J. Mycopathol. Res. 40(2) : 81–87 (2001)

Some studies on *Trichoderma* as biocontrol agent

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The genus *Trichoderma* is being extensively used as biocontrol agent against plant pathogens. In the present paper results of four strains distributed in two species have been presented. The various aspects for its growth like media, pH and temperatures were studied including their antagonistic potential against *Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani and R. bataticola*. The different substrates were also tested to select suitable media for mass multiplication.

Key words : *Trichoderma*, biocontrol, root pathogens

INTRODUCTION

Biological control is the reduction of inoculum density or disease producing activities of a pathogen or a parasite in its active or dormant state, by one or more organism accomplished naturally or through manipulation of the environment, host or antagonist or by mass introduction of one or more antagonists (Garrett, 1970 ; Cooke and Baker, 1983). Many fungi are used but *Trichoderma* sp. and *Gliocladium* sp. are widely used as biocontrol agent (Bandyopadhhayay, 2001).

Biocontrol agents are mainly used against soil borne fungi like *Fusarium* sp., *Sclerotium* sp., *Rhizoctonia solani* and *R. bataticola*, etc. Besides these, *Phytophthora* and *Pythium* sp. are also controlled by these agents.

The present study aimed to screen potential *Trichoderma* species/strains which is effective against major root pathogens *Rhizoctonia solani*, *Rhizoctonia bataticola*, *Sclerotium rolfsii* & *Fusarium oxysporum* and to formulate a product which has easy applicablity in the field.

MATERIALS AND METHODS

Collection of soil samples for isolation of *Trichoderma*

Soil samples as well as rotten cow dung were collected from different places and isolations were made on PDA by directly sprinkling fine pulverized samples on poured plates. The plates were incubated for two days at 25° ± 2°C and *Trichoderma* was isolated and purified from such plates. Species/strains of *Trichoderma* were also isolated from diseased roots of crops like soybean, gram/pulse crops. Preliminary differentiation of *Trichoderma* was made by seeing culture of colony, pigmentation, growth and sporulation. Later the isolates examined microscopically. Photomicrographs of the *Trichoderma* were also taken by Leica DMLB binocular microscope MPS-30 model camera.

Isolation of test fungi

Test fungi viz. Rhizoctonia *bataticola, R. solani, F. oxysporum* f.sp. *ciceri* and *Sclerotium rolfsii* were isolated from diseased roots/plants of chickpea and maintained on PDA.

Study of growth and sporulation of different species/strains of *Trichoderma*

All the 12 strains viz. H-14, B-1, G, B-4, B-9, B-

19, B-29, B-11, H-21, M-1, M-7, M-9 etc. were grown on PDA out of which 4 species/strains of *Trichoderma* viz. H-14, B-29, G and M-7 were selected being highly sporulating and produce more pigment. The testing was done on two media : PDA and *Trichoderma* specific medium (TSM).

Testing of suitable strains active against root pathogens

The four selected strains of *Trichoderma* were tested against the four soil borne plant pathogens. This test was done to examine the efficacy of each test pathogen and also to see which strain of *Trichoderma* is most effective in checking the growth of which test fungus. For this test dual culture methods on PDA was used by placing two fungi (*Trichoderma* and test pathogen) opposite to each other. Three replications were maintained in each case, control was kept in each case. The plates were incubated at 25° ± 1°C and observations were recorded on inhibition or overlaping growth, the pigmentation by the antagonistic fungal strains was also recorded through change of the colour of the medium on reverse side of petri plates.

Studies on factors affecting growth and sporulation

Effect of medium

The four *Trichoderma* isolates were grown on two media viz. non-synthetic : PDA (Potato dextrose agar) and synthetic medium : TSM (*Trichoderma* specific medium). Then it was observed that on which medium the fungal growth, sporulation and pigmentation were best.

Effect of pH on growth and sporulation of *Trichoderma*

pH is one of the most important factors that affect the fungal growth. pH of liquid medium was adjusted at 5, 6, 7, 8 and 9 by addinng 1 N HCL or 1 N NaOH solution as required. Then for 300 ml medium 6 g agar was added to each flask and the medium was sterilized in autoclave at 121°C for 15 min. The plates were inoculated using 6 mm disc of 5 day old inoculum. The observations were recorded after four days.

Effect of Temperature

The temperature effect was seen at 15°, 20°, 25°, 30° and 35°C on Potato dextrose agar medium.

Selection of most suitable strain of *Trichoderma*

Selection of most suitable strain for mass multiplication was made on the basis of very fast growth, abundant sporulation and reasonably good pigmentation/metabolites production besides the strain which inhibit the growth of all four fungi most effectively.

Counting of CFU

The colony forming unit (CFU) of the most suitable strain of *Trichoderma* was determined by making suspension in sterilized water and using Haemocytometer.

Testing of medium for mass multiplication

For mass multiplication of the suitable strain different media were used. For this purpose linseed, mustard, sesamum cake, niger cake, sorghum, bajara grain, sawdust, wheat straw, farm yard manure and sago were used. The cakes were first crushed to make fine powder, 100 g of these crushed cakes and other media were put in the special thermo tolerant transparent bags. To each bag 20-25 ml water was added to make the cakes just moist. Grain of jowar and bajara were half boiled then 0.1% $CaCO_3$, was added 100 g of these were put in the bags. Farmyard manure was also partially sterilized. Sago was treated with 1% yeast, peptone and sucrose solution for 1 min. Sago with simple distilled water with $CaCO_3$ were also kept in some bags. All the bags were provided with cotton plug. The spore suspension was prepared by suspending desired number of discs to 300 ml of water. The suspension was checked for CFU and bags were inoculated with 5 ml of above spore suspension. After 4-5 days all the inoculated substrate were checked for growth and sporulation of *Trichoderma* without clotting.

RESULTS

Selection and identification of species/strains of *Trichoderma*

Trichoderma spp. / strains were isolated from different sources. It was identified species/strains of *Trichoderma* based on different growth and color viz brown (B), medium brown (M) and hyaline (H). One strain was isolated from guava dead tree trunk hence designated as G. The growth and sporulation of these strains have been presented in Table 1.

On the basis of growth and sporulation B-29, M-7, H-14 and G are the best sporulating and fast growing among all the strains, hence were selected for further investigation. Followings are the identification of *Trichoderma* spp. /strains in the present study.

1. *T. viride* strain -*1*
2. *T. pseudokoningii*
3. *T. viride* strain -*2*
4. *T. viride* strain -*3*

The morphological characters of each are given below.

***Trichoderma viride* Pers.ex Fries,. Syst. Mycol. 3:315:1829 Rifai Mycol. Pap. 116:1-26,1969: M-7 Isolate -1.**

Colonies fast growing, greenish conidiophores sparingly branched at time, at regular interval these are in groups or clusters ; phialides flask shaped, narrowed at the base and tapering above, occasionally forming whorls, 7.75-9.3 x 3.1 µm, phialospores remains in gloeoid mass at the mouth of the phialides in groups of 5-10 conidia, subglobose to ovate, finely ornamented, 3.1-4.03 x 3-3.3 µm.

***Trichoderma pseudokoningii,* Rifai Mycol. Pap. 116,1-26,1969: H-14 Isolate**

Colonies rapidly growing on potato dextrose agar covering an area of 90 mm in 72 h. Conidiophores erect or straggling, solitary or frequently branching aggregated into floccose tufts, hyaline, septate, branching irregularly, weakly or strongly verticillate. Sporogenous cells or phialides borne singly or in clusters, hyaline, ovate to flask-shaped when aggregated into tufts, 15.5-24.8 × 3.1-4.6 µm in size. Phialospores sub-hyaline or green, non-septate, gathering in balls at the mouth of the phialides, 3.1-4.65 × 2.33-3.1 µm in size.

***Trichoderma viride* Press ex Fries., G: Isolate-2**

Colonies fast growing, greenish, conidiophores branched, phialides remain singly or in groups of 2-3, flask-shaped, 10.85- 2.4 × 3.87 -4.18 µm. Phialospores globose to subglobose, 3.1 - 3.87 × 3-3.5 µm.

***Trichoderma viride* Press ex Fries., B-29 : Isolate -3**

Colonies on potato dextrose agar fast growing, spreading, fruiting areas appear in tufts, white at first but becoming green with age. Condiophores detrichotomously branched with phialides singly or in clusters, bottle shaped, 6.2 – 8.5 × 3.1 µm. Phialospores globose to subglobose, finely ornamented, 3.1-3.8 x 3-3.6 µm in size.

Testing of suitable strains active against root pathogens

All the spp./strains have checked the growth of *Sclerotium rolfsii*, *C* and *Fusarium oxysporum* upto the same extent. Sporulation and pigmentation also from very good to excellent. It was found that more or less all the spp./strains have checked the growth of *Rhizoctonia bataticola* whereas, *Trichoderma* G and H-14 isolates have shown more growth suppressing of *R. solani* in comparison to M-7 and B-29.

Effect of media

Four species/strains were grown on two media (1) synthetic (TSM : *Trichoderma* specific medium, (2) non synthetic (PDA : potato dextrose agar medium). All the strains showed full growth (i.e. 90 mm.) in both the media. M-7 showed medium sporulation whereas H-14 very good sporulation. Pigmentation was medium in all the 4 strains on PDA whereas no pigmentation was observed in synthetic medium.

Effect of pH on growth and sporulation

Five pH ranges viz. 5, 6, 7, 8 and 9 were tested for growth, sporulation and pigmentation of four *Trichoderma* species/strains. It was found that growth on all the pH and of all the strains was good except H-14 at pH-7 (60 mm) and M-7 at pH-6 (70 mm). Sporulation was very good in B-29 at pH-5 and in H-14 at pH-5 and 6 whereas no sporulation was observed in M-7 strain except medium at pH-8. Incosnsistent results were observed with regard to pigmentation in G strain and M-7 strain, whereas it was medium in B-29 strain at pH 7 to 9 and at pH 5 and 6 of strain H-14. Pigmentation was absent on other pH of the two strains.

Table 1 : Growth and sporulation of different strains of *Trichoderma.*

Trichoderma strains	Sporulation*	Pigmentation
H05	+++	-
H07	+	+
H08	+++	-
H14	++++	++
H17	+++	++
H19	+++	++
H27	++	++
M01	++	+
M02	+++	+
M05	++	-
M07	+++	+++
M13	+++	++
M16	+++	+
M20	+++	++
B01	++	+++
B04	++	+
B07	+++	++
B08	+++	++
B09	+++	++
B11	++++	++
B14	+++	+
B19	++	+
B23	+++	++
B26	++	+
B29	++++	+++

+ (Medium), ++(Good), +++(Very Good), ++++(Excellent),
*Average of 3 replicates.

Effect of temperature

Five temperature ranges were tested for this purpose. Growth was minimum (20 mm) at 15°C of all the spp./strains while at rest of the temperatures it varied from 78 to 90 mm in all the strains i.e. M-7, H-14, G and B-29. Strain H-14 and G showed very good sporulatoion at 35°C & 30°C. No sporulation was observed in M-7 strain at 20°C. In other strains the effect of temperature on sporulation varied from medium to good. Abundant pigmentation was observed in G isolate at 30°C and in H-14 at 35°C.

Table 2 : Evaluation of suitable medium for mass multiplication of *Trichoderma.*

Substrate	Sporulation*
Sesame cake (sterilized)	+++
Sesame cake (unsterilized)	+++ (contamination)
Linseed cake (sterilized)	++
Linseed cake (unsterilized)	+++ (contamination of *Aspergillus*)
Niger cake (sterilized)	++
Niger cake (unsterilized)	Contaminated
Mustard cake (sterilized)	No growth, clumps formed
Mustard cake (unsterilized)	No growth, clumps formed
Jowar (boiled)+$CaCO_3$	++
Jowar (boiled)+$CaCO_3$	++
Bajra (boiled)+$CaCO_3$	No growth (bacterial contamination) too wet
Bajra (boiled)+$CaCO_3$	No growth (bacterial contamination) too wet
Farmyard manure (sterilized)	No growth (bacterial contamination)
Farmyard manure (unsterilized)	No growth (bacterial contamination)
Wheat straw + YPS	No growth (bacterial contamination)
Wheat straw without YPS	+
Sawdust (sterilized)	No growth (bacterial contamination)
Sawdust (unsterilized)	No growth (bacterial contamination)
Sago + $CaCO_3$ +YPS	++++
Sago + $CaCO_3$ –YPS	No growth (bacterial contamination)
Sago + Distilled water	No growth
Sago (1m water) + 1g soapstone	No growth
Sago (2.5m water) + 2g soapstone	No growth
Sago (5m water) + 3g soapstone	No growth
Sago (1m YPS) + 1g $CaCO_3$	++++
Sago (1m YPS) + 2g $CaCO_3$	++++
Sago (1m YPS) + 1g soapstone	++++
Sago (1m YPS) + 2g soapstone	+++ (contamination)
Sago (2.5m YPS) + 1g $CaCO_3$	++++
Sago (2.5m YPS) + 2g $CaCO_3$	+++
Sago (2.5m YPS) + 1g soapstone	++++
Sago (2.5m YPS) + 2g soapstone	+++
Sago (5m YPS) + 1g $CaCO_3$	Little growth, clumps formed, too wet
Sago (5m YPS) + 2g $CaCO_3$	+(clumps formed)
Sago (5m YPS) + 1g soapstone	Little growth, clumps formed, too wet
Sago (5m YPS) + 2g soapstone	+(clumps formed)

1m—treatment for one minute, 2.5m—treatment for 2.5 minutes, 5m—treatment for 5 minuts
YPS—1% Yeast + peptone + sucrose solution.
+ (medium), ++ (good), +++ (very good), ++++ (excellent)
* Average of 3 replicates.

From the above results it was found that the H-14 strain control all the test pathogens better than other

strains. It was also found that in both the media the growth and sporulation of H-14 was good. The strain also showed good growth and sporulation at all the pH and temperatures tested. Therefore, the strain was selected as the most suitable for mass multiplication.

Testing of medium for mass multiplication

It is clear from the Table 2 that among the cakes, sterilized sesamum cake gave the best sporulation of *Trichoderma*. Unsterilized sesamum cake showed growth and contamination together. Sterilized niger cake and linseed cake also produced good growth. Unsterilized linseed cake produced good sporulation but it became contaminated with *Aspergillus niger*. Unsterilized niger cake produced contamination only. Mustard cake both sterilized and unsterilized produced no growth of *Trichoderma.*

Jowar seeds both treated with $CaCO_3$ and untreated showed good sporulation whereas bajra seeds with or without $CaCO_3$ treatment showed no sporulation of *Trichoderma*. Bajra seeds produced bacterial contamination too. Sterilized and unsterilized farmyard manure and sawdust showed no growth and sporulation of *Trichoderma*.

Sago treated with distilled water and soapstone (sterilized) show no growth. Sago treated with 1% yeast peptone sucrose solution for 1 minute plus 1 g or 2 g $CaCO_3$ or 1 g or 2 g sterilized soapstone produced very good sporulation (nearly 100 % in all the cases except in 1 g $CaCO_3$ where 75% growth was found) of *Trichoderma*. Sago treated with 1% yeast-peptone-sucrose solution for 2.5 minutes plus 1 g or 2 g $CaCO_3$ showed good sporulation. This sago of 2.5 minutes when treated with 2 g sterilized soapstone gave less sporulation than earlier treatment, but this 2.5 minutes treated sago with 1% yeast-peptone-sucrose solution when treated with 1 g sterilized soapstone, produced fast growth, good sporulation (75 % growth) too. Sago treated with 1 % yeast-peptone-sucrose for 5 minutes plus 1 g $CaCO_3$ and 2 g $CaCO_3$ showed no sporulation and little sporulation respectively. This 5 minutes treated sago with 1% yeast peptone sucrose solution when treated with 1 g and 2 g sterilized soapstone, produced little and medium sporulation of *Trichoderma.*

DISCUSSION

As biocontrol agent *Trichoderma* is most effective against soil borne plant pathogens, as these are very difficult to manage by traditional method of control. In the present study, the several spp./strains tested, the four spp./strains (isolate) of *Trichoderma* checked the growth of *Sclerotium rolfsii, Fusarium, oxysporum, Rhizoctonia solani* and *R. bataticola.* The results are in accordance with Muthamilan and Jeyarajan (1996), Dutta and Das (1999) and Zaki and Ghaffar (1998). This cellulolytic fungus also reported to control *Sclerotinia sclerotiorum* (Sharma *et al.*, 1999), *Phytophthora* spp. (May and Kimati, 1999), *Colletotrichum coccodes* (Okhovat *et al.*, 1996) and *Cochliobolus sativus* (Biles and Hill, 1998).

In the present study all the four strain viz. M-7, B-29, H-14 and G controlled the growth of *Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani* and *R. bataticola.* In the two *Rhizoctonia* spp., *Trichoderma* overgrw and covered the colony. The results are in agreement with Silveria *et al.* (1994), and Biswas (1999).

Of the two media tested for growth, sporulation and pigmentation of *Trichoderma* spp. potato dextrose agar (PDA) was the most suitable. The growth and sporulation of *Trichoderma* was also found to be good in *Trichoderma* specific medium. The results confirmed the finding of Biswas (1999) who isolated *T. harzianum* on *Trichoderma* specific medium but the pigmentation by all the four strains of *Trichoderma* was nil in *Trichoderm* specific medium where as in potato dextrose agar medium the strain showed abundant pigmentation. Some unknown substance in the natural medium may be the cause of abundant pigmentation.

In test for pH it was found that all the four strains could grow at pH range between 5-9. Strain M-7 of *Trichoderma* showed best growth and sporulation at pH-8. This strain may be the native of alkaline soil and thus this became adaptive to high pH conditions and grow well in the alkaline pH. The result is in contradiction with those of Jackson *et al.* (1991) who found that *Trichoderma* isolate produced optimum biomass at acidic pH range between 4.6

and 6.3. Strain G also showed good growth, sporulation and pigmentation at pH 6. The results confirmed the findings of Singh *et al.* (1998).

The strains B-29 and H-14 also showed good growth at the pH range between 5 to 7. Das *et al.* (1995) also reported that *T. harzianum* more effectively reduce sheath blight pathogen of rice in acidic soil (pH 6.8) but it also reduce the infection in neutral soil (pH 7) too. This acidic pH requirement of strain may be because of natural good sporulating tendency of most fungi of most fungi at pH range of 5-6 (Lilly and Barnett, 1951). Although different strain showed different temperature requirement for growth and sporulation, but if we take a common temperature than it was found that all the strain showed medium to good growth and sporulation at a temperature around 25°C. The relatively narrow range of temperature permitting reproduction suggests that this phase involve some chemical and physical processes which are not necessary for vegetative growth and which are more exacting in their temperature pigment than are those which suffice for the vegetative phase (Hawker, 1996).

Of the nine substrates used in the present study as mass multiplication medium, it was found that very good growth and sporulation was found in sesame cake, followed by linseed and niger cake. No growth was found in saw dust, farmyard manure, bajra seed and mustard cake.

Poor growth or no growth is due to poor aeration clumps formation. Shamarao *et al.* (1998) found that for mass multiplication/production of *Trichoderma. Pongamia* was the best medium followed by neem cake and groundnut cake. Kaur and Mukhopadhyay (1992) showed wheat bran mixed with saw dust gives good potentiality as medium for *Trichoderma harzianum*. Best growth in sesame cake was found because after mixing this cake with water, clumps were not formed and the cake remained in powdered condition, this favoured aeration a condition needed for good sporulation.
The good growth and sporulation of *Trichoderma* was found on calcium carbonate treated and untreated grains of jowar. The result is in accordance with Laranjeira *et al.* (1996) who used sorghum grains as food base for growth and sporulation of *Trichoderma* and found good results in it. This may be because of presence of needed nutrients for growth and sporulation of *Trichoderma* in jowar seed, but sorghum grain easily get colonized by *Fusarium* and *Curvularia* being seed borne and responsible for head mould under various soil conditions when temperature is high, which may inhibit the growth of *Trichoderma.*

In sago treated with 1% yeast-peptone-sucrose and $CaCO_3$ or sterilized soapstone, best growth and sporulation of *Trichoderma* was found whereas in sago treated with simple water, no growth and sporulation was found. This may be because in simple sago there is no nutrient available for germination of spores hence *Trichoderma* could not grow in sago treated with water, but yeast-peptone-sucrose solution provides nutrient to sago and the sago treated with this solution becomes nutrient rich, *Trichoderma* showed best growth and sporulation in this medium. Sago treated with 1% yeast-peptone-sucrose solution for 1 minute and 2.5 minutes showed better growth as compared to 5 minute treated sago, no clump was formed and good aeration was there and thus good growth and sporulation occurred. But in 5 minute treatment, clumps were formed and less aeration was there and no sporulation was found in this case. Again, in this 5 minute treatment sago became too wet and it may be contaminated with bacteria too. Thus yeast-peptone-sucrose enriched sago is a better base for *Trichoderma* than sorghum. It is not easily colonized by other fungi.

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(Accepted for publication September 2 2002)