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Rapid Detection of *Ganoderma* Disease of Coconut by using ITS-PCR and Assessment of Inhibition Effect of Various Control Measures by Fungicides and Bioagents

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Two biochemical and molecular methods have been applied for detecting the *Ganoderma,* a basidiomycete fungi causing basal stem rot disease (BSR) of coconut in the early stage itself. Leaf, stem/bark and root extracts recorded highest optical density values than their corresponding healthy tissues. Increased transmittance values observed from the extracts of diseased leaf, stem, bark and root extracts compared healthy tissues. For polymerase chain reaction (PCR) test, the primer generated from the internal transcribed spacer region one (ITS 1) of ribosomal DNA gene of *Ganoderma*, which produced a PCR product of 160 bp in size is used for early detection. Different systemic, non-systemic and bioagents were screened against *Ganoderma* isolates for *in vitro* management of the disease. Among the non-systemic fungicides tried *in vitro*, captan, mancozeb and thiram at 0.2 per cent completely inhibited the growth of the fungus. In case of systemic fungicides trideomarphm, hexaconzole and carbendazim at 0.1 per cent recorded highest per cent inhibition of mycelium growth. Dual culture study carried out with three different species of *Trichoderma* revealed that *T. harzianum* and *T. koningii* were found effective.

Key words: Biochemical, Molecular, diagnosis, *Ganoderma*, Bioagents, fungicides.

Coconut is an important oil seed as well as plantation crop in India with an area of 1.8 million hectares and an annual production of 54 billion nuts[1]. In India, basal stem rot disease (BSR) caused by *Ganoderma lucidum* (Leys) Karst is a major limiting factor in coconut production. The disease is also referred as Thanjavur wilt, bole rot, *Ganoderma* disease and Anabe. The incubation period of this disease has been determined to be several. The visible disease symptoms appear at a very late stage of infection when more than half of the root tissues have been decayed, leaving no chance for the grower to cure the infected palms. Basal stem rot disease of coconut can be contained by management practices if the disease is detected in the early stages. A few methods have been reported to be useful to identify the diseased palms even before expression of symptoms; though the methods are non-specific for BSR[2].The polymerase chain reaction (PCR) technology has revolutionized the field of plant pathology in diagnosing various plant pathogens. The internal transcribed spacer (ITS) regions of ribosomal RNA gene (rDNA) have been selected as specific targets for PCR detection of *Ganoderma*[3].

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Ganoderma wilt disease can be contained by management practices, if the disease is detected in the early stages. A few methods have been reported to be useful for early detection of the disease, though the methods need further refinement. One bottleneck is to assess the initial stages of infection as visual symptoms are always elusive. Further, by the time dependable visual symptoms appear, much damage has been done to the tree already, making the revival a real problem. Therefore, attempts are made to detect at early stage infection by different biochemical staining methods or techniques.

MATERIAL AND METHODS

There are different biochemical early diagnostic techniques which are used to detect *Ganoderma* root rot disease viz., colorimetric method using EDTA and KOH , iodine-potassium iodide staining technique, alkaline copper sulphate test using leaf, root and bark/stem extracts and molecular method using PCR based diagnosis. The biochemical methods were repeated in the laboratory to validate their efficient response for detecting the presence of causal pathogens or disease in the collected samples.

Colorimetric method

10 ml of saturated potassium hydroxide was added to 5g of root or stem tissues and autoclaved for 30 minutes at 1.1 kg/cm^2. The solution was decanted and the tissues were treated with 5ml of 95 per cent ethanol. One ml of ethanol extract was made up to 10 ml with the same solvent and read in spectrophotometer at 425nm. The optical density of stem tissues increased with the increase in disease intensity from 0.445 in healthy to 1.002 in severely diseased palms and healthy sample served as control[2].

Iodine – Potassium Iodide staining technique

Bark and root tissues from diseased and healthy palms were autoclaved separately with saturated KOH at 1.1 kg/cm^2 and washed repeatedly with 90 per cent ethanol and stained with iodine- potassium iodide solution (0.2 g iodine + 2.0 g potassium iodide in 100 ml of water). The stained tissues were examined under microscope. The healthy bark and root tissues stained yellow and the colour intensity increased with increase in disease severity[4].

EDTA method

Leaf or root tissues were extracted with 0.3 M EDTA solution. The optical density at 400 nm increased with increase in disease severity[2].

Orthophenanthroline reagent method

In this method root tissues from diseased and healthy palms were autoclaved separately and washed repeatedly with 90 per cent ethanol and sterile water. Then the solution was decanted and the tissues were treated with 5ml of 95 per cent ethanol. One ml of ethanol extract was made up to 10 ml with the same solvent and read in spectrophotometer at 570 nm. The optical density of root tissues increased with the increase in disease intensity from 0.182 to 0.187 in severely diseased palms[4].

Alkaline copper sulphate (CuSO4) test

In this test different concentrations of alkaline copper sulphate solution *viz*., 1, 2, 3, 4 and 5 per cent were prepared. Then 1ml of copper sulphate solution was taken and added to 1ml of sap of both diseased and healthy leaves of coconut palms[2].

Molecular diagnosis

PCR based early detection method of *Ganoderma* was also carried out by isolating the suspected samples (roots) from the trees. In this method both infected and healthy roots were collected for isolation of pathogen and genomic DNA was extracted. In polymerase chain reaction (PCR), the DNA region used for the molecular determination of the fungus is the gene cluster that codes for the ribosomal RNA gene, in which internal transcribed spacers region is used for the identification of *Ganoderma* fungus. Template DNA was extracted from coconut roots. The PCR buffers, nucleotide mix and Taq polymerase were used[3]. The Gan1 and Gan2 primers 18 primers chosen as primers are 5' – TTG ACT GGG TTG TAG CTG – 3' (forward primer) and 5' – GCG TTA CAT CGC AAT ACA – 3' (Reverse primer). The application of this primers generated from the ITS1 sequence proved to be useful for the specific detection of plant pathogenic *Ganoderma*[3].The expected DNA fragment product size is 167 bp. The thermocycler was programmed as follows; 5 min preheating at 95°C followed by 48 cycles consisting of denaturation at 94°C for 40 s, annealing at 52°C for 40 s and extension at 72°C for

45 s with a final 12 min extension at 72°C. The PCR products were analysed by electrophoresis on a 1.6% agarose gel followed by visualized under UV light, photographed and documented gel doc unit.

Based on the observations made from all the isolates and clusters of isolates definite keys were be prepared for different variants of *Ganoderma* by developing similar and dissimilar characters.

In vitro* assay of fungicides and biocontrol agents against *Ganoderma lucidum

Six systemic fungicides *viz.*, Calixin (Tridemorph 80 EC), Bavistin (Carbendazim 50 WP), Hexaconazole (Contaf 5 EC), Sprint (Carbendizim 25 WS +Mancozeb 50% WS), Tebuconazole (Raxil 25 DS), Vitavax power (Thiram 37.5 + Carboxin 37.5 WP) at 0.05, 0.075, 0.1% concentration and six non-systemic Captaf (Captan 50WP), Dithane M-45 (Mancozeb 75 WP), Blitox-50 (Copper Oxychloride 50WP), Thiram (Thiram 75 WP) and Zineb (Indofil Z- 78 WP) @ at 0.1, 0.15 and 0.2% were tested for their efficacy against the pathogen on PDA medium by poisoned food technique[5]. The fungicides in the required concentration were incorporated into the medium and the plates were inoculated with five mm disc of *G. lucidum* in three replications. Radial growth were recorded and compared with the growth in control plates. Similarly *Bacillus subtilis, Pseudomonas fluorescens, Paecilomyces lilacinus, Trichoderma harzianum, T. koningii, T. viride, T. virens* were screened against *G. lucidum* using dual plate techinique. Both the pathogen, *G. lucidum* and the antagonistic fungi were grown on PDA medium. Petri plates inoculated only with *G. lucidum* served as control. Each experiment was replicated three times. Observation on mycelia growth of the pathogen was recorded upto 15 days of incubation. The inhibition per cent was calculated[6].

$$I = \frac{(C - T)}{C} \times 100$$

Where,
I = Per cent inhibition over control
C = Radial growth in control
T = Radial growth in treatment (fungicide/bioagent)

RESULTS AND DISCUSSION

Iodine Potassium Iodide staining technique

Out of twelve samples only six samples 1, 3, 5, 8, 10, 11 gave positive results. After examining the bark tissues under the microscope the tissues were stained brown colour. It was observed that healthy tissues were stained light brown where as infected tissues stained dark brown. In the colorimetric method (KOH test), the difference between healthy and diseased tissues were examined by recording the optical density values at 425nm. There was a significant difference in O. D. values of stem and root tissues which ranged from 0.410 to 0.420 in root tissues, 0.480 to 0.486 in stem tissues. O. D. values ranging from 1.230 to 1.236 and 1.160 to 1.176 were obtained in diseased root and stem tissues, respectively.

EDTA test

Leaf and root tissues were used to see the difference in O. D. values at 400nm. In leaf and root tissues the O. D. value was ranged from 0.840 to 0.845 and 1.980 to 1.996 in healthy and diseased tissues, respectively.

Table 1. Validation of different tests for early detection of samples for diseased conditions

Table 1a. Iodine potassium iodide Staining technique

Isolate	No. of samples tested	No of samples which gave positive reaction	Reaction	
			Healthy	Diseased
SMG-1	12	1	-	Dark brown
		3	Light brown	Dark brown
		5	-	Dark brown
		8	Light brown	Dark brown
		10	-	Dark brown
		11	-	Dark brown

Table 1b. Biochemical methods

SMG-I isolate	Number of samples tested	Colorimetric method O. D. value at 425nm		EDTA- Test O. D. value at 400nm		Orthophenopthlene test O. D. value at 570nm		Alkaline copper test Concentration (%)					
		Root	Stem/Bark	Leaf	Root	Leaf	Root	1	2	3 Root	4	5	Untreated
Healthy	2	0.410-0.420	0.480-0.486	0.840-0.845	0.210-0.214	0.165-0.175	0.100-0.104	Slight green	Slight green	Slight green	Light green	Light green	Light brown
Diseased	12	1.230-1.236	1.160-1.176	1.980-1.996	1.420-1.428	0.312-0.317	0.180-0.186	Light brown mix light green	Light brown mix light green	Brown mix green	Brown mix green	Brown mix dark green	Brown

Table 2. Evaluation of fungicides against *Ganoderma lucidum*

Non-systemic fungicides	Inhibition (%) Concentration (%) 0.1	0.2	0.3	Mean	Systemic fungicides	Inhibition (%) Concentration (%) 0.05	0.075	0.1	Mean
Captan	100.00 (90.05)*	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)	Hexaconazole	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)
Copper oxychloride	77.17 (61.40)	82.59 (65.38)	88.52 (70.23)	82.72 (65.67)	Trideomarph	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)
Kavach	66.29 (54.54)	73.33 (58.94)	83.70 (66.23)	74.44 (59.90)	Carbendazim	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)	100.00 (90.05)
Mancozeb	85.56 (67.70)	93.52 (75.36)	100.00 (90.05)	93.02 (77.70)	Bavistin	71.85 (57.99)	74.07 (59.42)	85.93 (68.01)	77.28 (61.80)
Thiram	92.31 (73.95)	94.91 (77.01)	100.00 (90.05)	95.74 (80.34)	Tebuconazole	58.52 (49.93)	61.30 (51.55)	84.81 (67.10)	68.21 (56.19)
Zineb	31.48 (34.14)	41.85 (40.33)	42.59 (40.76)	38.64 (38.41)	Vitavax power	88.15 (69.90)	88.15 (69.90)	88.52 (70.23)	88.27 (70.01)
	F	C	FxC				F	C	FxC
S.Em	0.7	0.99	0.41		S.Em		0.85	1.21	0.49
CD @ 1%	2.1	3.56	1.25		CD @ 1%		3.21	4.21	1.47

*Arc sin transformed values

Orthophenopthlene test

O. D. values were recorded at 570nm from the leaf and root tissues which are ranged from 0.165 to 0.175, 0.100 to 0.104 and 0.312 to 0.317, 0.180 to 0.186 in healthy and diseased tissues of leaf and root portions, respectively.

Alkaline copper test

Root tissues were subjected for five different concentrations of 1, 2, 3, 4 and 5 per cent and difference in colour changes was recorded. Change in colour difference was observed in both healthy and diseased tissues and the colour intensity varied with increasing concentrations compared to untreated tissues. In healthy tissue, colour intensity varied from slight green to light green compared to untreated sample where intensity was light brown colour. Higher colour intensity was observed at 5 per cent concentration where in alkaline copper sulphate solution treatment showed more colour intensity in diseased tissues (brown mix dark green) than the healthy tissues (pure brown colour).

PCR techniques

For early detection of disease, the apparently healthy looking root samples from infected palms were collected cultured and DNA was isolated from them. All the collected *Ganoderma* isolates showed the amplifications with both the primers. Gan1, Gan2 produced a 167 bp DNA fragment and GanET ITS-3 produced a 320 bp DNA fragment. Other workers[3] suggested similar things as noticed in the present study to detect the presence of *Ganoderma*. Detection of *Ganoderma* at early stages of infection and

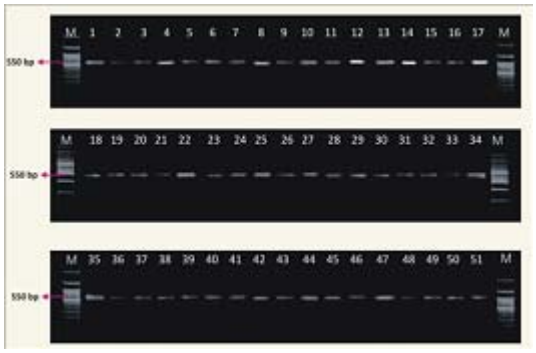

Fig. 1. ITS1 and ITS4 region amplification of *Ganoderma* samples (Lane 1-51, 51 isolates). Lanes M 100bp at both the sides

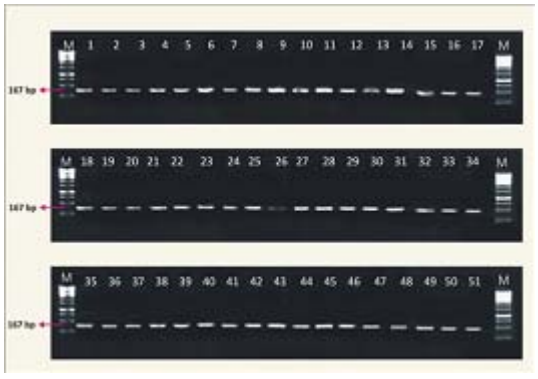

Fig. 2. Specific amplification of *Ganoderma* samples using Gan1 and Gan2 primers (Lane 1-51, 51 isolates). Lanes M 100bp at both the sides

Table 3. Evaluation of bioagents against *Ganoderma lucidum*

Sl. No.	Treatments	Inhibition (%)
1	*Trichoderma harzianum*	100.00 (90.05)*
2	*T. koningii*	100.00 (90.05)
3	*T. virens*	94.44 (76.41)
4	*T. viride*	64.44 (53.43)
5	*Pseudomonas fluorescens*	61.85 (51.88)
6	*Bacillus subtillis*	58.33 (49.82)
7	*Paecilomyces lilacinus*	59.07 (50.25)
	Mean	76.88 (65.98)
	S.Em±	0.12
	CD at 1%	0.45

*Arc sin transformed values

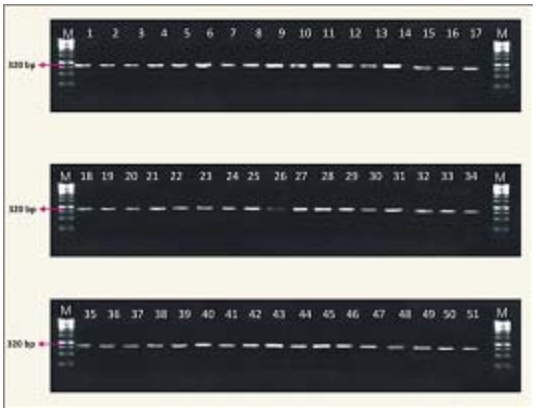

Fig. 3. Specific amplification of *Ganoderma* samples using GanET and ITS3 primers (Lane 1-51, 51 isolates). Lanes M 100bp at both the sides

managing the disease by using various treatments; can be monitored by PCR technology[7]. Both infected roots and *Ganoderma* isolates showed 167 bp amplification, which confirms the presence of *Ganoderma*. ITS1 region of *Ganoderma* is flanked by highly conserved sequences and no variation was noticed from generated amplificate within the other pathogenic *Ganoderma* species surveyed[8]. The present molecular study on *Ganoderma* also has similar kind of observations.

However, in the present investigation leaf, stem /bark and root samples showed similar trend, i.e. increase in optical density in the diseased tissues when compared to corresponding healthy tissues. These early diagnostic techniques give a definite indication on the usefulness of these methods, studies with large number of samples showing different disease intensity and palm with suspected symptoms and palms without symptoms is necessary before any one of these tests employed on a field scale[8]. It is also possible by using PCR techniques for early detection of the incidence of *Ganoderma* root rot in plantations. The transmittance values of diseased spindle leaf extract decreased while that of diseased root extract increased when compared to the extracts from corresponding healthy tissues.

Disease management

In vitro* evaluation of fungicides against *Ganoderma lucidum

In vitro evaluation of new molecules of fungicides is very much necessary before they are tried under field conditions. Among the non-systemic fungicides which are commonly used for other disease management are captan, mancozeb and thiram which at 0.2 per cent concentration showed cent per cent inhibition of mycelial growth of fungus followed by copper oxychloride and the least inhibition was recorded in Zineb with 0.1 per cent. Drenching of 0.4% Copper oxychloride at the rate of 15lit/tree are effective to combat basal stem rot of coconut (Table 2). In the present investigations options are more to use either Copper oxychloride or other fungicides[9].

Among the systemic fungicides, tridemorph, hexaconazole and carbendazim showed cent per cent inhibition of mycelial growth of all *Ganoderma* isolates fungus and was followed by vitavax power and raxil at 0.1 per cent concentration while, least per cent inhibition of mycelial growth was recorded in sprint (Table 2). Similarly[10] least mycelial inhibition of *Ganoderma applantum* with copper oxy chloride (0.3%), bordeaux mixture (1%), calixin (0.1%) and hexaconazole (0.1%); root feeding and soil drenching of calixin and hexaconazole were more effective and also some other fungicides like propiconazole, tridemefon, cyproconazole and penconazole were effective against basal stem rot disease in plantation crops[11]. There are no such studies in arecanut root rot. The effectiveness of the triazole fungicides like hexaconazole may be attributed to their interferance with the biosynthesis of fungal sterols and inhibit the ergosterol biosynthesis, which is essential to the structure of cell wall and its absence cause irreparable damage to cell wall leading to death of fungal cell. A similar study was reported for the effectiveness of triazoles, which inhibit the sterol biosynthesis pathway in several fungi. At higher concentration most of the fungicides inhibited maximum mycelial growth but decreased with reduced concentration. Effectiveness of systemic and non systemic fungicides and combi products suggest their practical use alternatively one after the other to delay or reduce fungicidal resistance.

In vitro* evaluation of bioagents against *Ganoderma lucidum

Under biological control of plant diseases, various antagonistic organisms have been identified, which fight against the pathogens by different mechanisms viz., competition, lysis, antibiosis, siderophore production and hyper parasitism[12]. Among the bioagents tried *Trichoderma harzianum* and *T. koningii* was the best in inhibiting mycelial growth (100%) of many *Ganoderma* isolates followed by *B. subtillis*, *P. lilacinus* and the least per cent inhibition of mycelial growth was observed in *Pseudomonas fluorescens* (Table 3). *Trichoderma* sp. is the most effective antagonist, to inhibit the *Ganoderma lucidum*[13].

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