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In vitro and Field Efficacy of Native Biocontrol Agents on Stem Bleeding Pathogen of Coconut *Thielaviopsis paradoxa*

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A B S T R A C T

Stem bleeding disease caused by *Thielaviopsis paradoxa* is one of the major diseases of coconut in almost all the coconut growing regions of Andhra Pradesh. Bioefficacy of native bioagents *viz., Trichoderma* spp and Pseudomonas fluroscens on stem bleeding pathogen *Thielaviopsis paradoxa* under *in vitro* conditions revealed that all the three isolated native *Trichoderma* spp were found inhibitory to the mycelia growth of *Thielaviopsis paradoxa* on Potato Dexose Agar media. Maximum percent inhibition of mycelia growth of *Thielaviopsis.paradoxa* was obtained by *Trichoderma viride* (69.35%) followed by *Pseudomonas fluroscence* to 69.32 % as against only 62.90% growth inhibition of *Thielaviopsis paradoxa* by *Trichoderma harzianum.* Twelve substrates tested for mass multiplication of biocontrol agents *viz., T.viride, T.harzianum* and *T.hamatum* under *in vitro* conditions noticed that maximum mycelial growth of *Trichoderma* spp was found on 30% neem cake and 7% neem cake. Evaluation of native biogents under field conditions revealed that among the eleven treatments imposed, maximum decrease in bleeding patch (13.11 cm) was obtained with T_8 *i.e.,* basal application of *T. harzianum* (50 g) + neemcake (5kg)/year) + smearing of talc formulation of *T.harzianum* paste on stem bleeding patches) closely followed by T_7 treatment (11.55 cm) *i.e.,* basal application of *T. viride* (50 g) + neemcake(5kg)/year) + smearing of talc formulation of *T. viride* paste on stem bleeding patches) and T_9 treatment *i.e.,* basal application of *T.hamatum* (50 g) + neemcake (5kg)/year) + smearing of talc formulation of *T.hamatum* paste on stem bleeding patches) with a reduction in perimeter by 11.55cm and 8.51 cm respectively. On the other hand, un treated control palms showed an increase of 2.68 cm of perimeter of the bleeding patch. Thus, soil application of *Trichoderma* spp. along with neem cake and smearing of *Trichoderma* spp paste to the stem bleeding patches was effective in controlling the stem bleeding disease under field condition.

Introduction

Stem bleeding of coconut is a debilitating disease and is prevalent in all coconut growing regions in the tropics. The disease was first reported from Srilanka (Petch, 1906) and later reported in India (Sundararaman, 1922) and other countries. The disease is caused by a fungal pathogen, *Thielaviopsis paradoxa* (de Seynes) von Hohnel. The disease has been found to occur in all soil types, but more in laterite soils and sandy soils on the seashore or backwater areas (Nambiar, 1994). Stem bleeding disease on coconut recorded up to 15% in Andhra Pradesh (Srinivasulu *et al.,* 2005). The pathogen is a soil borne pathogen and enters the plant through growth cracks present on the stem and causes cortical decay. The disease is characterized by development of dark brown patches appearing at the basal portion of the trunk. A dark reddish brown liquid exudes from the longitudinal growth cracks present on the stem bark and form irregular streaks of exudation. These streaks may coalesce and form larger lesions. No oozing is seen from old lesions. The exudates eventually dry up to form black encrustations with brownish orange margins. The tissues beneath the discolored patch show decay. As the decay progresses, the tissues become black and fibrous. As a result of this, cavities are formed from which liquid comes out, when the bark is pressed. Severe infection may lead to reduced yield and death of young palms. Symptoms also occur on crown region. The outer whorl of leaves becomes yellow rather prematurely, droop and finally dry up. The trunk gradually tapers towards the apex and the crown size is reduced. The bleeding patches spread spirally about half way up the stem and sometime reach the crown and cause the death of palms. In severe cases the bleeding patches reach the crown and kill the palm.

Soil drenching with calixin 0.1% (Radhakrishnan, 1990) and root feeding with Bavistin 5% or Calixin 5% (Ramanujam *et al,* 1993), have been found to reduce the disease to some extent. Sudarshan *et al*.(2019) reported that Difenoconazole 25% EC @ 0.1%,Propiconazole 25% EC@0.1%, Tebuconazole 25.9% EC @ 0.15% and Thiophanate Methyl 70%WP @ 0.28% has recorded cent per cent inhibition. Though chemical treatments inhibit the pathogen, but biological control is an eco-friendly, long-lasting, highly effective method for the elimination of soil borne pathogens and a good alternative for the chemical and physical treatments. The literature revealed that the pathogen can be successfully controlled by *Trichoderma viride* under *in vitro* conditions (Jayaratne *et al.,* 2015; Ranjana Chakrabarty *et al.,* 2013; Tapwal1 *et al.,* 2011).

Several authors reported about the soil application of neem cake will reduce the intensity of pathogen (Kartikeyan *et al.,* 2005; Hoitink *et al*., 2006; Darmono and Purwantara, 2006). But there is very sparse literature on field efficacy of native bioagents in control of stem bleeding disease in coconut. The present study was carried out to investigate the role of bioagents in inhibiting the growth of the fungus in *in vitro* and field condition in controlling the disease.

Materials and Methods

Isolation of coconut pathogens

The disease symptom of stem bleeding caused by *Thielaviopsis paradoxa* is depicted in Plate 1. The stem portion of the infected palm where bleeding symptoms were conspicuous was chiseled out and surface sterilized with 0.1% sodium hypochlorite followed by 3 washes in sterilized distilled water (SDW) and then the stem bits were plated on Potato Dextrose Agar (PDA) media plates for

Thielaviopsis paradoxa. The plates were then incubated for three days at 29 + 1 0c and the test pathogen was isolated by purification .

Isolation and identification of antagonistic fungi from rhizospheric region of coconut

Soil samples were collected from rhizospheric region of coconut in Iragavaram and Undaraivaram mandals of West Godavari district, Andhra Pradesh. Serial dilution and plate count method was used for isolation of antagonistic fungi. The collected soil samples were subjected to serial dilutions using sterile distilled water and 0.5 ml of each sample at 10^{-3} and 10^{-4} dilutions were spread on petri-dishes containing *Trichoderma* specific medium (TSM) (Elad and Chet, 1983). Two plates were maintained for each dilution. The plates were then incubated at 28^{o}C and were examined after four days. Hyphal tip method was adopted for pure culture of organisms. The isolated antagonistic fungi were identified up to the level of genus or species based of growth, color, philides characters on PDA medium.

Isolation and identification of antagonistic bacteria

Samples were serially diluted and 0.1 ml of sample was spread on plates containing King's B medium. The isolate was purified by streaking and was maintained further. Identification of bacterial bioagent was made as per the description and physiological status suggested by Hilderband *et al.*, (1992) and identified as *Pseudomonas fluroscence.*

In vitro antagonism on fungal pathogens of coconut

Dual cultures of the fungal antagonists and the test pathogen were prepared by inoculating PDA discs from the growing margins of fresh fungal cultures on to petri dishes containing PDA (Gams *et al.*, 1980) and incubating them .The dual cultures were observed for antibiosis and agar blocks from the regions where the colonies merged were observed for typical interactions under the light microscope.

In case of bacterial antagonists, 8 mm mycelia discs of the pathogens were placed individually at the center of the plates and bacterial strain was streaked at three positions 2 cm away from edge of the petri plates with PDA medium and incubated. The mycelia growths of the test pathogens were measured at 48 hrs and subsequently one week after incubation (Nandakumar *et al.*, 2000).

Mycoparasitism of test pathogen isolates by fungal antagonists was studied using the dual culture technique developed by Dennis and Webster (1971) described by Sanchez *et al.*, 2007. The antagonists were grown on PDA for a period from 0 to 25 days and their effect on growth of test pathogens were tested by exposing inverted plates of freshly inoculated pathogens to plates containing antagonists cultures and sealing together by cello tape. The pathogen growth was measure after 4 days of incubation in both the cases at 29 ± 1^{o}c and percent inhibition was calculated by using the formula as given by Vincent (1947).

$$\% \text{ inhibition} = \frac{\text{Mean growth in control} - \text{Mean growth in treatment}}{\text{Mean growth in control}} \times 100$$

Mass multiplication of *Trichoderma* spp

The antagonistic fungi *viz.*, *T.viride, T.harzianum* and *T.hamatum,* were tested for mass multiplication on coconut leaf bits, coconut coir, coconut dry leaf powder, oil palm stem bits, oil palm leaf bits, farm yard manure, redgram, bajra, bengal gram, wheat

grain, 30% neem cake and 7% neem cake. One hundred gram of each substrate was weighed in conical flasks (500 ml) containing 2% sucrose solution and kept undisturbed for 24 hours. Later, the excess water was drained out and the flasks were autoclaved and subsequently seeded with mycelial bits of *Trichoderma* spp. The flasks were then incubated at 28 ± 1°c for 7 days and the observations were recorded.

Assessment of *Trichoderma* spp population in neemcake

One gram of sample from developed talc formulation in neemcake was derived at regular intervals and the population (CFU) of *Trichoderma* spp. in neemcake were conducted at 10, 20, 30, 40, 50, and 60 days intervals after preparation by serial dilution plate technique in selective media for bioagents.

Talc formulation of *Trichoderma* spp

Talc formulation of native *Trichoderma* spp was prepared. Potato dextrose broth was prepared and sterilized by autoclaving at 15 PSI (121.6 °c) for 15 minutes. Eight mm diameter mycelial discs of antagonist was inoculated and incubated at 28 ± 1°c for 7 days. The homogenate (1 x 10^8 spores/ml) was mixed with talc powder at 1 : 2 ratio along with 0.5% carboxy methyl cellulose and dried in shade, following the method described by Jayarajan *et al.*, (1994) with slight modification. The product was used for soil application studies.

Field evaluation of native *Trichoderma spp.*

A field experiment was conducted at Iragavaram village, West Godavari district of Andhra Pradesh during 2014 to 2016 for two years, by imposing ten treatments along with untreated control in coconut cultivar East Coast Tall. The palms were well managed with regular package of practices given by DrYSRHU, Venkaramannagudem. Ten treatments were imposed along with untreated control in Randomized Block Design with three replications and three palms per replication.

Treatment details were given in Table 1.

The talc powder formulations of the bioagents contained a spore load of 625 x 10^3 cfu g^{-1} powder was used for study. Treatments containing basal application of either neem cake alone or in combination with *Trichoderma* spp were imposed by making basins at a diameter of 2m from the stem at a depth of 15 cm and were immediately covered with soil and irrigated. Talc formulations were also smeared on the bleeding patches. Bleeding patches of nearly equal size were selected for the palms and perimeter of the bleeding patches was taken into account for judging the degree of disease incidence and one conspicuous bleeding patch was selected for each palm imposing treatments on the stem keeping in view the chances of appearance of more than one bleeding patch on the same stem. Initial perimeter on the bleeding patches was recorded prior to imposing the treatments and subsequent observations were made at monthly intervals. The efficacy of the treatments was determined by comparing the reduction in the perimeter of the bleeding patch after recording the final observations after 12 months.

Results and Discussion

Isolation of coconut pathogens

Isolation carried out from the diseased tissues yielded a pathogenic isolate of *Thielaviopsis paradoxa* (Plate 1). The hyphae were pale brown with cylindrical to oval endoconodia. Chalamydospore production is terminal, in

chain and they are thick walled, obovate to oval, *p. palmivira* mycelium is typical non-septate and hyaline, intercellular in the tissue drawing its nutrients through haustoria. It develops rapidly to cover the host tissue with a cottony growth, especially during highly humid condition (Plate 2). The sporangiophores are simple or branched and the sporangia are pear-shaped, with prominent papillae. They are formed singly at the tips of conidiophores, and are hyaline and thin walled, measuring 38-72 x 33-42µ. They germinate by releasing motile zoospores through the papillary opening.

In vitro antagonism of *Trichoderma spp* and *Pseudonomas fluroscence* on coconut stem bleeding pathogens

The results on *in vitro* antagonism of biocontrol agents on coconut stem bleeding disease pathogen *Thielaviopsis paradoxa* (Fig 1) revealed that the percent inhibition of *Thielaviopsis paradoxa* ranged from 62.90 to 69.35 % . It was observed that significantly maximum growth inhibition of *Thielaviopsis paradoxa* were observed with *Trichoderma viride* to a percent inhibition of 69.35 followed by *Pseudomonas fluroscence* to 69.32 % (Plate 3a & Plate 3b).The least growth inhibition of *Thielaviopsis paradoxa* to 62.90 % was observed with *Trichoderma harzianum.* The results are in corroboration with earlier workers who reported the potential of biocontrol agent against coconut pathogens (Jayaratne *et al.,* 2015; Tapwal1 *et al.,* 2011). Sudarshan *et al.,* 2019 reported that *Trichoderma viridae* was found to be most effective on *Thielaviopsis paradoxa* with 61.62% inhibition followed by *T. harzianum* and *T. virens* with 60.80 and 59.49 per cent inhibition respectively. *Trichoderma viridae* produces several groups of antibiotics, toxins and then the growth of the pathogen is inhibited (Eziashi *et al.,* 2010). Also it can inhibit or reduce the growth of the pathogen through competition for space, nutrients or oxygen. Priya *et al.,* 2012 reported *Pseudomonas fluroscene,* a potential inhibitory biocontrol agent against *Gnanoderma* under *in vitro* conditions. The inhibition of mycelial growth of the pathogen by *Pseudomonas fluroscence* may be due to the production of antibiotics. Production of antibiotics HCN, pyrrolnitrin, phenazine and 2, 4-diacetyl phloroglucinol and lytic enzymes by *Pseudomonas fluroscence* against fungal pathogens were reported by many workers (Ramamoorthy *et al* 2002; Saravanakumar *et al*., 2008).

Substrate for mass multiplication of *Trichoderma* spp

Twelve substrates were tested for mass multiplication of biocontrol agents *viz., Trichoderma viride, T.harzianum* and *T.hamatum* (Table-2). Among the substrates tested, maximum mycelial growth of *Trichoderma* spp. was found on 30% neem cake, 7% neem cake, oil palm stem bits, oil palm leaf bits and wheat grains followed by FYM and coconut fresh leaf bits. However, slight mycelial growth of *Trichoderma* spp was recorded on redgram and bajra, whereas no mycelial growth was noted on coconut coir and coconut dry leaf powder. The results were in tune with the findings of Bhasakaran, 1990, Mohiddin *et al.,* 2017,). Rini and Sulochana (2007) reported that pre-boiled sorghum grains, coir pith + neem cake (1:1), cow dung + neem cake (1:1) + wheat flour (10%) maintained high populations of *T. harzianum* and *T. viride* within 10 days of inoculation. Ajay Tomer *et al.,* (2016) noticed very high level of population dynamics and quite longer shelf life of *T. harzianum i.e.* for 150 days in a substrate of mixture of de-oiled cakes of neem, jatropha, mahua and karanjaes with sorghum grains and wheat bran. Several workers also reported that neem cake encouraged the saprophytic soil microflora especially *Trichoderma* in coconut basins.

Assessment of *Trichoderma* spp population in neem cake

To test the population built-up of *Trichoderma* spp. under *in vitro* conditions, the effective bioagents were mixed with sterilized and unsterilized neemcake separately and heaped for different days. The population of bioagents was assessed at 10, 20, 30, 40, 50 and 60 days interval and the results showed that 20 days incubation period buildup good population of *Trichoderma* spp. in both sterilized and unsterilized neem cake. Maximum number of colonies of *Trichoderma* spp was observed more in sterilized neem cake than unsterilized neem cake (Table 2). The results were on par with the findings of Bhaskaran (1990),who reported that *T.harzianum* and *T.hamatum* were found to be antagonistic to *G.lucidum* and application of neem cake (5 or 10 kg / palm / year) encouraged the saprophytic soil micro flora especially *Trichoderma* in coconut basins and was effective in the control of *Ganoderma* wilt.

Table.1 Substrate for mass multiplication of *Trichoderma* spp

Substrate	Mycelial growth (after 7 days)		
	T.viride	***T.harzianum***	***T.hamatum***
30% neem cake	+++	+++	+++
7% neem cake	+++	+++	+++
Farmyard manure	++	++	++
Coconut coir	--	--	--
Coconut dry leaf powder	--	--	--
Coconut fresh leaf bits	++	++	++
Oil palm stem bits	+++	+++	+++
Oil palm leaf bits	+++	+++	+++
Wheat grains	+++	+++	+++
Bengal gram	++	++	++
Red gram	+	+	+
Bajra	+	+	+

-- No growth; + Slight growth; ++ Moderate growth; +++ Maximum growth

Table.2 Population load of biocontrol agents in neem cake under sterilized and unsterilized conditions

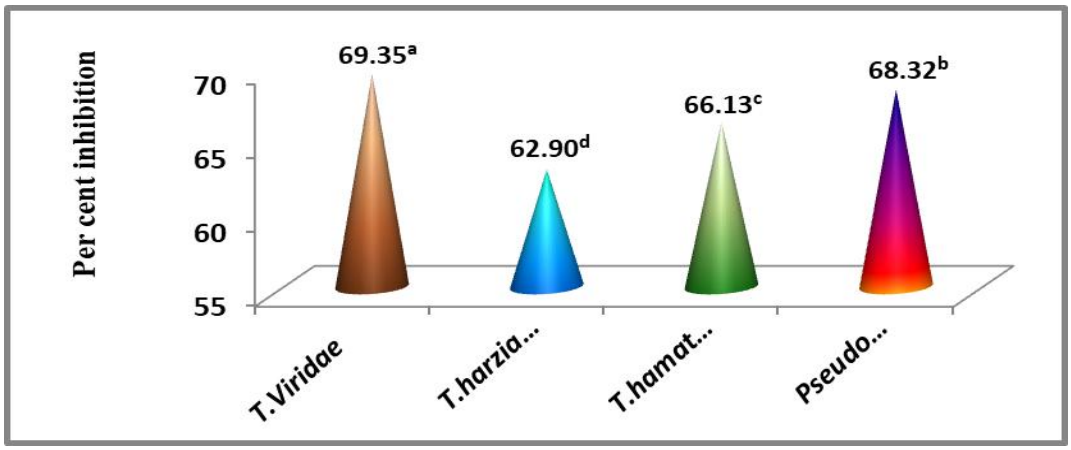
Isolate	Neem cake unsterilized (Cfu x 10^{-4})						Neem cake sterilized (Cfu x 10^{-4})					
	Day's intervals											
	10	**20**	**30**	**40**	**50**	**60**	**10**	**20**	**30**	**40**	**50**	**50**
T.viride	4	31	22	9	5	2	20	48	43	29	17	11
T.harzianum	5	28	24	8	6	0	19	44	37	26	20	10
T.hamatun	7	32	22	10	5	2	23	51	42	31	26	14

Table.3 Field efficacy of *Trichoderma* spp on stem bleeding disease in coconut

Treatments		**Perimeter of the exudation patch(cm)**		
		Initial	**Final**	**Decrease/Increase**
T_1	Basal application of *T. viride* (50 g) + neem cake (5kg/palm)	8.11	2.22	-5.88
T_2	Basal application of *T. harzianum* (50 g) + neem cake(5kg/palm)	8.70	3.88	-4.88
T_3	Basal application of *T. hamatum* (50 g) + neem cake(5kg/palm)	6.22	2.77	-3.44
T_4	Smearing of talc formulation of *T.viride* paste on stem bleeding patches	8.22	0.77	-7.44
T_5	Smearing of talc formulation of *T.harzianum* paste on stem bleeding patches	7.77	0.22	-7.55
T_6	Smearing of talc formulation of *T.hamatum* paste on stem bleeding patches	8.22	4.77	-3.44
T_7	Basal application of *T. viride* (50 g) + neemcake (5kg/palm) + Smearing of talc formulation of *T.viride* paste on stem bleeding patches	14.77	3.22	-11.55
T_8	Basal application of *T. harzianum* (50 g) + neem cake(5kg/palm) + Smearing of talc formulation of *T.harzianum* paste on stem bleeding patches	15.77	2.66	-13.11
T_9	Basal application of *T. hamatum* (50 g) + neem cake(5kg/palm)+ Smearing of talc formulation of *T.hamatum* paste on stem bleeding patches	13.84	5.33	-8.51
T_{10}	Neem cake 5 kg / palm/year	12.26	4.0	-2.26
T_{11}	Untreated control	5.68	8.36	+2.68
			CD (p=0.05)	**1.32**

(- = Decrease or reduction) ,(+ = Increase)

Fig.1 *In vitro* antagonism of native fungal and bacterial agents on coconut stem bleeding pathogen



* Numbers in each column followed by the different letter are significantly different. Values represent the means of 6 replicates.

Plate.1 Stem bleeding patch on the trunk of coconut palm

Plate.2 Stem bleeding disease pathogen

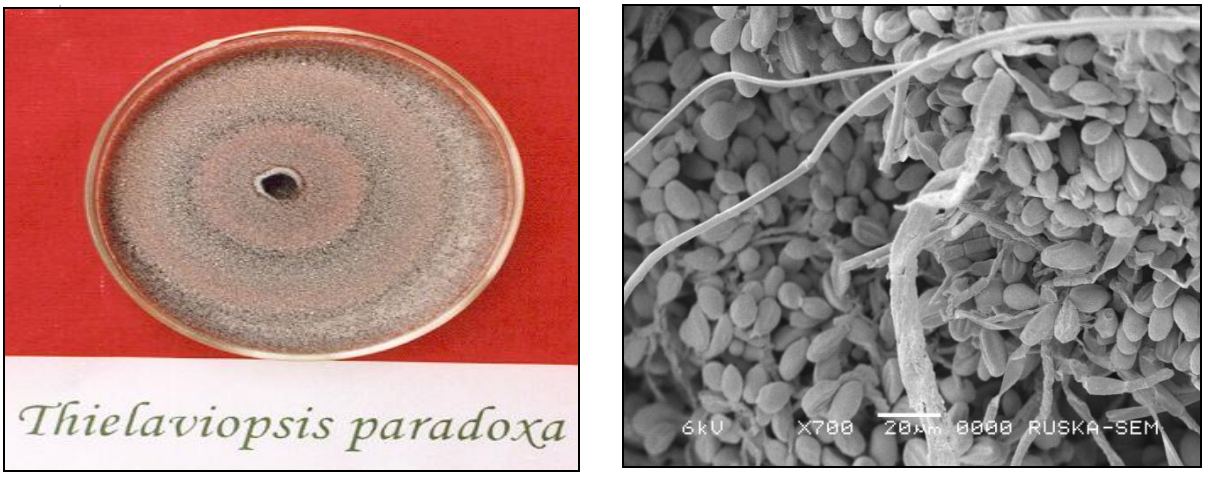


Plate.3a *In-vitro* efficacy of *Trichoderma* spp on *T. paradoxa*

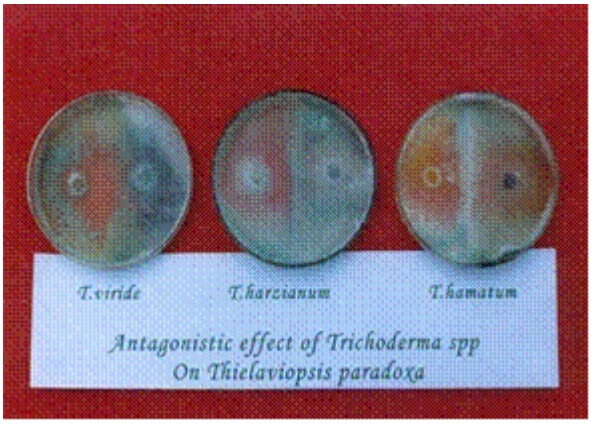


Plate.3b *In-vitro* efficacy of *P.fluorescens* on *T. paradoxa*

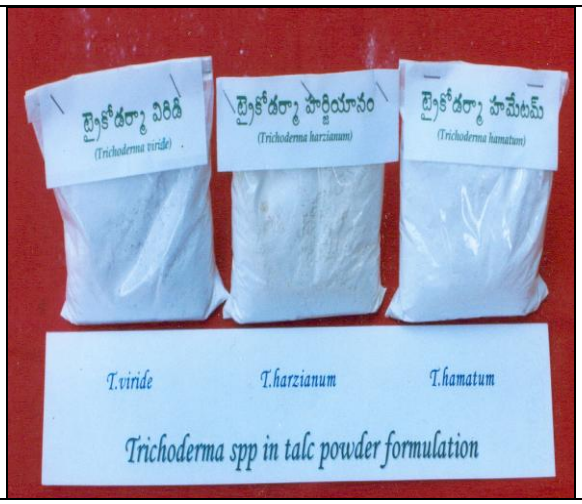


Plate.4 Talc formulation of *Trichoderma* spp.

Plate.5 Smearing of talc formulation of *T.viride* paste on stem bleeding patches

Plate.5 Basal application of *T. viride* (50 g) + Neemcake (5kg)/year

Field efficacy of native bioagents on coconut stem bleeding pathogen *Thielaviopsis paradoxa*

The results of the field experiment indicated that all three *Trichoderma spp* were effective against stem bleeding disease. It was observed from the table 3 and plate 4 & 5, that there was decrease of perimeter of the exudation patch(cm) in all the treatments except in T_{11}-untreated control where the perimeter of the exudation patch increased by 2.68 cm. The reduction of exudation patch(cm) ranged from minimum of 2.26cm with T_{10} treatment *i.e.,* application of neem cake @ 5kg/pal/year) to maximum of 13.11cm with T_8 treatment *i.e.,* basal application of *T. harzianum* (50 g) + neem cake (5kg)/year + smearing of talc formulation of *T.harzianum* paste on stem bleeding patches).Among the different treatments imposed, maximum decrease in bleeding patch (13.11 cm) was obtained with T_8 *i.e.,* basal application of *T. harzianum* (50 g) + neemcake (5kg)/year) + smearing of talc formulation of *T. harzianum* paste on stem bleeding patches) closely followed by T_7 treatment (11.55 cm) *i.e.,* basal application of *T. viride* (50 g) + neemcake (5kg)/year) + smearing of talc formulation of *T. viride* paste on stem bleeding patches) and T_9 treatment *i.e.,* basal application of *T.hamatum* (50 g) + neemcake (5kg)/year) + smearing of talc formulation of *T.hamatum* paste on stem bleeding patches) with a reduction in perimeter by 11.55cm and 8.51 cm respectively. The results were in tune with the finding of Srinivasulu and Raghava Rao (2009), who reported that the application of *Trichoderma* spp. caused lysis of mycelium of Ganoderma lucidum. Furthermore, they have found that the application of T. harzianum/T. viride/T. hamatum pasted over bleeding patches and soil application of the bioagents @ 50 g in 5 kg neem cake has reduced the perimeter of the Ganoderma wilt patches on coconut trees.

Neeraja *et al.,* (2018) soil application of talc based formulation of 125 g each of *Trichoderma reesei* and *Pseudomonas fluorescens* + 5 kg of neemcake/palm at yearly interval was effective in managing the Basal stem rot disease (Ganoderma wilt). Soil application of 125 g of each *Trichoderma reesei* and *Pseudomonas* sp along with neem cake 5 kg per palm per year reduced the disease incidence and increased the nut yield of coconut (Manjunath *et al.,* 2019).

In the present study, the decrease of bleeding patches was observed and the reason attributed may be when the biocontrol agents applied to the bleeding patches, they established on the rotted region at the expense of the pathogen and sporulate there by caused amelioration of the tissue from further rotting by the stem bleeding pathogen *Thielaviopsis paradoxa. Trichoderma* spp had shown inhibitory effect on stem bleeding disease pathogen *Thielaviopsis paradoxa* by production of volatile and non-volatile metabolites that are antagonistic to the pathogen besides mycoparasitism. Soil application of *Trichoderma* spp along with neem cake favours the population built up of *Trichoderma* spp. in the soil, thereby causing reduction in the *Thielaviopsis paradoxa* population and subsequently the disease spread. This is evident from the fact that all the treated palms have contained only one spot and no further appearance of the bleeding patches except in control palms where the number of bleeding patches increased from 1 to 4 minute specks. The reason attributed may be the treatments containing *Trichoderma* spp either by smearing of *Trichoderma* spp. paste on stem bleeding patches along with soil application with neem cake was effective in controlling the further spread of the disease. Neem cake is suitable substrate for multiplication of *Trichoderma* spp (Srinivasulu *et al.,* 2004a) and is also inhibitory to the growth of *T.paradoxa*

(Ramanujam *et al.,* 2002). However, neem cake when applied alone was effective in checking the inoculums load of *Thielaviopsis paradoxa* in an indirect way probably through increasing the population load of the existing antagonistic mycoflora thereby preventing the further spread of the diseased perimeter of the bleeding patch. On the other hand, control palms recorded an increase in the disease severity as indicated by an increase in perimeter of the bleeding patch. The results of the fields experiment offer a scope for an easy and effective management of the stem bleeding disease at field level by the coconut farmers.

It is concluded that in nutshell, native biocontrol agents *viz., Trichoderma viridae, Trichoderma.hamatum* and *Trichoderma harzianum* screened for antagonism under *in vitro* are effective against mycelia growth of stem bleeding pathogen *Thielaviopsis paradoxa.* Under field conditions, basal application of *T.harzianum* (50 g) or *Trichoderma viridae* (50g) with neem cake (5kg/palm) in combination with smearing of talc formulation of *Trichoderma harzianum* or *Trichoderma viridae* paste on stem bleeding patches were effective in controlling the stem bleeding of coconut.

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