Research Article

Biological Control Agents for the Management of Basal Stem Rot Disease in Coconut

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Abstract

Ten isolates of each antagonistic *Pseudomonas* and *Trichoderma* were isolated from coconut roots and rhizospheric soil and their inhibitory potential against *Ganoderma lucidum* was ascertained through dual culture method. Among the isolates, P10 of *Pseudomonas* and T5 of *Trichoderma* exhibited the maximum inhibition to mycelial growth of fungus. The isolates exhibiting maximum inhibition were compatible with each other. The isolates P10 of *Pseudomonas* sp and T5 of *Trichoderma* were identified as *Pseudomonas fluorescens* and *Trichoderma reesei*. Further, these two isolates were evaluated under field conditions for their efficacy against basal stem rot disease of coconut. It was observed that 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.

Key words: Coconut, neem cake, *Pseudomonas fluorescens,* and *Trichoderma reesei*

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Coconut is primarily a small holder's crop cultivated throughout the humid tropics. It provides nutritious drink, many edible nutritious products, oil for edible and non edible uses, and fibre of commercial value, shell for fuel and a variety of miscellaneous products. The crop is aptly associated with Indian culture and heritage and hence, looking its multifarious usages, it is described as Kalpatharu. Indian subcontinent is in third place with respect to acreage and first in production and productivity accounting for 22.34 per cent of the world's coconut production and is one of the major players in the world's coconut trade. More than 90 per cent of coconut grown in India is seen in four Sothern states *viz*., Karnataka, Kerala, Tamil Nadu and Andhra Pradesh (CDB, 2017).

The palm though is hardy in nature; it is being debilitated by an array of plant pathogens. Of the pathogens inciting variety of diseases in coconut, Basal stem rot disease caused by a fungus *Ganoderma lucidum* (Leys) Karst is a major limiting factor in coconut production in India. This disease is also known as “Thanjavur wilt” in Tamil Nadu, "Ganoderma Wilt" in Andhra Pradesh and "Anabe Roga" in Karnataka. It is the most serious disease limiting coconut production in Tamil Nadu, Andhra Pradesh and Karnataka. It is also reported from Kerala, Maharashtra, Gujarat and Orissa. This disease was first reported on palms in India by Butler (1906). Venkatarayan (1936) studied the disease which affected both coconut and arecanut in Karnataka. The fungus has a wide host range infecting both monocots and dicots (Bhaskaran and Ramanathan, 1984).

Despite decades of research towards its management, basal stem rot (BSR) disease has remained and continued to be as a challenge for plant pathologists. Many methods comprising host resistance, chemical and biological approaches have been practiced from time immemorial. However, among several methods deployed for the control of the disease, chemical control has been widely practiced in many countries. Since, introduction of Calixin, a vast number of fungicides have appeared in the market and their potential has

been evaluated against BSR. Very recently, Hexaconazole is in recommendation for the management of BSR in coconut (POP, UHS 2015). Though azole group of fungicides have yielded a satisfactory results, there toxic nature and residual level in the coconut water has not been taken into account largely. Further, the non-judicial usage 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. A better strategy to avert the development of epidemics is to treat the pathogen when its level in the field is low and to prevent further increases over the growing season. Effective options include employing the pathogen's natural enemies as biological control agents, as less destructive or more environmentally friendly than chemical treatments. Many studies have reported antagonistic activity of fungi and bacteria against phytopathogens, and this is considered as a very appealing alternative to the use of chemical fungicides.

Trichoderma and *Pseudomonas* are the most widely employed ubiquitous biological control agents. The predominant antagonistic potential and the ease with which they can be mass multiplied under laboratory have virtually enhanced the scope for tapping of these microbes for disease management. Perusal of literature clearly implied the uncompariable antagonistic potential of these two microbes against a variety of plant diseases. Studies have also been conducted for the biological control of basal stem rot disease and stem bleeding diseases of coconut using *Trichoderma* spp and *Pseudomonas* sp antagonists (Srinivasulu and Rao 2007).

With backdrop of these points, the present study was contemplated to harness the potential of *Trichoderma* and *Pseudomonas* against basal stem rot disease of coconut and its impact on yield.

Materials and Methods

Isolation of pathogen. The Coconut palm depicting characteristic symptoms of basal stem rot disease was selected and the bleeding patches on stem portion was scraped with sterile sharp knife and brought to laboratory in polythene cover. The pathogen associated was isolated through tissue segment method of Rangaswamy (1958) using Potato Dextrose Agar medium. The pathogen was further purified and identified based on morphological and cultural characteristics and was stored in PDA slants for further usage.

Antagonistic bacteria and fungus. One gram of coconut rhizospheric soil sample was suspended in 99 ml of sterile distilled water. Samples were serially diluted and 0.1 ml of sample was spreaded on plates containing King's B medium and

Table 1. Bacterial isolates isolated from coconut

Isolate	Grams Reaction	Source
P1	Negative	Rhizosphere soil of coconut
P2	Positive	Rhizosphere soil of coconut
P3	Positive	Rhizosphere soil of coconut
P4	Negative	Rhizosphere soil of coconut
P5	Negative	Rhizosphere soil of coconut
P6	Negative	Rhizosphere soil of coconut
P7	Negative	Rhizosphere soil of coconut
P8	Negative	Rhizosphere soil of coconut
P9	Negative	Rhizosphere soil of coconut
P10	Negative	Rhizosphere soil of coconut

incubated at –80 C for 24 hrs. All isolates were purified twice and then stored at -80 C in Nutrient broth containing 15 per cent glycerol for further use. Identification of the different cultures of antagonistic bacteria was done as per the methods recommended in the laboratory guide for identification of plant pathogenic bacteria published by the American Phytopathological Society (Schaad 1992). The totals of ten bacterial isolates were isolated from the soil samples collected from different places (Table 1).

In addition to this, ten *Trichoderma* isolates were also isolated from the coconut rhizospheric soil using *Trichoderma* selective medium (Elad and Chet 1983) through serial dilution method (Table 2).Identification was done based on morphological and cultural characteristics on PDA medium. The purified isolates of *Trichoderma* were stored in refrigerator for further usage.

Table 2.*Trichoderma* isolates isolated from coconut

Isolate	Source
T1	Rhizosphere soil of coconut
T2	Rhizosphere soil of coconut
T3	Rhizosphere soil of coconut
T4	Rhizosphere soil of coconut
T5	Rhizosphere soil of coconut
T6	Rhizosphere soil of coconut
T7	Rhizosphere soil of coconut
T8	Rhizosphere soil of coconut
T9	Rhizosphere soil of coconut
T10	Rhizosphere soil of coconut

Antagonistic activity of bacterial and fungal antagonists

Bacterial antagonists. The antifungal activity of ten *Pseudomonas* isolates was tested by dual culture technique (Zivkovic et al 2009) using PDA medium. A mycelial disc (9 mm diam) of the pathogen was placed at one end of the Petri plate and the bacterial antagonists were streaked one cm away from the periphery of the Petri plate just opposite to the mycelial disc of the pathogen. The control plate was maintained without antagonist. The treatments were replicated thrice and incubated at 28±2 C for twenty days. The growth of the pathogen towards the bacterial colony and inhibition zone was measured after twenty days of incubation. The radial growth of pathogen was measured and per cent inhibition (PI) was calculated.

Fungal antagonists. Nine-mm actively growing PDA culture disc of the pathogen was placed on PDA medium 1.5 cm away from the edge of the Petri plate. On the opposite side of the medium in Petri dish a 9-mm culture disc of the fungal antagonist was placed. PDA medium inoculated with the pathogen alone served as the control. Three replications were maintained. The plates were incubated at room temperature (28 ± 2 C). When the control plate showed full growth of the pathogen, the mycelial growth of the pathogen in plate containing fungal antagonist was measured. The results were expressed as per cent inhibition of mycelial growth over control.

Compatibility studies. The compatibility of the *Trichoderma* isolate T5 with the *Pseudomonas* isolate P10 was tested by their mycelial overgrowth on the PGPR strains without any inhibition zone, using the dual culture technique (Dennis and Webster, 1971).

Preparation of talc-based formulation of *Pseudomonas* and *Trichoderma*

Pseudomonas. A loopful of bacterium was inoculated into the KB broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28 ± 2 C). After 48 h of incubation, the broth containing 9 $x10^8$ cfu ml/1 was used for the preparation of talc-based formulation. To 400 ml of bacterial suspension, 1 kg of the purified talc powder (sterilized at 105 C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose 10 g (adhesive) were mixed under sterile conditions following the method described by Vidhyasekaran and Muthamilan (1995). After overnight shade drying, it was packed in polypropylene bag and sealed.

Trichoderma. *Trichoderma* isolate was multiplied

in molasses-yeast broth (30 ml molasses; 5 g yeast; plus water to a total volume of 1000 ml) as per the procedure described by Jeyarajan et al (1994). The sterile broth was inoculated with an actively growing mycelial disc (9 mm) and incubated for 5 days. After multiplication, 500 ml broth 3 x 10^8cfu ml^{-1} (fungus) was mixed with one kg of Talcum powder. The mixture was shade dried for two days till moisture attains 20 per cent. For each kg of the talc powder, 5 g of carboxy methyl cellulose was added as adhesive material and the talc-based preparation was stored in polythene bags and used for further studies.

Field evaluation of *Trichoderma* and *Pseudomonas* strains. The elite isolates of *Trichoderma* and *Pseudomonas* were further evaluated for their efficacy against Basal stem rot disease in coconut under field conditions. The field experiment was conducted at Gandsi village in Arsikere Taluk of Karnataka during 2014 to 2017 for three years. The coconut variety Tipatur Tall was used and regular practices of coconut were done throughout experimental period as per package of practice of UHS, Bagalkot. A total of 12 different treatments were imposed with three replication under each treatment. Each palm around 30 years old aged constituted one replication. The different treatments were:

T1- 125g of *TR* + 1.25 kg NC/ palm quarterly

T2- 125g of *TR* + 2.5 kg of NC/ palm half yearly

T3- 125g of *TR* + 5 kg of NC/ palm/year

T4- 125g of *PF* + 1.25 kg of NC/ palm quarterly

T5- 125g of *PF* + 2.5 kg of NC/ palm half yearly

T6- 125g of *PF* + 5 kg of NC/ palm/year

T7- 125g of *TR* and *PF* +1.25 kg of NC/ palm quarterly

T8- 125g of *TR* and *PF* +2.5 kg of NC/ palm half yearly

T9- 125g of *TR* and *PF* + 5 kg of NC/ palm/year

T10- Root feeding of 1 ml of Hexaconazole in 100 ml water thrice in a year

T11- 125g of *TR* and *PF* + 5 kg of NC/ palm/year+ Root feeding with 1 ml Hexaconazole / 100 ml thrice in a year + Micronutrient application @1kg/palm/ Year and

T12- Control

where TR- *Trichoderma reesei*, PF- *Pseudomonas fluorescens* and NC- Neem cake.

All the treatments were applied as soil application.

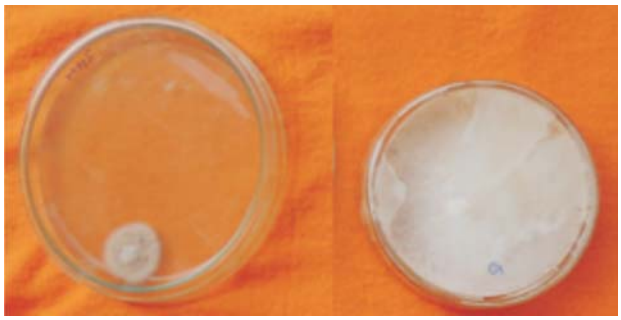
Disease Indexing. All the experimental palms in this trial were indexed for disease using formula Disease index= 23.6+17.4H+36.6R-0.6L where H-height of bleeding patch, R- reduction in leaf size (0-4 scale), L-Number of functional leaves.The disease index at the beginning and at quarterly interval was recorded in individual replication and the average was calculated.

Yield data. The experimental palms were also recorded with yield obtained under each harvest and total yield per year per palm was calculated.

Statistical analysis. The data were analysed independently for the studies under field conditions. The data were analysed as randomised block design (RBD) using the IRRISTAT version 92-1 programme developed by the biometrics unit at the International Rice Research Institute, Philippines.

Results and Discussion

In vitro* antagonism of *Pseudomonas* isolates against *Ganoderma. Ten bacterial and ten fungal isolates were isolated from rhizospheric soil of coconut (Table 1 and 2) and their antagonistic potential was tested. It was observed that, all the bacterial isolates had significant variation with respect to antagonism observed. The isolate P10 was effective in arresting the growth of pathogen to the tune of 77.77 per cent (Fig 1) and was followed by P9 (75.55). The least inhibition was observed in case of P2 with 33.88 per cent inhibition (Table 3). The results were in line with findings of Priya et al (2012) wherein she reported the inhibitory potential of fluorescent *Pseudomonas* against *Ganoderma* under *in vitro* conditions. The inhibition of mycelial growth of the pathogen by *P. fluorescens* may be due to the production of antibiotics. Production of antibiotics *viz.,* HCN, pyrrolnitrin, phenazine and 2, 4-diacetyl phloroglucinol and lytic enzymes by *P. fluorescens* against fungal pathogens were reported by many workers (Ramamoorthy and Samiyappan, 2001; Ramamoorthy et al 2002; Saravanakumar et al 2008).



P10 isolate Control

Figure 1. Growth suppression of *Ganoderma* by P10 isolate of *Pseudomonas*

Table 3. *In vitro* efficacy of bacterial isolates against radial growth of *Ganoderma*

Bacterial Code	*Mycelial growth (mm)	PIOC
P1	51.00	43.33
P2	59.50	33.88
P3	56.70	37.00
P4	25.00	72.22
P5	39.00	56.66
P6	56.95	36.72
P7	37.10	58.77
P8	52.00	42.22
P9	22.00	75.55
P10	20.00	77.77
Control	90.00	–
CD (P=0.05)	1.57	
SEm±	0.48	

*Mean of three replications; PIOC- Per cent inhibition over control

In vitro antagonism of *Trichoderma* isolates against *Ganoderma.* The species of *Trichoderma* constitute an important microbial population residing in soil and has been exploited tremendously for management of many soil borne diseases. In present study, among the ten *Trichoderma* isolates isolated from Rhizosphere soil, the isolate T5 exerted the maximum inhibition of 79.11 per cent under *in vitro* (Table 4, Fig 2). The results are in corroboration with earlier workers who reported the potential of *Trichoderma* sp. against *Ganoderma* (Srinivasulu et al 2001, Karthikeyan et al 2005). A large number of peptides and cyclic polypeptides antibiotics such as trichodermin, trichodermol, harzianum A and harzianolide are produced by *Trichoderma* species could be the possible reason for inhibition of pathogen under *in vitro* (Vinale 2005).

Table 4. *In vitro* efficacy of *Trichoderma* isolates against radial growth of *Ganoderma*

Isolate	*Mycelial growth (mm)	PIOC
T1	46.80	48.00
T2	64.35	28.50
T3	61.07	32.15
T4	31.45	65.06
T5	18.80	79.11
T6	79.07	12.14
T7	63.08	29.91
T8	31.28	65.24
T9	42.50	52.78
T10	78.50	12.78
Control	90.00	–
CD (P=0.05)	7.63	
SEm±	2.58	

*Mean of three replications; PIOC-Per cent inhibition over control

T5 isolate Control

Figure 2. Growth suppression of *Ganoderma* by T5 isolate of *Trichoderma*

Table 5. Influence of *Trichoderma* and *Pseudomonas* on BSR disease of coconut under field conditions

Treatment	Pre initiation	*Disease Index at different months after treatment												
		Sep14	Dec14	Mar15	June15	Sep 15	Dec1 5	Mar 16	June 16	Sep 16	Dec 16	Mar 17	June 17	Sep 17
T_1	42.00	41.40	30.30	30.30	37.27	37.5	36.15	49.13	50.30	48.24	49.15	49.18	50.33	51.78
T_2	50.10	49.40	45.90	45.90	39.00	38.75	38.00	50.92	48.55	45.82	43.51	42.23	43.59	45.97
T_3	43.20	43.50	53.00	48.60	38.50	37.5	36.82	51.55	50.52	48.02	49.00	48.12	46.87	45.98
T_4	38.20	38.20	43.60	41.80	39.00	38.50	37.12	52.43	49.19	46.95	47.20	47.00	44.40	47.49
T_5	37.00	34.70	37.90	37.90	39.00	36.5	36.00	48.16	45.07	41.78	40.18	40.00	45.50	45.75
T_6	15.50	16.40	15.10	15.10	37.99	38.50	38.00	35.53	35.24	35.59	34.65	34.00	37.00	41.86
T_7	63.80	63.20	68.90	68.90	38.40	37.75	36.15	67.22	64.41	63.98	63.00	62.15	65.12	64.83
T_8	24.90	24.90	14.90	14.90	37.50	41.5	41.67	35.41	39.41	36.76	37.15	38.10	41.30	42.30
T_9	38.20	38.80	14.60	14.60	38.75	35.87	35.00	36.09	35.33	29.68	27.21	26.28	28.05	27.33
T_{10}	43.20	43.20	58.30	56.50	38.75	39.00	40.00	35.80	35.33	38.71	37.13	37.00	38.26	38.18
T_{11}	53.50	52.30	67.10	67.10	39.05	39.27	38.00	36.80	36.15	34.48	33.08	33.00	37.86	39.08
T_{12}	31.40	32.60	56.70	56.70	61.00	88.75	69.15	71.74	68.91	66.27	68.27	69.13	68.75	71.17
	CD (P=0.05)	NS	NS	NS	1.55	1.31	1.79	8.43	9.76	7.40	5.15	4.98	2.78	1.97
	SEm±				0.51	0.43	0.59	2.87	3.32	2.52	1.68	1.66	0.95	0.67

*Mean of three replications NS- Non Significant; T1- 125g of *TR* + 1.25 kg NC/ palm quarterly,T2- 125g of *TR* + 2.5 kg of NC/ palm half yearly, T3- 125g of *TR* + 5 kg of NC/ palm/year,T4- 125g of *PF* + 1.25 kg of NC/ palm quarterly, T5- 125g of *PF* + 2.5 kg of NC/ palm half yearly,T6- 125g of *PF* + 5 kg of NC/ palm/year,T7- 125g of *TR* and *PF* +1.25 kg of NC/ palm quarterly, T8- 125g of *TR* and *PF* +2.5 kg of NC/ palm half yearly, T9- 125g of *TR* and *PF* + 5 kg of NC/ palm/year, T10-Root feeding of 1 ml of Hexaconazole in 100 ml water thrice in a yearT11-125g of *TR* and *PF* + 5 kg of NC/ palm/year+ Root feeding with 2 ml Hexaconazole/100ml thrice in a year+ Micronutrient application@1kg/palm/year and T12- Control where TR- *Trichodermareesei*, PF- *Pseudomonas fluorescens* and NC- Neem cake. All the treatments were applied as soil application.

Efficacy of *Pseudomonas* and *Trichoderma* on BSR disease incidence and yield. In most of the research, to date, biocontrol agents are applied singly to combat the growth of the pathogens. Although the potential benefits of a single biocontrol agent application has been demonstrated in many studies, it may also partially account for the reported inconsistent performance because a single biocontrol agent is not likely to be active in all kinds of soil environment and all agricultural ecosystems (Raupach and Kloepper 1998). These have resulted in inadequate colonization, limited tolerance to changes in environmental conditions and fluctuations in production of antifungal metabolites (Weller and Thomashow 1994). Thus, more emphasis was laid on the combined use of two or more strains of biocontrol agents, which turned out to be more successful than either of them alone, as reported by several workers (Thilgavathi et al 2007; Senthilraja et al 2010). Mixtures of biocontrol agents will also have the advantage of exercising a broad spectrum activity, enhancing the efficacy and reliability of biological control generally and ensuring greater induction of defense enzymes over individual strains (Latha et al 2009). Keeping view of these facts, in this study, one most potent *Pseudomonas* isolate P10 and one *Trichoderma* isolate T5 were assessed for their compatibility under *in vitro* and were observed that they were compatible with each other. After knowing their compatibility, they were mass multiplied in the talc powder and were mixed with neem cake for the delivery in field. The combination of *Pseudomonas* and *Trichoderma* along with neem cake were applied to the infected palm at various interval. It was observed that, during initial six months there was no difference among the treatments imposed with respect to disease index. However, after six months, it was noted that, there was significant variation with respect to disease among the various treatments. The treatment T9 *i.e* application of 125g of *Trichoderma reesei* and *Pseudomonas fluorescense* along with 5 kg of Neem cake per palm per year was most effective recording the lowest disease index of 27.33 per cent after three years as against

Table 6. Influence of *Trichoderma* and *Pseudomonas* on nut yield of coconut per palm per year

Treatment	*Nut Yield Per Palm		
	Sept 14 to Aug15	Sep 15 to Aug 16	Aug 16 to Sep 17
T1	52.67	60.33	60.67
T2	52.33	63.00	68.00
T3	50.00	59.67	65.00
T4	49.67	67.00	84.33
T5	49.33	56.33	45.50
T6	57.33	70.00	69.67
T7	45.00	68.33	75.13
T8	48.67	71.33	76.33
T9	46.33	85.75	98.00
T10	45.00	73.00	86.67
T11	45.67	49.67	84.00
T12	49.33	52.33	58.00
CD (P=0.05)	NS	15.85	21.52
SEm±		5.38	7.33

*Mean of three replications

the 71.17 in control (Table 5). In addition to the disease incidence, the observation on nut yield was also documented in various treatments. It was recorded that, during first year of the experiment, the yield of coconut had not varied statistically. However, during second and third year, the treatment T9 recorded the highest nut yield per palm during Sept 15 to Aug 16 (85.75) and Sept16 to August 17 (98.00) (Table 6). The results are in agreement with findings of many earlier workers who reported the potential of both *Pseudomonas* and *Trichoderma (*Karthikeyan et al 2006, Srinivasulu et al 2005). The role of neem cake in disease management has been elucidated by Gunashekar et al (1986) wherein he observed the increased population of antifungal microbes in neem cake. Furthermore, Krishnamurthy and Bhaskaran (1993) reported the enhanced population of *Trichoderma* spp. due to addition of neem cake into the soil. The reason attributed for the reduction in the disease index of coconut and increased yield after application of *Pseudomonas* and *Trichoderma* along with neem cake is production of antibiotics, metabolites and siderophore by *Trichoderma* and *Pseudomonas*, respectively (Daniel Jebaraj et al 2012). Hence, it is concluded that, the basal stem rot disease can be managed by application of 125 g *Pseudomonas fluorescens* and *Trichoderma reesei* along with 5 kg neem cake per palm per year and in addition the nut yield can also be increased.

Compliance with ethical standards

Conflict of interest. The authors declare that they have no conflict of interest.

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