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In Vitro Screening and Evaluation of Different Substrates and Carrier Materials for Mass Multiplication of *Trichoderma* against Dry Root Rot in Acidlime incited by *Fusarium solani*(Mart.) Sacc.

A Hari Krishna and M Reddi Kumar

Department of Plant Pathology, S.V.Agricultural College, Tirupati -517502. * Presently Senior Scientist (Plant Pathology), Regional Agricultural Research Station, Tirupati.

ABSTRACT

Among 14 isolates of *Trichoderma* spp. NT_2, NT_4 and NT_6 were characterized as very fast growing, while NT_5, NT_7, NT_9 and CT_{13} are fast growing and remaining isolates as moderate growing. In dual culture technique, the isolates NT_2, NT_6 and NT_4 have shown maximum inhibition of 81.57 per cent, 78.68 per cent and 75.10 per cent against the test pathogen *Fusarium solani* while the least inhibition (56.97 %) was observed in the isolate CT_{14}. Among various substrates tested for mass multiplication, wheat bran- vermiculite supported maximum growth followed by molasses -yeast medium. The shelf life period was high in talc based formulation when it was stored both at room and refrigerator temperature.

Key words: Acidlime, *Trichoderma*, biological control, dry root rot, mass multiplication.

Corresponding author

E-mail: reddi_kumar01@yahoo.com

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INTRODUCTION

Acid lime (*Citrus aurantifolia S*wingle) is one of the important citrus fruits, constitutes nearly 20 per cent of the total citrus production in India. Andhra Pradesh is one of the important citrus cultivated states in the country with a total area of about 1.66 l ha and an annual production of 2.23 l t. Acid lime is affected by several soil -borne pathogens viz., *Fusarium, Rhizoctonia,* and *Diplodia* species causing root-rot diseases both in nursery and main field affecting life and production of acid lime. Among these, *Fusarium solani* causes dry root-rot in acid lime seedlings and also in the developed plants. Control of plant diseases by chemicals can be spectacular but this is relatively a short term measure and moreover, the accumulation of harmful residues some times causes serious ecological problems. Biological methods, on the other hand can be economical, long lasting and free from residual side effects. Some fungi naturally occurring in soils and rhizosphere zone of various crops serve as a potential biocontrol agent against various plant pathogens. Fungi in the genus Trichoderma are of increasing interest as bioprotectant against plant diseases (Papavizas, 1985). Mass multiplication, shelf life and delivery of bioagent are the three important aspects for production and commercialization of biological products. The efficient biocontrol agent should have long shelf life without loosing its efficacy. This study was initiated for development of a cheap suitable substrate for mass multiplication and to assess the shelf life of potential native *Trichoderma* spp. based biopesticide for the management of dry root rot disease in acidlime.

MATERIALS AND METHODS

The laboratory and pot culture experiments pertaining to the present research work were conducted in the Department of Plant Pathology, S.V. Agricultural College, Tirupati. A roving survey was conducted for the occurrence of dry root-rot incidence in acidlime orchards and rhizospheric root and soil samples were collected for isolation of test pathogen and native *Trichoderma* sp. in major acidlime growing areas of two districts viz; Potti Sree Ramulu Nellore and Chittoor districts of Andhra Pradesh. The pathogen was isolated from the infected roots by tissue segment method [10] on *Fusarium* specific medium. Axenic culture of the pathogen was obtained by single hyphal tip method and maintained on PDA throughout the present investigation. The pathogen was identified as *Fusarium solani* based on its mycelial and conidial characters [1]. Sand -Sorghum medium (SSM) was used for developing mass production of inoculum for carrying out the pathogenicity tests. Different isolates of *Fusarium solani* were multiplied on SSM. The SSM was prepared by mixing 100 g sorghum grain flour, 50 g dry sand, 50 ml distilled water in 500 ml conical flask and sterilized at 15 psi for one hr. for three consecutive days. Flasks were subsequently inoculated with 8 mm diam discs of culture of the test fungus and incubated at 28±2 C. Pathogenicity of the *Fusarium* isolates was distinguished on the basis of the relative percentage of plants killed. Different isolates of *Fusarium solani* were multiplied on Sand Sorghum medium and added to soil in pots at 7.5 percent (W/W) by soil infestation following the procedure given by Haware [5]. Soil mixture without inoculum was served as control. Each pot was planted with acid lime seedlings and symptoms development was observed. Seedling mortality percent was recorded at 45 DAS. Based on the percent mortality of seedlings, virulent isolate was identified. Three replications were maintained.

Serial dilution technique was used to isolate antagonistic mycoflora from rhizosphere soil of citrus plants. Three days old colonies of mycoflora were picked up and purified by single spore isolation method. Antagonistic mycoflora were identified based on mycological keys described by Barnett *et al.* [1] and it was further confirmed by sending the cultures to Indian Type Collection Centre (ITCC), Indian Agricultural Research Institute (IARI), New Delhi. The cultures were maintained by periodical transfer on to PDA for further studies. The antagonistic activity of isolated mycoflora against pathogen was determined by dual culture technique under *in vitro* conditions [8]. Among all the antagonistic mycoflora, the one potential antagonistic fungus which was found to be effective against *Fusarium solani* has been selected and used for further studies.

For mass multiplication of *Trichoderma* [11], solid substrates viz; sorghum grain flour (Sorghum grain flour comprising of 50 g was taken in each of the 250 ml conical flasks and 10 ml tap water was added for adjusting moisture to 50 percent (w/v))and vermiculite-wheat bran (*Trichoderma* was multiplied on Molasses-yeast medium for 10 d. Vermiculite comprising of 100 g and wheat bran 33 g were mixed together and sterilized in an oven for three days at 70 C. Then 20 g of fermented biomass and 17.5 ml of 0.05 N HCl were added, mixed well and dried in shade) and liquid substrates viz; molasses –yeast medium (Molasses comprising of 30 g was taken in one lt conical flask and 5 g Brewer's yeast were added to this. Then adjusted to one lt by adding distilled water), potato jaggery broth (200 g of Potato slices were boiled and squeezed the slices after boiling. Jaggery 20 g was mixed with the filtrate obtained and made up the final volume to one lt by adding distilled water) were used. All the substrates were inoculated with 2 to 3 discs of 7 d old culture of potential *Trichoderma* spp. separately into conical flasks and incubated at temperature 28 ± 2C. Observations regarding population levels on different substrates were taken after 7 d of incubation by dilution plate count technique. The best suitable medium for proliferation of the antagonist was identified based on highest number of cfu/g formed by the antagonist on each substrate.

Carrier materials and shelf life

The substances evaluated as carrier material for bio-formulation were Talc powder (*Trichoderma* culture biomass along with medium 1 lt, Talc powder (300 mesh, white colour) 2 kg, Carboxy Methyl Cellulose / Gum Arabic powder 10 g) and Gypsum (*Trichoderma* culture biomass along with medium 1lt, Gypsum powder 2 kg, Carboxy Methyl Cellulose / Gum Arabic powder 10 g). Preparation of powder formulation was carried by using stationary culture method. The biomass from 15 d culture of *Trichoderma* cultivated in flasks was used for preparation of formulation. The biomass along with medium in conical flasks was mixed with a carrier in the ratio of 1:2. The mixture was air dried in shade for 3 to 4 d and blended to have a flowing powder to which 0.5% sticker (CMC/ Gum Arabic) was added. The formulation thus prepared was stored in white polythene bags at room temperature and in refrigerator (4^0 C). The formulation prepared using stationary culture method was periodically estimated for shelf life at 15 d interval upto 90 days. One gram of the formulation was drawn from respective formulation at 0, 15, 30, 45, 60, 75 and 90 d of storage and cfu /g of the formulation was estimated by serial dilution technique and plating on *Trichoderma* specific medium. The population estimation was done at regular intervals

using the formula: cfu/g = cfu/plate X dilution factor X 10 ml of water/ amount plated X weight of inoculum taken

RESULTS and DISCUSSION

In the present study, survey was conducted in three acid lime growing mandals of P. S. R. Nellore *viz.,* Nellore Rural, Podalakur and Rapur and the average percentage of disease incidence was 13.72, 19.94 and 23.36 per cent, respectively. The highest average per cent disease incidence was recorded in Rapur mandal (23.36%) of P. S. R. Nellore district. In Chittoor district, survey was conducted in B. N. Kandriga and G. D. Nellore mandals and the average per cent incidence recorded were 12.76 and 15.91 per cent, respectively. Among these maximum disease incidence was observed in Nellore district. Among the two districts, the highest mean per cent disease incidence was 23.36 per cent recorded in Rapur mandal of P. S. R. Nellore district, might be due to the presence of calcareous soils, high soil pH which predisposes the acid lime trees to dry root-rot infection. Vijay Kumar [13] reported that dry root-rot disease was more in deep red sandy loam soils. This may be attributed to the hard pan of stone or gravel in the sub soil, which are impervious to root system to develop and disturb the root system.

The pathogen was isolated from the root-rot affected acid lime plants by tissue segment method on *Fusarium* selective medium [10]. White, cottony and fluffy growth with smooth margin colonies were observed on 4^{th} d after inoculation [2].A sum of nine *F. solani* isolates were isolated using *Fusarium* selective medium. The isolates were designated as NF_1 to NF_6 for the isolates of *F. solani* isolated from P. S. R. Nellore and CF_7 to CF_9 for Chittoor *F. solani* isolates. Soil inoculation method [5] was followed to establish the association of pathogen with disease. The studies on pathogenicity test in pot culture revealed that per cent disease incidence was more in NF_4 isolate collected from Degapudi of P. S. R. Nellore district followed by CF_3 isolate of Chittoor district. Relationship between virulence of the pathogen and disease development in acid lime seedlings was observed.

In the present investigation, a total of 102 rhizosphere soil samples were collected from healthy plants of diseased acid lime orchards of P. S. R. Nellore and Chittoor districts. Serial dilution technique was used to isolate antagonistic mycoflora from rhizosphere soil. Colonies of *Trichoderma* spp., were obtained on *Trichoderma* selective medium (TSM) on 7^{th} day after inoculation. The antagonistic *Trichoderma* spp. was identified based on mycological keys described by Barnett and Hunter [1]. All the antagonistic cultures were sent to IARI, Pusa, New Delhi for further confirmation.

A sum of 22 *Trichoderma* spp. were isolated and studied for their growth rate on PDA medium at 12 hr interval for three days using PDA medium. Depending on the growth rate, the isolates were categorised into three groups i.e., very fast (75-90 mm diam within 48 hr of incubation), fast (75-90 mm diam within 60 hr of incubation) and medium (75-90 mm diam within 72 hr of incubation) growing colonies. Based on the full growth obtained by the isolates within 72 hr, 14 isolates were selected for further studies. These results were in confirmation with Kavitha [7] as she categorized the *Trichoderma* isolates based on their faster growth within the stipulated time. All 14 isolates of *Trichoderma* spp. were screened for their efficacy against test pathogen. All the fourteen isolates of *Trichoderma* inhibited the growth of *F. solani* in dual culture. However, the isolate NT_2 showed maximum

percentage of inhibition (81.57) followed by NT_6 (78.68) and NT_4 (75.13). However, both were found to be on a par with each other in inhibiting the pathogen. The inhibition percentage of other isolates were NT_9 (72.63), NT_7 (71.84), NT_5 (70.92), CT_{13} (70.17), NT_1 (68.15), CT_{10} (66.23), NT_3 (64.92), NT_8 (64.03), CT_{11} (62.25), CT_{12} (59.67) and CT_{14} (56.97). Least percentage inhibition was observed in the isolate CT_{14} (56.97). The isolate NT_2 was found to be more effective due to its more percentage inhibition and it was used for mass multiplication studies. In a similar study, Selvarajan and Jeyarajan [12] showed the effect of eight fungal antagonists against *F. solani*. Among which, isolates of *T.viride* and *T.harzianum* showed maximum per cent of inhibition against *F. solani*.

The isolates of *Trichoderma* after testing the efficacy in dual culture assay were classified into three groups. The isolates which inhibit the pathogen for more than 75 percent were grouped as highly effective and the isolates that are showing the inhibition percentage between 70-75 percent were pooled in moderately effective group whereas the isolates which inhibited the pathogen less than 70 percent were kept under less effective group. Based on the results obtained, the isolates NT_2 (81.57), NT_6 (78.68) and NT_4 (75.13) were grouped under highly effective category and the isolates NT_9 (72.63), NT_7 (71.84), NT_5 (70.92) and CT_{13} (70.17) were placed in moderately effective group whereas the isolates NT_1 (68.15), CT_{10} (66.23), NT_3 (64.92), NT_8 (64.03), CT_{11} (62.25), CT_{12} (59.67) and CT_{14} (56.97) were pooled in the less effective group (Plate 1).The grouping of the isolates based on the efficacy in dual culture assay were in coincidence with the results obtained by [3].

Table 1: *In vitro* screening of *Trichoderma* isolates against *F. solani* by Dual culture Method

S.No.	Isolates	Radial growth of *Fusarium solani* (mm)*	Percent inhibition over control
1	NT_1	24.2	68.15 (55.67)**
2	NT_2	14.0	**81.57 (64.63)**
3	NT_3	26.66	64.92 (53.76)
4	NT_4	18.90	75.10 (60.28)
5	NT_5	22.10	70.92 (57.86)
6	NT_6	16.20	78.68 (62.73)
7	NT_7	21.40	71.84 (58.14)
8	NT_8	27.33	64.03 (53.21)
9	NT_9	20.80	72.60 (58.54)
10	CT_{10}	25.66	66.20 (54.57)
11	CT_{11}	28.69	62.25 (52.17)
12	CT_{12}	30.42	59.97 (50.76)
13	CT_{13}	22.60	70.17 (56.48)
14	CT_{14}	32.70	56.97 (49.03)
15	Control	76.00	00.00 (00.00)

S.Ed.	3.65
S.Em±	2.58
C.D.(0.05)	7.46
C.V. (%)	8.53

* Mean of three replications

** Figures in parentheses are angular transformed values

NT- Nellore *Trichoderma;* CT – Chittoor *Trichoderma*

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Four solid and liquid substrates viz; wheat bran-vermiculite, sorghum grain flour, molasses-yeast and potato dextrose broth were used for the mass multiplication of the NT_2 isolate which showed an inhibition percentage of 81.57 per cent against test pathogen, *F. solani*. Among all the four substrates, wheat bran-vermiculite substrate was found to be best in respect of colony forming units compared to other substrates (Table 2). The mean population was highest in wheat bran-vermiculite (3.56 x 10^8 cfu/g) followed by molasses-yeast medium (3.03 x 10^8 cfu/g), sorghum grain flour (2.46 x 10^8 cfu/g) and potato dextrose broth (2.06 x 10^8 cfu/g). Highest mean population was recorded in wheat bran-vermiculite (3.56 x 10^8 cfu/g) and the lowest mean population (2.06 x 10^8 cfu/g) was recorded in potato dextrose broth. The mean population in all the above substrates was recorded after 7 d and 14 d after incubation and these readings were mean of three replications (Table 2).

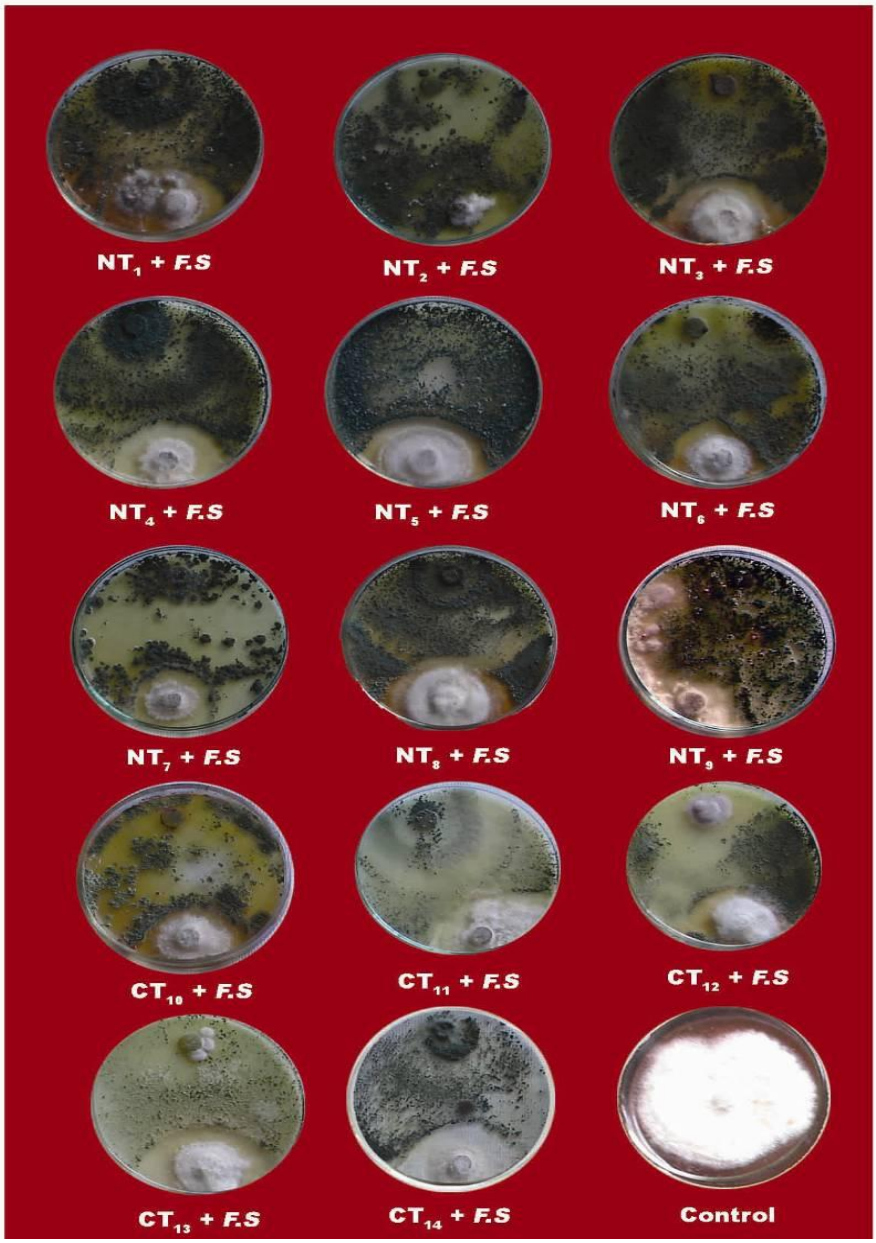

Table 2: Influence of various solid and liquid substrates on mass multiplication of potential *Trichoderma* isolate.

S.No.	Substrate medium	Colony forming units (Cfu/g) x 10^8	
		7 DAI	14 DAI
	Solid substrate		
1.	Sorghum grain flour	1.45	2.46
2.	Vermiculite-wheat bran	1.62	3.56
	Liquid substrate		
1.	Molasses yeast medium	1.57	3.03
2.	Potato Dextrose Broth	1.21	2.06

	Substrate	Interval	Interaction
S.Em±	0.031	0.022	0.045
C.D. (0.05)	0.094	0.067	0.134
C.V. (%)	3.8663		

Two carrier materials i.e., talc and gypsum and the sticker, gum arabic were used to prepare the powder formulation. The biomass along with culture was added to the carrier materials in the ratio of 1:2 along with 5 to 10 g of sticker material. The formulated product was shade dried for a week and packed in a white transparent polythene cover and stored at room and refrigerator temperature. The results from Table 3 revealed that the maximum shelf life was recorded in the carrier Talc both at room and refrigerator temperature. At room temperature, in Talc formulation the population increased steadily upto 60 d (6.0 x 10^8 cfu/g) and it was declined to 2.9 x 10^8 cfu/g at 90 DAI. Highest mean population (6.0 x 10^8 cfu/g) was recorded at 60 d after storage and the lowest mean population (2.9 x 10^8 cfu/g) was recorded at 90 d after storage at room temperature. With regard to Gypsum formulation, maximum mean population was highest (3.6 x 10^8 cfu/g) at 15 DAI and there after it was declined to 2.81 x 10 cfu/g at 30 DAI (2.81 x 10^8 cfu/g) and finally at 90 DAI the population recorded was very low (7 x 10^6 cfu/g). In Talc at room temperature, maximum population was observed at 60 DAI (6.0 x 10^8 cfu/g) and after that the decline in population was found. While at freezing temperature the population was in increasing trend even after 90 DAI [6].

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