Research Article

doi : 10.25081/jpc.2024.v52.i3.9735
Journal of Plantation Crops, 2024, 52(I) : Page 39-46

In vitro evaluation of bioagents for the management of basal stem rot disease of coconut

K .K .Greena[1,2*], Keerthana Jayarajan[1,2], Vinayaka Hegde[1], N.Lakpale[2], Daliyamol[1], V. H.Prathibha[1]

[1]*ICAR-Central Plantation Crops Research Institute, Kasaragod- 671124, India*
[2]*Indira Gandhi Krishi Vishwavidyalaya, Raipur- 492012, India*

(Manuscript received: 14.11.2024, Revised:19.02.2025, Accepted: 20.02.2025)

Abstract

Basal stem rot caused by *Ganoderma* spp. is an important disease affecting coconut. Being asoil-borne pathogen, it can persist in coconut ecosystem for longer periods. Therefore, an eco-friendly remedy for the management of *Ganoderma* is essential. Following soil sample collection and isolation, the current study evaluated the efficacy of fluorescent *Pseudomonas* and *Trichoderma* spp. against basal stem rot pathogen *Ganoderma gibbosumin vitro*using dual culture assay. Among the 15 isolates of fluorescent *Pseudomonas*evaluated, the inhibition varied between 41% and 80 %. *P. aeruginosa* (isolate P13) exhibited maximum inhibition of 80.36%. All the isolates of *Trichoderma* showed greater antagonistic capacity with percentage inhibitions ranging from 71% to 87 %. *T. harzianum* isolate T20 exhibited a maximum inhibition of 87.92%.The present study identified several efficient antagonists of*Ganoderma* spp thatcould be further utilized for the management of the pathogen in field conditions.

Keywords:Basal stem rot, *Ganoderma* spp.,Coconut,*Pseudomonas, Trichoderma*

Introduction

Coconut (*Cocos nucifera* L.) is an important multi-purpose crop of the tropics and has proven to be a reliable source of income for farmers. The palm has been variously called the '*Kalpa Vriksha'* or 'The tree of life' or 'The tree of wealth' as every part of the palm is useful, providing livelihood to millions.India is one of the largest producers of coconut with an area and production of 21 lakhs ha and 19,309 million nuts, respectively (CDB, 2021).Coconut cultivation has been traditionally confined to southern states, which contribute to more than 80% of production.

Among the various fungal pathogens infecting coconut, Basal Stem Rot (BSR) caused by *Ganoderma* spp. is a devastating disease. Major symptoms include discolouration and decay of roots, stem bleeding from the basal portions of the trunk, yellowing and wilting of leaves which subsequently leads to drying and drooping. Following the death of the palm, fruiting body of *Ganoderma* appears on the base of the trunk (Bhaskaran *et al.,*1982). It is apathogen that most commonly affects palm trees between the ages of 10 and 30 years.Maximum incidence of *Ganoderma* spp. (up to 62.50 %) was observed in coconut gardens raised in sandy and red soils in the coastal district of Andhra Pradesh (Srinivasalu *et al.*,2003).

The pathogen is soil-borne, persistingfor a prolonged duration in soil by inhabiting dead and decaying or living hosts. Despite the potential of fungicides in managing the disease, their negative environmental impact cannot be ignored.Currently, for the management of the disease, hexaconazole is widely recommended (Subramanian*et al*., 2018). However, the adverse effects of azole group fungicides on beneficial microbes and their residual presence in coconut water have not been considered thoroughly(Hubballi *et al*., 2019). Thus, it is essential to identify potential biocontrol agents which can manage the disease effectively.

Several studies conducted using bioagents such as *Pseudomonas* and *Trichoderma* were proven to be effective for the management of basal stem rot (Bhaskaran *et al*., 1988;Rajappan *et al*., 2010; Ranjana and Sarma, 2017; Neeraja *etal*., 2018).They are easily mass multiplied in the laboratory and have a dominant antagonistic potential, which increases their scope as disease management agents.In this context, the present study was designed to exploit the potential of

**Corresponding Author: greenakk40@gmail.com*

Pseudomonas and *Trichoderma* against*Ganoderma*.

Materials and methods

Isolation of *Ganoderma* spp

Samples of sporocarps, barks from infected stem and infected roots were collected from coconut gardens showing symptoms of basal stem rot.Collected fresh basidiocarps were rinsed with sterile distilled water, and after being air-dried, were cut into small pieces and surface sterilized using 0.1% $HgCl_2$ followed by washing three times in sterile water to eliminate any $HgCl_2$ residue. The samples were kept in polypropylene bags along with wet cotton at room temperature for about 3-4 days. Upon observation of mycelial growth, the samples were transferred to Potato Dextrose Agar (PDA) medium or to *Ganoderma* Specific Medium (GSM) (Ariffin and Idris, 1991).

Upon successful isolation, pure culturing of the isolates was carried out by hyphal tip culture method by transferring the tip of a single hyphae growing from the colony to a fresh PDA plate and was maintained thereafter at 28±1°C.

Collection and isolation of bioagents

Soil samples were collected from the rhizosphere of healthy coconut palms growing in disease-affected gardens located majorly in Kerala, Karnataka and Tamil Nadu. The samples were taken at a depth within 15cm from the surface of the soil. Serial dilutions were carried out for the isolation of *Trichoderma* spp. and *Pseudomonas fluorescens*. For isolation of *Trichoderma* spp., 10^{-2} to 10^{-4}dilutions were spread onto *Trichoderma* Specific Medium (TSM)(Elad *et al*., 1981) plates and were incubatedat 28±1°C.*Trichoderma* colonies were identified based on morphological and cultural characteristics.

For the isolation of *Pseudomonas*, 10^{-4} to 10^{-7}dilutions were utilized and 0.1ml of respective dilutions were spread on petri plates containing specific media (King's Bmedium)(King *et al*., 1954) and were incubated at 28 ± 2° C for 24-48 hrs. Colonies were observed for fluorescence under UV illuminator and pure culturing of such colonies were carried out by streak plate method.

In vitro efficacy of fluorescent *Pseudomonas* against *Ganoderma*

Dual culture tests were carried out to estimate the efficacy of 15 isolates offluorescent *Pseudomonas*isolated from the collected soil samples. The bipartite interactions were carried out using a simple confrontation assay technique developed by Kotasthane *et al*., 2017. The technique involves using the edges of a 75 mm glass funnel for transferring *Pseudomonas* isolates into petriplates which are pre-inoculated with fungal pathogen. The funnel was sterilized by alcohol dipping followed by flame sterilization. Petri plateswere pre-inoculated with 0.9 cm discs of seven days old fungal cultureat the center. The edge of the sterilized funnel was dipped into 3 days old *Pseudomonas* culture dispensed in sterile petri plates. The bacterial culture was inoculated by gently placing the funnel edge on pre-inoculated petri plates of *Ganoderma*.The inoculum was carefully transferred almost equidistant from the centrally placed fungal mycelium disc.The plates were incubated at 28±2°C and observations were taken.

In vitro efficacy of *Trichoderma* spp. against *Ganoderma*

In vitro antagonistic studies were carried out using 20 isolates of *Trichoderma* spp. (19 isolated from collected soil samples and anisolate viz., *T. harzianum* (T20) obtained from ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala. Dual culture technique was carried out by co-culturing both pathogen and antagonist on opposite sides of the same petri plate. Plates inoculated with *Ganoderma*alone served as control. The distance between the margins of growth of *Ganoderma* and *Trichoderma* was used to determine the inhibition.

The percentage inhibition was calculated based on the formula by Vincent, 1947.

$$percentage\ inhibition\ (\%) = \frac{growth\ of\ pathogen\ in\ control - growth\ of\ pathogen\ in\ treatment}{growth\ of\ pathogen\ in\ control} * 100$$

Molecular characterization of selected isolates

The molecular characterization of effective *Pseudomonas* and *Trichoderma* isolates based on the 16S rRNA and ITS conserved regions respectively were carried out. DNA extraction was performedusingHiPurA®Bacterial Genomic DNA Purification KitandHiPurA® Fungal DNA Purification Kit following the manufacturer's protocol.Amplification of 16S rRNA and ITS gene region was carried out using bacterial universal primers 27F&1492R (Heuer *et al*., 1997) and fungal universal primers ITS1 and ITS4 (White *et al*., 1990). Details of primers are given in Table 1.

Table 1. Details of primers used

	Primers	Primer sequence	References
Bacterial universal primers	27F	52 -AGAGTTTGATCCTGGCTCAG-32	Heuer *et al*., 1997
	1492R	52 -TACGGYTACCTTGTTACGACTT-32	
Fungal universal primers	ITS1F	5'- TCCGTAGGTGAACCTGCGG -3'	White *et al*., 1990
	ITS4R	5'- TCCTCCGCTTATTGATATGC – 3'	

The DNA amplification reactions were performed in a Prima-96™ Thermal Cycler (Himedia®). The PCR conditions are given in Table 2.Amplified PCR products were confirmed using1.2% agarose gel electrophoresis. DNA banding patterns were visualized, and images were acquired with agel documentation system (BIO-RAD GelDoc Go Imaging System) and the expected band of 1.5 kb and 600 bp were observed respectively for 16S rRNA and ITS gene region.PCR products were sent for sequencing to Biokart India Pvt. Ltd. Bangalore. The FASTA sequences thus obtained were subjected to homology search using BLASTN analysis.

Table 2. PCR conditions for primers

Step	Universal primer ITS		Repeats	Bacterial universal primers		Repeats
	Temp(^{0}C)	Duration		Temp(^{0}C)	Duration	
Initial Denaturation	94	3 min	1	94	4 min	1
Denaturation	94	30 sec	35	94	45 sec	30
Annealing	55	30 sec		55	45 sec	
Primer Extension	72	1 min		72	1 min	
Final Extension	72	10 min	1	72	10 min	1
Hold	4	5 min	1	4	5 min	1

Statistical analysis

Statistical analysis of data was done using R Studio version 2023.12.0+369 (RStudio Team, 2020). Analysis of variance (ANOVA) was performed at two significance levels ($P<0.05$ and $P<0.01$). Comparison of means by Tukey's test was also performed.

Results and Discussion

The bioagents were tested for their efficacy against thefast-growing isolate of *Ganodermagibbosum* (Accession no. PQ439679). Fifteen isolatesof fluorescent *Pseudomonas* and 19 isolates of *Trichoderma* spp. were isolated from soil samples obtainedfrom different

localities (Table 3). Antagonistic activity of these isolates was tested *in vitro* against*Ganoderma*.

In vitro efficacy of fluorescent *Pseudomonas* against *Ganoderma*

Reduction in mycelial growth and inhibition zones were evaluated as indicators of antagonist efficacy.Inhibition was clearly observed by limiting or suppressing the growth of mycelium in the area where the isolates and pathogens were in contact (Fig. 1).

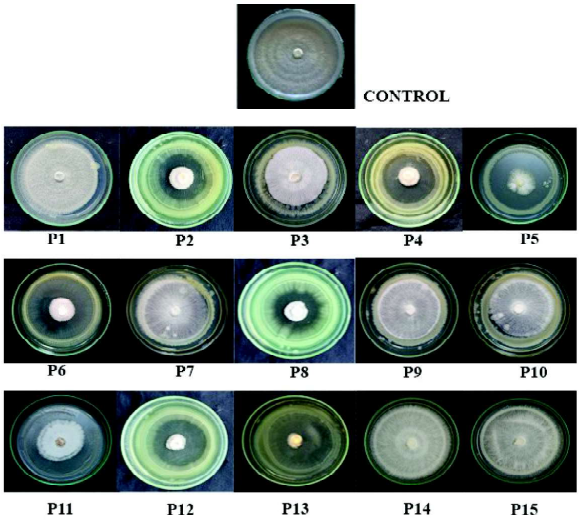

Fig.1. Confrontation assay for testing the efficacy of *Pseudomonas* isolates against *Ganoderma*

Some of the isolates showed no antagonistic activity. A total of 8 isolates out of 15 clearly showed suppression of mycelial growth of *G. gibbosum*. Among the efficient isolates of *Pseudomonas,* the inhibition varied between 41 to 80 %. The highest activity was shown by isolate P13 (80.36%) followed by P12 (74.11%) over control at 5 days after incubation (Fig. 2).

The antagonistic ability of *Pseudomonas* can be attributed to the production of antifungal metabolites(Ramamoorthy *et al*., 2002; Saravanakumar *et al*., 2008).*In vitro* studies conducted to evaluate the efficacy of 156 *Pseudomonas* isolates against *G. applanatum* showed inhibition percentages between 39% and 73% (George *et al*., 2012).

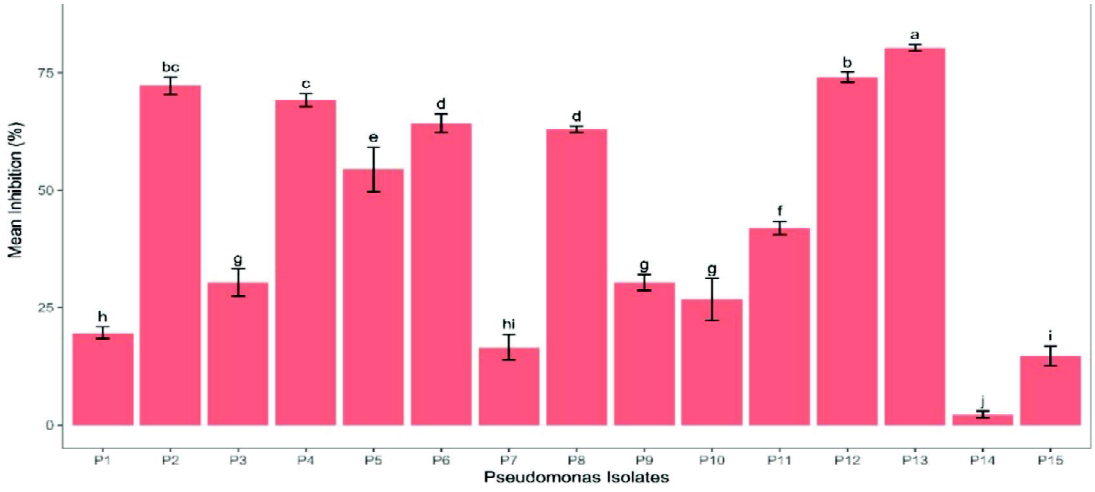

Fig.2. Bar graph representing the efficacy of *Pseudomonas* isolates against *Ganoderma*

Table3. List & place of collection of *Pseudomonas* and *Trichoderma* isolates usedin the study

Pseudomonas Isolates	Place of Collection	*Trichoderma* isolates	Place of Collection
P1	Kasaragod, Kerala	T1	Kasaragod, Kerala
P2	Ernakulam, Kerala	T2	Hassan, Karnataka
P3	Kasaragod, Kerala	T3	Hassan, Karnataka
P4	Erode, Tamil Nadu	T4	Thiruvananthapuram, Kerala
P5	Kasaragod, Kerala	T5	Thiruvananthapuram, Kerala
P6	Malappuram, Kerala	T6	Assam
P7	Malappuram, Kerala	T7	Thiruvananthapuram, Kerala
P8	Thiruvananthapuram, Kerala	T8	Thiruvananthapuram, Kerala
P9	Erode, Tamil Nadu	T9	Kasaragod, Kerala
P10	Malappuram, Kerala	T10	Kasaragod, Kerala
P11	Kasaragod, Kerala	T11	Kasaragod, Kerala
P12	Tiruppur, Tamil Nadu	T12	Malappuram, Kerala
P13	Thiruvananthapuram, Kerala	T13	Kasaragod, Kerala
P14	Kasaragod, Kerala	T14	Tiruppur, Tamil Nadu
P15	Malappuram, Kerala	T15	Thiruvananthapuram, Kerala
		T16	Lakshadweep
		T17	Shimoga, Karnataka
		T18	Hassan, Karnataka
		T19	Tumkur, Karnataka
		T20	CPCRI

***In vitro* efficacy of *Trichoderma* spp. against *Ganoderma* spp.**

There was a noticeable inhibition in the growth of fungal mycelium in the area of contact between the isolates and fungal pathogens (Fig. 3). All the isolates of *Trichoderma* showed greater antagonistic capacity against *Ganoderma* with percentage inhibitions ranging from 71% to 87 % after 6 days of incubation.

T. harzianum isolate T20 showed maximum inhibition of 87.92% followed by isolates T5 (87.55%) and T14 (86.04%). Minimum inhibition of 71.32% was exhibited by isolate T6 (Fig. 4). Similar results were obtainedinearlier studies which showed the efficacy of *Trichoderma* spp. against the pathogen (Srinivasalu *et al*., 2001, Karthikeyan *et al*., 2005).Bhaskaran *et al.* (1988) analyzedand

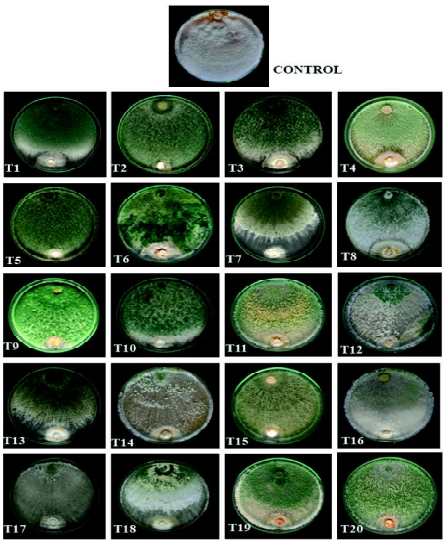

Fig. 3. Dual culture assay for testing the efficacy of *Trichoderma* isolates against *Ganoderma*

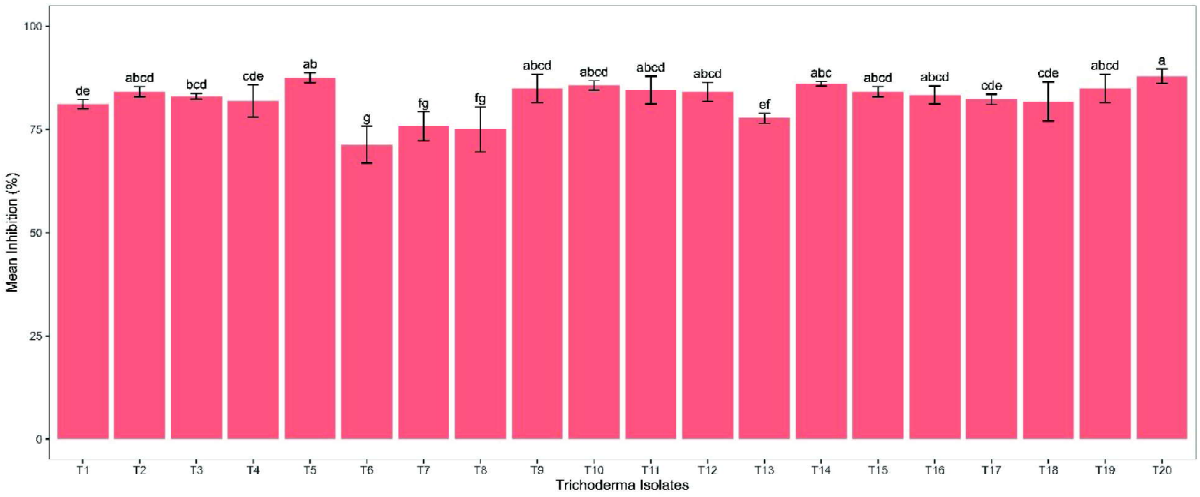

Fig.4. Efficacy of *Trichoderma* isolates against *Ganoderma*under *in vitro* conditions

reported the antagonistic efficacy of several *Trichoderma* spp. against *G. lucidum*and *G. applanatum.*

Trichoderma species produce various peptides and cyclic polypeptide antibiotics, including trichodermol, trichodermin, harzianolide and harzianum A which may be responsible for the inhibition of pathogen development under *in vitro*conditions (Vinale, 2005).Several studies have shown that secondary metabolites produced by *Trichoderma* can suppress the growth of pathogens while enhancing plant growth (Kullnig *et al*., 2000; Kubicek *et al*., 2001).

Molecular analysis of the most efficient isolate P13 confirmed its identity as *P. aeruginosa*(Accession no. PQ524515). With the exception of certain strains that are harmful to humans (He *et al*., 2004), *P. aeruginosa* is a versatile and ubiquitous bacterium that has been recognized as an effective antagonist of a number of bacterial and fungal plant pathogens (Chandra *et al*., 2020).*Pseudomonas* strain produces numerous compounds which aid in disease control. These inhibitory substances include siderophores, HCN, antibiotics like pyrrolnitrin, pyoluteorin, and phenazine, as well as degradative extracellular enzymes including chitinase, protease, cellulose and β-1,3 glucanase (Dowlingand O'Gara, 1994; Haasand Défago, 2005; Deshwal*et al*., 2011).

Other efficient *Trichoderma* isolates such as T5 and T14 were identified as *T. virens*(Accession no. PQ510471) and *T. harzianum* (Accession no. PQ510480) respectively,based on molecular analysis of their ITS regions.Prior research showed that under *in vitro* conditions, *Trichoderma* species such as*T. viride*, *T.harzianum*, and *T. hamatum*, inhibited basal stem rot infections to an extent of 63 to 84% (Srinivasulu*et al*., 2002).In addition to being effective at reducing basal stem rot in oil palm, *T. harzianum* (strain FA1132) was found to increase chlorophyll concentration and root and leaf weights(Naher *et al*., 2012). Recent studies have revealed that NRPS (Non-Ribosomal Peptide Synthetases) Tex2 of *T. virens* causes the assemblage of 11- and 14-module peptaibols, which have potent antimicrobial properties(Mukherjee *et al*., 2011).

Conclusion

As an initial guiding system for identifying potential biocontrol agents, *in vitro* screening has proved to be very beneficial. Several potent fungal antagonists were identified in the present investigation which were highly efficient and could be further utilized for the management of the coconut pathogens in field conditions. While the results are promising, more studies are needed to confirm their efficacy as biocontrol agents. Their ability to prevent disease development has to be investigated on coconut seedlings in field conditions.

Acknowledgement

The authors acknowledge the Division of Crop Protection, ICAR-Central Plantation Crops Research Institute, Kasaragod, 671124, Kerala, India for providing assistance and guidance for the research program.

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