated, identified, characterized and screened for this effectiveness to begin in this direction. Since there has been no systematic and widespread work on *Trichoderma* spp. of Nagaland, the present work has been carried out with the objective of isolating *Trichoderma* spp. from soil of Nagaland, studying their effectiveness and finally testing a laboratory-made formulation.

MATERIAL AND METHODS

The present work was carried out in the Department of Plant Pathology, School of Agricultural Sciences and Rural Development (SASRD), Nagaland University, situated at 25°45′ 43″ N latitude and 93° 53′ 04″ E longitude at an elevation of 310 m above mean sea level.

The soil samples were collected from different fields, namely virgin, *Jhum*, cultivated, and fallow land of Medziphema block of Nagaland state.

The *Trichoderma* selective medium (TSM) was used for the isolation of *Trichoderma* spp. and Potato Dextrose Agar (PDA) medium was used for purification of *Trichoderma* spp., isolation of soil-borne plant pathogens, maintenance of all the microorganisms and for performing dual culture technique.

TSM was prepared following the composition Akrami (2011) ($MgSO_4$ - 0.2 g, K_2HPO_4 - 0.9g, KCl - 0.15g, NH_4NO_3-1g, D-glucose anhydrous - 3g, Rose Bengal - 0.15g, Agar-agar - 20g). These constituents were added into 950 mL of distilled water and autoclaved at 121 ^{0}C for 20 minutes. The biocidal ingredient, chloramphenicol, of 0.25g quantity was mixed in 50 mL of sterilized distilled water and added to a cool molten autoclaved basal medium.

The PDA medium was prepared by following the composition described by Ricker and Ricker (1936) (Peeled potato - 200g, Dextrose - 20g, Agar-agar - 20g, Distilled water - 1000mL, pH – 7.0).

Isolation of Trichoderma spp. by serial dilution-agar plating method

Nine millilitres sterilized distilled water blanks were labeled as 1, 2, 3, 4 and 5 and sterilized plates as 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} with a marker. One gram sample of finely pulverized, air dried soil was added into the first water blank to make 1:10 dilution. Dilution was shaken vigorously to obtain uniform suspension of microorganisms. One millilitre of suspension was transferred from the first tube into the second water blank with the help of a sterile pipette under aseptic condition and shaken well. Further dilutions were made as 10^{-3}, 10^{-4} and 10^{-5} by pipetting one millilitre suspension into additional water blanks (3, 4 and 5). Aliquots (0.1 mL) were transferred from each 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions into three sterile plates containing solid autoclaved media and were spread with the help of 'L' shaped spreader. All the plates were incubated at 28±1^{0}C for seven days.

Purification of fungi

The colonies of *Trichoderma* isolates were picked up aseptically from seven days old culture and streaked on streptomycin supplemented PDA slants with the help of a sterilized needle. Cultured slants were incubated at 28±1 ^{0}C for seven days and preserved in a refrigerator for further use. Purified *Trichoderma* isolates were sub-cultured in Petri plates for desired tests

Identification of Trichoderma isolates

The *Trichoderma* isolates were studied under compound microscope and identified on the basis of morphological characters such as colonial growth, colour, shape and size of conidiophores, spores and phialides, in consultation with relevant literatures. Shape and size were recorded with the help of a compound microscope (magnification=450X). The size was measured with the use of a stage and ocular micrometer and expressed in micron (µ).

Growth rate of Trichoderma isolates on PDA medium

The culture discs (seven days old) of all the *Trichoderma* isolates were cut separately with the help of a sterile cork borer (5 mm diameter). The discs were aseptically transferred in cool, autoclaved medium with the help of a sterile needle and incubated at 28±1 ^{0}C.

In-vitro observations on growth rate were recorded at 24 hours as the first observation and ed at 12 hours intervals till plateful growth of the isolates. Radial growths of pathogens were measured in terms of millimetre (mm).

Testing the effectiveness of *Trichoderma* spp. on soil-borne plant pathogens

The effectiveness of the 10 isolates of *Trichoderma* spp. was tested against *Fusarium solani*, the wilt pathogen of solanaceous crops and *Sclerotium rolfsii,* the stem rot pathogen of soybean, by dual culture technique under *in-vitro* condition. The experiment was conducted following a Completely Randomized Block Design (CRD) with three replications. In the control plates, only the pathogens were inoculated separately. Per cent inhibition of the growth of the pathogens is calculated using the following formula:

$$\text{Per cent inhibition} = \frac{\text{Radial growth in control(C)} - \text{Radial growth in the treatment(T)}]\times 100}{\text{Radial growth in control(C)}}$$

In-vitro observations on the inhibition effect were recorded at 24 hours intervals till the growth of the pathogens was full in the control plates. Radial growths of pathogens were measured in terms of millimetre (mm).

Mass multiplication and formulation of the most effective Trichoderma sp.

Trichoderma isolate 1 was observed to be most effective against both tested plant pathogens; thus, it was mass multiplied and formulated as described below. Colony-forming units were counted from final formulation by serial dilution method.

Medium for mass multiplication

The Potato Dextrose Broth was used for mass multiplication of *Trichoderma* isolate 1 (Composition: Peeled potato – 200 g, Dextrose – 20 g, Distilled water - 1000mL, pH – 7.0)

Mass multiplication Procedure

The culture discs (seven days old) of *Trichoderma* isolate 1 were cut with the help of a sterile cork borer (5 mm diameter). The discs were aseptically transferred in a conical flask containing autoclaved cool broth (200 mL) with the help of a sterile needle and incubated at 28±1 ^{0}C for seven days.

Formulation

The formulation was prepared by following the composition described by Jeyarajan *et al*. (1994). Mass multiplied *Trichoderma* isolate 1 was mixed with sterile talc powder in a ratio of 1:2 and dried under shade. Shade dried formulation was transferred in sterile polythene bags and stored in refrigerator for further tests.

Colony forming unit (cfu) count of the formulation

To determine the quality and shelf life of the product the colony forming units were counted thrice; first counting was done on the same day of formulation, the second counting was done one month after formulation and the last counting was done six months after formulation by serial dilution method; where nine millilitres sterilized distilled water blanks were labelled as 1, 2, 3, 4, 5 and 6 and sterilised plates as 10^{-4}, 10^{-5} and 10^{-6} with marker. The one-gram final formulation was added into the first dilution blank to make 1:10 dilution. Dilution was shaken vigorously to obtain a uniform suspension. One millilitre of suspension was transferred from tube number one into water blank number two with the help of a sterile pipette under aseptic conditions and shaken well. Further dilutions were made as 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} by pipetting one millilitre suspension into additional water blanks (three, four, five and six). Aliquots (0.1 mL) were transferred from each 10^{-4}, 10^{-5} and 10^{-6} dilutions into three sterile plates containing solid autoclaved PDA medium and spread with the help of 'L' shaped spreader. All the plates were incubated at 28±1 ^{0}C. Colony forming units was calculated by the following formula:

$$\text{cfu} = \frac{\text{Number of colonies in three replications} \times \text{dilution factor}}{\text{Number of replications}}$$

Analysis of variance was done by using Fisher's method. To determine the significance or non-significance, respective 'F' value was calculated and the calculated value was compared with the corresponding table value at five percent probability level.

The standard error of differences of mean (S. Ed.) was calculated by using the following formula:

$$\text{S. Ed.} = \frac{\sqrt{2 \times \text{Error mean square}}}{\text{Number of replications}}$$

Critical difference (C. D.) for the significance 'F' value were calculated by multiplying the standard error of difference with corresponding 't' value at the probability level. The formula is given below

$CD_{0.05}$ = S. Ed × $t_{0.05}$ for error degree of freedom.

Where,

$t_{0.05}$ = table value for error degree of freedom at five per cent level of significance.

The percentage values were transformed into corresponding value by Arc sine.

Results and Discussion

Isolation and identification of Trichoderma spp. from soil samples

In the present work, ten *Trichoderma* isolates have been obtained and named *Trichoderma* isolate 1 to *Trichoderma* isolate 10 (Table 1). Identification of *Trichoderma* isolates was done based on characters with the help of available literature (Nagamani *et al*., 2009 and Rahman *et al*., 2011). However, species' identification and differentiation is problematic because of the homoplasy of morphological characters.

Characteristics of Trichoderma isolates

All *Trichoderma* isolates exhibited different morphological characteristics such as colonial growth, number of concentric rings, colour, structure, size, and shape of conidiophores, phialides, and spores.

The mycelial growth of all the *Trichoderma* isolates was studied on Potato Dextrose Agar (PDA) medium. The data obtained from the study showed that (Table 2), all the isolates utilized the PDA media efficiently with the best mycelia growth and abundant sporulation. All the *Trichoderma* isolates attained plateful growth within 72 to 84 hours. The difference in growth rate was observed. Among the tested isolates, the *Trichoderma* isolate 7

showed the fastest growth, followed by *Trichoderma* isolate 5 and *Trichoderma* isolate 8. *Trichoderma* isolate 2 was relatively the slowest. However, the rate of mycelial growth among the isolates was non-significant.

The present work's findings conform with the report of Mustafa *et al*. (2009). They found that the PDA medium was best for all types of growth of *Trichoderma* species, and the growth rate was maximum in the case of *T. viride* due to the inherent ability of *Trichoderma* spp. to utilize the culture media.

Testing the effectiveness of Trichoderma spp. on soil-borne plant pathogens

In the present work, ten *Trichoderma* isolates were screened to testify their ability to inhibit mycelial growth of *Fusarium solani* and *Sclerotium rolfsi*i by dual culture technique.

Effectiveness of Trichoderma isolates on Fusarium solani

The data obtained from the present work showed that (Table 3), the ability of different isolates of *Trichoderma* to inhibit the radial growth of *F. solani* varies between isolates and also showed statistically significant inhibition (P≤0.05) to the control plate. Per cent inhibition of the *F. solani* by the *Trichoderma* isolates was calculated 120 hours after incubation when the growth of the pathogens was full (90mm) in the control plates. Results of the test showed that all the isolates were highly effective against the pathogen with 80.19-92.96 per cent growth inhibition. Among the tested isolates, the *Trichoderma* isolate 1 showed excellent antagonistic activity against *F. solani* with 92.96 per cent of inhibition, followed by *Trichoderma* isolates 2 with 91.11 per cent of inhibition. However, lowest inhibition was observed in the case of *Trichoderma* isolates 6 with 80.19 per cent. The recorded data also showed that *Trichoderma* isolates 5, *Trichoderma* isolates 4 and *Trichoderma* isolates 10 were effective against the pathogen with 87.78 per cent, 87.22 per cent and 85.37 per cent of inhibition, respectively.The present work's findings conform with the report of Marquez *et al*. (2002), where they recorded inhibition per cent of *Fusarium oxysporum* f.sp. *dianthi* by *Trichoderma* spp. was as high as 89 per cent. Waghmare and Kurundkar (2011) also studied the effectiveness of fifty *Trichoderma* isolates against *F. oxysporum* f. sp. *carthami* causing wilt of safflower by dual culture method where *Trichoderma* isolate 29 inhibited maximum mycelia growth of the pathogen followed by *Trichoderma* isolate 33. Ommati and Zaker (2012) also reported 62.00 to 84.32 inhibition per cent on the causal organism of potato wilt disease (*F. oxysporum*) by *Trichoderma* spp. by dual culture technique.

In addition, Karunanithi and Usman (1999) reported that the *T. viride* was highly effective in reducing radial growth against *F. oxysporum* f.sp. *sesami* in dual culture followed by *T. harzianum* by producing non-volatile compounds. However, Mishra *et al*. (2011) reported that *T. viride* isolate Tr 8 was effective against *F. solani* with 69.3 per cent growth inhibition. Hari Krishna and Reddi Kumar (2013) also reported 57 to 81.57 per cent growth inhibitions of *F. solani* by 14 isolates of *Trichoderma* spp. in dual culture technique.

Effectiveness of Trichoderma isolates on Sclerotium rolfsii

The data obtained from the present work showed that (Table 4), the ability of different isolates of *Trichoderma* to inhibit the radial growth of *S. rolfsii* varies between isolates and led statistically significant inhibition (P≤0.05) to control plate. Per cent inhibition of the *S. rolfsii* by the *Trichoderma* isolates was calculated 144 hours after incubation when the growth of the pathogen was full (90mm) in the control plates. Results of test showed that all the isolates were highly effective against the pathogen with 72.04-88.52 per cent growth inhibition. Among the tested isolates, the *Trichoderma* isolate 1 showed excellent antagonistic activity against *S. rolfsii* with 88.52 per cent of inhibition followed by *Trichoderma* isolates 6 with 85.00 per cent growth inhibition on *S. rolfsii*. However, the lowest inhibition was observed in case of *Trichoderma* isolates 8 with 72.04 per cent. The recorded data also showed that *Trichoderma* isolates 2, *Trichoderma* isolates 3, *Trichoderma* isolates 4, *Trichoderma* isolates 7 and *Trichoderma* isolates 9 were effective against the pathogen with 83.33 per cent, 84.63 per cent, 83.15 per cent, 83.89 per cent and 83.70 per cent of inhibition, respectively. Moreover, sclerotia were not noticed in treatment plates, but in control plates 10-13 sclerotia were recorded. The present work's findings conform with the report of Garcia-Nunez *et al*. (2012), where they recorded high per cent inhibition of *Sclerotinia* spp. by *Trichoderma* strains. Ali and Javaid (2015) also reported that *T. viride* has the better antagonistic potential than the *T. harzianum* against *S. rolfsii* that causes of collar rot disease of chickpea.

In addition, Mishra *et al*. (2011) reported that *T. viride* isolate Tr8 inhibited 68.2 per cent growth of *S. rolfsii*. Castillo *et al*. (2011) reported that *Trichoderma* strains inhibited 45-63.80 and 50.90-81 per cent mycelia growth of *Sclerotinia sclerotiorum* and *Sclerotium cepivorum*, respectively.

Ramanujam *et al*. (2002) observed mycoparsitism and lysis of plant pathogenic fungi by *Trichoderma harzianum* and *T. viride*. Michrina *et al*. (1995) and Calistru *et al*. (1997) reported that different mechanisms of inhibition are involved, like competition and production of antibiotics (volatile and non-volatile compounds).

Mass multiplication and formulation of Trichoderma sp.

Trichoderma species as a successful biological control agent relies on the isolation, identification, characterization and antagonism and on the successful mass multiplication and formulation of the *Trichoderma* spp. in laboratory. Among the tested *Trichoderma* isolates, *Trichoderma* isolate 1 was the most effective against both pathogens. Therefore, *Trichoderma* isolate 1 was mass multiplied in Potato Dextrose Broth (PDB) and formulated by using talc powder. The *Trichoderma* isolate 1 formed white cum green thick mat of mycelia and spores, indicating that the growth and sporulation were good in PDB.

Trichoderma isolate 1 formulated by using talc powder was good with enough conidia. It was observed that the colony forming units decreased from 13 × 10^6 cfu to 11.66 × 10^6 cfu after one month to 5.66 × 10^6 cfu after six months of storage from the initial formulate. This indicates that even after six months of storage, the Trichoderma isolate 1 formulated by using talc powder still contained a good number of viable conidia. Since the viable conidia of *Trichoderma* isolate 1 in formulation were reduced by 57 per cent after six months of storage, it can be used before five months. The formulation was also compared with the six months old formulation obtained from Bio-control Laboratory, Medziphema, Nagaland. It was observed that both formulations were equivalent.

The findings of present work are in concordance with the report of Jeyarajan (1994) that the talc-based formulations of *Trichoderma* have a shelf life of 3 to 4 months. Viability of *Trichoderma* in talc formulation is reduced to 50% after four months of storage (Sankar and Jeyarajan, 1996).

CONCLUSION

It is important to assess spatial and temporal crop conditions to understand the impact of wagering monsoon, pest and disease incidence, and management practices, which directly affect the crop yield and, subsequently, the market. Although the estimation of crop area could be achieved with higher precision through remote sensing, obtaining information on crop conditions through the growing season is always a constraint due to high spatially and temporally variability. This issue has been overcome with the development of an -source tool coded using Python scripting with extended GDAL and OGR libraries to process Spatio-temporal satellite data and vector files for effectively delineating crop conditions from the backscattered values. The tool can generate statistics from farm level to State or country level in a quicker time, and the outputs are presented in both image and excel formats, which can serve as a base for understanding the crop condition spatially and temporally. The automated tool developed is of open source type and is specific to rice crop condition assessment from temporal SAR satellite data, while there exists scope for extending to other crops by analyzing backscatter signatures and the crop growth cycle. Some of the hard-coded parameters for assessing the crop condition can be made as user input to make the tool more dynamic.

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Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflict of interest in the publication of this content

Author contributions

Idea conceptualization-CDR, SB, Experiments- CDR ,Guidance -SB, Writing original draft – CDR, Writing-reviewing &editing -SB.

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