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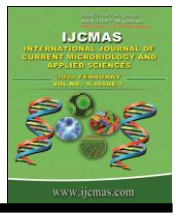

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Studies on *in vitro* Antagonism of Native Biocontrol Agents on Coconut Stem Bleeding and Bud Rot Disease Pathogens

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A B S T R A C T

In vitro antagonism of native biocontrol agents on coconut pathogens revealed that all the three isolated native *Trichoderma* spp were found inhibitory to the mycelia growth of *Thielaviopsis paradoxa* and *Phytophthora palmivora.* Maximum percent inhibition of mycelia growth of *Thielaviopsis paradoxa* and *Phytophthora palmivora* was obtained by *Trichoderma viride* followed by *Trichoderma hamatum* and *Trichoderma harzianum. Pseudomonas fluorescens* fared well against the coconut pathogens with the percent inhibition of mycelial growth of pathogen to an extent of 68.32 to 69.38%. Volatile metabolites of *Trichoderma* spp suppressed the mycelia growth of *Thielaviopsis paradoxa* and *Phytophthora palmivora.* In case of non volatile metabolites, significant inhibition of mycelia growth of test pathogens was noticed with *Trichoderma* spp at 100% concentration. Studies on the production of volatile and non volatile metabolites of *Pseudomonas fluorescens* against coconut pathogens revealed that the test pathogens *Thielaviopsis paradoxa* and *Phytophthora palmivora* were inhibited significantly. These *in vitro* studies offers scope for effective management of disease at field level.

Introduction

Diseases play an important role in palm loss and reduced yields of coconut in India. Even though coconut palm is hardy in nature and adaptable to varied climatic conditions, it is affected by many diseases (Nambiar, 1994, Henry Louis, 2002). Root (wilt)

(*Phytoplasma*), basal stem rot (BSR) (*Ganoderma spp*.), bud rot (*Phytophthora palmivora*), stem bleeding (*Thielaviopsis paradoxa*), leaf blight (*Lasiodiplodia theobromae*) and grey leaf spot (*Pestalotiopsis palmarum*) are the major diseases of coconut in India. Though a few management practices were available for combating the disease, none of them were effective in eliminating the problem completely. Several group of fungicides have yielded a satisfactory results, but their toxic nature and residual level in the coconut water has not been taken into account largely. Further, indiscriminate use of chemical pesticides or fungicides to cure or prevent plant diseases has caused soil pollution and detrimental effects in humans. Additionally, it eliminates the beneficial soil and biocontrol microorganisms. Biocontrol agents are another alternative for managing the destructive diseases of perennial crops such as basal stem rot disease of coconut (Srinivasulu *et al.,* 2005). Some species of *Trichoderma* spp. such as *T. asperellum*, *T. atroviride*, *T. virens*, and *T. harzianum* are widely used as biological control agents of many of plant pathogens in coconut and oil palm (Druzhinina *et al.,* 2011). Soil application of *Trichoderma viride* and *Pseudomonas fluorescens* talc formulations at the rate of 200g each palm in combination with 50kg farm yard manure was found effective against basal stem rot in coconut (Kartikeyan *et al.,* 2005). Darmono and Purwantara (2006) reported that an isolate of *T. harzianum* having biofungicidal activity against basal *Ganoderma spp*. an important pathogen in oil palms. Manjunath *et al.,* (2019) reported that isolates of *Trichoderma* spp mainly *Trichoderma reesei* and *Pseudomonas spp* especially *Pseudomonas fluroscence* were effective antagonists against basal stem rot disease of coconut. Biological control of plant diseases though gaining importance in the present day agriculture, its adoption in horticultural crops in general and plantation crops in particular is yet to be exploited for many other diseases in coconut. Hence, the present study was taken to exploit native biocontrol agents against stem bleeding and bud rot disease pathogens of coconut and also to study *in vitro* efficacy of native biocontrol agents against these pathogens of coconut.

Materials and Methods

Isolation and identification of antagonistic fungi from rhizospheric region of coconut

Soil samples were collected from rhizospheric region of coconut in Iragavaram and Undaraivarammandals 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 and identified as *T. viride*, *T. harzianum, T. hamatum* (Plate-3).

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.*

Isolation of coconut pathogens

The disease symptom of stem bleeding caused by *Thielaviopsis paradoxa* and bud rot caused by *Phytoptera palmivira* are 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*. Similarly, the bud rot pathogen, *Phytophthora palmivora* from infected bud tissues were isolated on PDA and were maintained. The plates were the incubated for 3 days at 29 + 1 0c and the test pathogen was isolated by purification (Plate 3& 4).

In vitro antagonism on fungal pathogens of coconut

Dual cultures of the fungal antagonists and the test pathogens 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).

The fungal antagonists that have shown inhibition in dual culture studies were grown on Potato dextrose broth to test the effect of the culture filtrates (nonvolatile metabolites) on the test pathogens by food poisoning technique (Khara and Hadwan, 1990). The culture filtrates were purified either by autoclaving at 15 PSI for 15 min. The sterilized filtrate was incorporated in the medium for observing fungal growth and inhibition at different concentration (10%, 20%, 50% and 100%). The PDA mixed filtrate were poured (20 ml each) into sterilized petri-dishes and the plates were inoculated with fresh disc of the test pathogens individually.

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 technique allows the researchers to understand the overall effect of biological control agents. 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}cand percent inhibition was calculated by using the formula as given by Vincent (1947).

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

Results and Discussion

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

The results on *in vitro* antagonism of biocontrol agents on coconut stem bleeding disease pathogen *Thielaviopsis paradoxa* and

bud rot disease pathogen *Phytophthora palmivora* revealed that the percent inhibition of *Thielaviopsis paradoxa* ranged from 62.90 to 69.35 % and *Phytophthora palmivora* ranged from 69.38 to 82.03 % by various biocontrol agents (Table 1). 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 3) whereas *Trichoderma viridae* was effective in arresting the growth of *Phytophthora palmivora* to 82.03 % closely followed by *Trichoderma hamatum* to 75.39 % (Plate 4). The least growth inhibition of *Thielaviopsis paradoxa* & *Phytophthora palmivora* to 62.90 % and 71.87 % respectively was observed with *Trichoderma harzianum.* The results are in corroboration with earlier workers who reported the potential of biocontrolagent against coconut pathogens (Srinivasulu *et al.,* 2001; Karthikeyan *et al.,* 2005). Palanna *et al.,* (2013) reported that among the 17 biocontrol agents screened, native *Trichoderma* spp. (V2) recorded 81% reduction over control in dual culture studies against *G. applanatum. Trichoderma spp,* constitute an important microbial population residing in soil and has been exploited tremendously for management of many soil borne diseases ((Jayaratne *et al.,* 2015; Tapwal *et al.,* 2011). *Trichoderma viridae* produces several groups of antibiotics, toxins and then the growth of the pathogen is inhibited (Eziashi *et al.,* 2010). Apart from that the direct attack is called mycoparasitism which kills the pathogen by mechanical and chemical means. Also *Trichoderma* species can inhibit or reduce the growth of the pathogen through competition for space, nutrients or oxygen. *Trichorderma* is fast growing and has the ability to colonize on a wide variety of substrates that makes the organism efficient soil colonizers and bio-control agents. (Sanchez *et al.,* 2007).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). George *et al.,* (2012) screened 156 fluorescent pseudomonads against *G. applanatum* and found that 8% of the total fluorescent pseudomonads showed antagonism towards *G. applanatum* with inhibition ranging from 39 to 73%.

Effect of volatile and non-volatile metabolites of *Trichoderma* spp on coconut pathogens

It is noticed from the table 2 that the mycelial growth of *Thielaviopsis paradoxa* was suppressed when exposed to 25-day-old cultures of *Trichoderma hamatum* and *Trichoderma harzianum* with percent inhibition of 70.96 and 75.86 respectively, while *Trichoderma viride* did not inhibit the mycelial growth of *Thielaviopsis paradoxa.* None of the three *Trichoderma* spp were effective against *Thielaviopsis paradoxa* at zero and 15 days (age) of their exposure. In case of *Phytophthora palmivora*, maximum suppression was obtained with 25 days old cultures compared to zero days. *Trichoderma harzianum* of 25 days old cultures suppressed the highest percent (58.00 %) followed by *Trichoderma viride* (56.15%) and *Trichoderma hamatum* (54.00 %) while minimum suppression was noticed with zero days cultures of *Trichoderma harzianum* (16.00%).

Table.1 *In vitro* antagonism of native fungal and bacterial agents on coconut pathogens

Biocontrol agents		Per cent inhibition	
		Thielaviopsis paradoxa	*Phytophthora palmivora*
1	*T.viride*	69.35[a]	82.03[a]
2	*T. harzianum*	62.90 [d]	71.87 [c]
3	*T. hamatum*	66.13[c]	75.39 [b]
4	*P.fluorescens*	68.32[b]	69.38[d]

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

Table.2 *In vitro* efficacy of *Trichoderma* spp for production of volatile metabolites against coconut pathogens

Biocontrol agents		Per cent volatile inhibition					
		Thielaviopsis paradoxa			*Phytophthorapalmivora*		
		Days before exposure			Days before exposure		
		0	**15**	**25**	**0**	**15**	**25**
1	*Trichoderma viride*	0	0	0[c]	16.25 [c]	25.00[c]	56.15 [b]
2	*Trichoderma harzianum*	0	0	70.94 [b]	16.00 [b]	26.25 [b]	58.00 [a]
3	*Trichodermahamatum*	0	0	75.86 [a]	18.00 [a]	27.00[a]	54.00 [c]

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

Table.3 *In vitro* efficacy of *Trichoderma*spp for production of nonvolatile metabolites against coconut pathogens

Biocontrol agents		Per cent inhibition							
		Thielaviopsis paradoxa				*Phytophthora palmivora*			
		Concentration of culture filtrate (%)				Concentration of culture filtrate (%)			
		10	**50**	**70**	**100**	**10**	**50**	**70**	**100**
1	*T.viride*	4.76[b]	23.80 [b]	35.30 [b]	45.56 [b]	8.32 [b]	23.56 [b]	49.28 [b]	52.00[a]
2	*T. harzianum*	3.80 [b]	20.00 [c]	23.33 [c]	46.57 [b]	6.86 [c]	20.00 [c]	48.00 [c]	49.00 [b]
3	*T. hamatum*	21.11 [a]	37.78 [a]	41.11 [a]	76.67 [a]	12.36 [a]	35.25 [a]	50.25 [a]	51.00 [a]

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

Table.4 Effect of volatile metabolites of *P. fluorescens* on coconut pathogens

Age of antagonist (days)	Per cent inhibition of coconut pathogens	
	T. paradoxa	*P. palmivora*
0	0	0
2	0	0
4	0	0
6	17.91[a]	19 [a]
10	50 [b]	60 [b]

*Values with different superscripts are significantly different

Table.5 Effect of non- volatile metabolites of *P.fluorescens* on coconut pathogens

Age of antagonist (days)	Per cent inhibition of coconut pathogens	
	T. paradoxa	*P. palmivora*
10	5^{a}	8^{a}
20	10^{b}	10^{a}
50	40^{c}	43^{b}
100	50^{d}	52^{c}

*Values with different superscripts are significantly different

Plate.1 Disease symptom of stem bleeding (*Thielaviopsis paradoxa*) on coconutpalm

Plate.2 Disease symptom of bud rot (*Phytophthora palmivora*) on coconut

Plate.3 Native isolates of *Trichoderma spp.from* soil

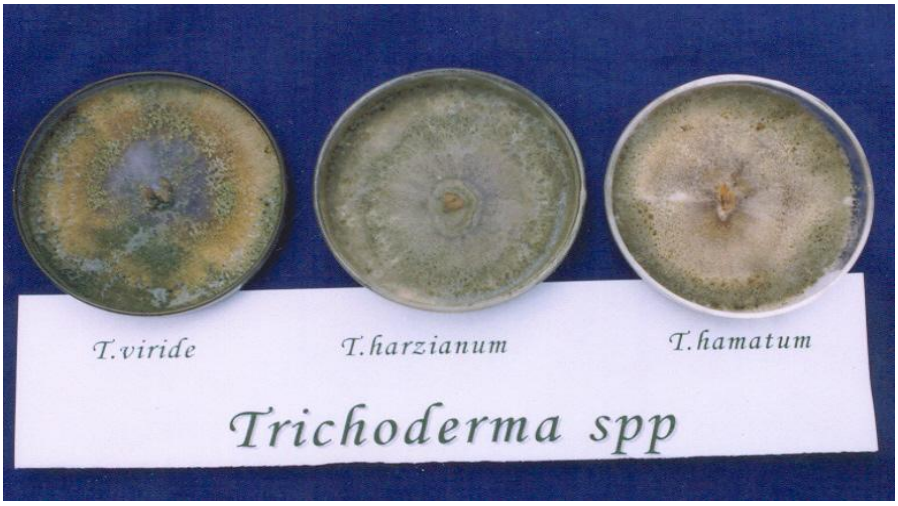
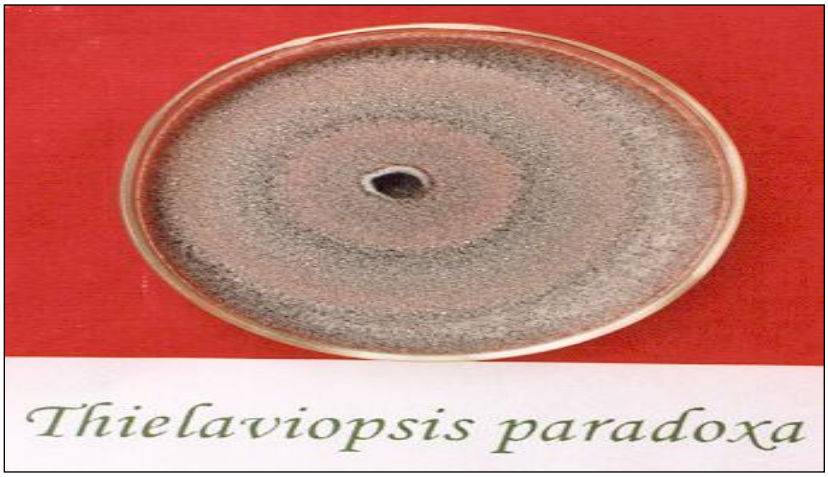
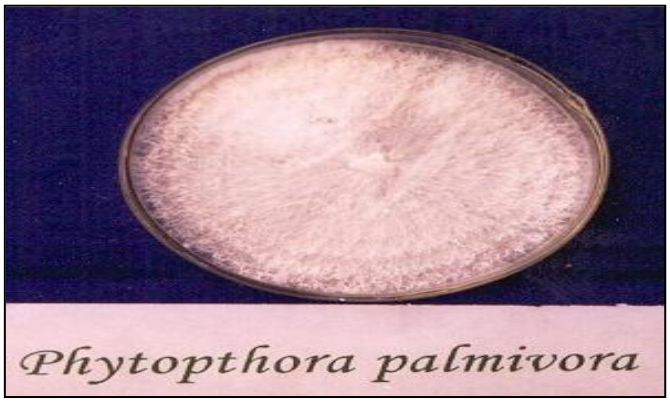


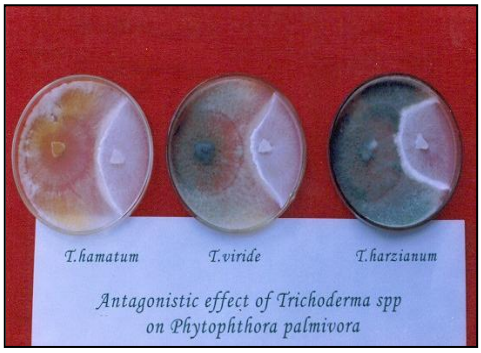
Plate.4 *In vitro* efficacy of *Trichoderma spp* and *Pseudomonas fluroscence* on *Thielaviopsis paradoxa*

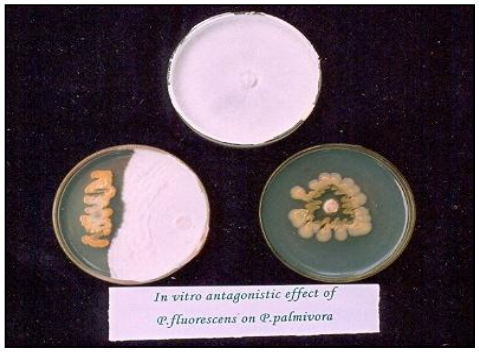



Antagonistic effect of *Pseudomonas fluroscenceon Thielaviopsisparadoxa*

Plate.4 *In vitro* efficacy of *Trichoderma spp* and *Pseudomonas fluroscence* on *Phytophthora palmivora*







A positive correlation was also obtained between inhibition of the pathogen growth and increased age of the antagonist before exposure to the pathogen. Hyphae from the exposed cultures of the test pathogens when transferred to fresh medium did not grow. The reason attributed may be due to the production of volatile metabolites by the *Trichoderma* spp *i.e. T. viride, T. harzianum* and *T. hamatum* which are both fungicidal and fungistatic (Claydon *et al.,* 1987).

In non volatile metabolites, significant inhibition on mycelial growth of *Thielaviopsis paradoxa* and *Phytophthora palmivora* pathogens was noticed with all three spices of *Trichoderma* spp at 100% concentration of culture filterate (Table 3). The mycelial inhibition of *Thielaviopsis paradoxa* ranged from minimum with *Trichoderma harzianum* (3.80%) at 10 % concentrate of culture filterate to maximum inhibition (76.67 %) at 100 % concentrate of culture filterate by *Trichoderma hamatum.* Similar observations were noticed with the inhibition of mycelial growth of *Phytophthora palmivora.* Minimum inhibition of mycelial growth of *Phytophthora palmivora* was noticed with *Trichoderma harzianum* at 10 % concentration of culture filterate (6.86) whereas maximum with 100% concentrate of culture filterate of *Trichoderma viridae* (52.00%). A positive correlation was observed between an increase in the concentration of the culture filters of *Trichoderma* spp and the per cent inhibition in mycelial growth of test pathogens The observation of this study is agreeable with Bourguington (2008) who stated that species of *Trichoderma* produces nonvolatile metabolites, such as antibiotics and enzymes, which involve in inhibiting growth of pathogenic fungi and spore germination.

Effect of volatile and non-volatile metabolites of *Pseudomonas fluorescens*

Studies on the production of volatile metabolites by *Pseudomonas fluorescens* against coconut pathogens revealed the both the pathogens *Thielaviopsis paradoxa*and *Phytophthora palmivora* were inhibited significantly when were exposed to different age old cultures of *Pseudomonas fluorescens*. The inhibition of *Thielaviopsis paradoxa* and *Phytophthora palmivora* was noticed moderately when 6 day old bacterial antagonist was used. The volatile metabolites produced by the antagonist was significantly against *Thielaviopsis paradoxa* and *Phytophthora palmivora* with 10 day old bacterial antagonist (Table 4). The non-volatile metabolites of the P*seudomonas fluorescens* against the coconut pathogens was also notice when 50% concentration and above of the culture filtrate was fortified in the medium. Significant reduction of the coconut pathogens *viz*., *Thielaviopsis paradoxa* and *Phytophthora palmivora* was noticed to a tune of 40% to 60% at a concentration of 50% culture filterate and above concentration of the bacterial antagonist (Table 5). However, the non-volatile metabolite production was not significant when 20% and less concentration of the culture filtrate of the antagonist was fortified in the medium.

In nutshell, native biocontrol agents *viz., Trichoderma viridae, T.hamatum* and *T.harzianum* screened for antagonism under *in vitro* are effective against mycelia growth of *Thielaviopsis paradoxa* and *Phytophthora palmivora* of coconut. Production and inhibitory effect of volatile substances by the antagonists found a positive correlation between inhibition of the pathogens growth and with usage of aged cultures of the antagonists before exposure to the pathogens. Antagonistic effect of culture filtrate (non-volatile metabolites) of *Trichodrema* spp against *Thielaviopsis paradoxa* and *Phytophthor apalmivora* is by poisoned food technique and inhibition of mycelia growth of test pathogens noticed with all the three species of *Trichoderma* at 100% concentration. It offers wide scope for effective management of disease at field level.

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