Research Article

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Bioefficacy of certain chemical and biofungicides against *Hypoxylon* spp. causing wood rot disease in tea

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Abstract

Wood rot disease caused by *Hypoxylon serpens* is the most widespread and serious stem disease in tea. Among the 350 bacterial and 35 fungal biocontrol isolates collected from several tea growing regions of southern India, three bacterial isolates produced higher antagonistic potential against this fungal pathogen. Two of the efficient strains were identified as *Bacillus* sp. (HBCWR-3 and WR46-2) and third one was *Pseudomonas* sp. (WR5-4). In case of fungal biocontrol agents, the type culture *Trichoderma viride* procured from Microbial Type Culture Collection (MTCC) performed better in controlling the pathogen over *T. harzianum.* Five systemic fungicides, hexaconazole, carbendazim, tebuconazole, tridemorph, benomyl and a contact fungicide, copper oxychloride were evaluated for studying their bioefficacy against wood rot pathogen. In this study, benomyl 50% WP or copper oxychloride at the lowest concentration (0.01%) completely inhibited the growth of the fungus *in vitro*. Moreover, bioefficacy of certain plant aqueous extracts of *Azadirachta indica,* acetone extracts of *Pongamia pinnata*, *Cinnamom, Artemisia nilagirica*, *Lantana camera, Ageratum conyzoides* and a bryophyte, *Heteroscyphus argutus* were also studied against *H. serpens*. Among them, *A. nilagirica* followed by *H. argutus* and *A. indica* were effective in controlling the wood rot pathogen. In the case of liquid biofungicides tested, 'Expel' controlled the tea pathogen efficiently. The present study revealed that, chemical fungicide (Benomyl or copper oxychloride at 0.01%), botanical extracts at 10% (*A. nilagirica*, *H. argutus*, *Azadirachta* and 'Expel') and biocontrol agents (*Bacillus* sp., *Pseudomonas* sp. and *T. viride*) were effective in controlling wood rot pathogen under *in vitro* condition.

Keywords: Biocontrol agents, biofungicides, fungicides, *Hypoxylon* spp., tea, wood rot

Introduction

Wood rot disease in tea, which has been reported from several countries including India, Kenya, Malawi, Zimbabwe and Sri Lanka (Arulpragasain and Balasuriya, 1991). Wood rot is the second most important tea disease in Kenya and it has become the most widespread and serious stem disease in tea crop. In Sri Lanka, the natural field incidence of wood rot was around 60-90 per cent and over 90 per cent could bring about 27 per cent and 36 per cent yield reductions respectively, showing that the disease can cause significant crop loss to the tea industry (Balasuriya and Adikaram, 1998). Presently, wood rot is noticed in several tea estates of southern India. The symptoms of wood rot normally appear when the bushes are getting old. Consequently, the foliages from the affected branch show wilting and scorching followed by black fructification of wood rot fungi (stroma). The pathogen spreads mainly through pruning knives and the infection progress from pruning cuts during wet weather. This fungus is able to multiply rapidly and spread over the main stem that causes death of the tea bushes. Large areas of the tea plantation in southern India are affected by this disease, because the pathogen is soil borne and has fast spreading ability. No concrete control measures have been developed against wood rot disease in tea. Research conducted during recent years focused on the selective use of botanical fungicides, as they are safer and eco friendly. Plant extracts have been known for their antifungal properties for many years

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in agricultural sector. Apart from chemical fungicides, certain plant extracts were also used as alternatives in controlling many fungal diseases. Generally, the plant diseases are effectively controlled by bacterial and fungal antagonists. *Trichoderma* and *Pseudomonas* group of colonies showed higher antagonism against wood rot and grey blight pathogens in tea *in vitro* (Vidhya Pallavi *et al.*, 2010). Evaluation of chemical, biological and botanical fungicides will be helpful to develop an appropriate control strategy as integrated schedule for wood rot disease control. In the present study, attempts were made to identify efficient antagonistic microorganisms present in the tea ecosystem and chemical and botanical control measures of the wood rot disease were critically assessed under *in vitro* condition.

Materials and methods

Isolation of wood rot pathogen

Field survey was conducted in the tea growing areas of southern India *i.e.,* Anamallais and Nilgiris (in Tamil Nadu), High Range, Central Travancore and Wayanad (in Kerala) and Koppa (in Karnataka) for the isolation of wood rot pathogen from the infected specimens. The diseased plant parts were collected and washed in tap water and then dried by placing them between folded filter papers. Isolation of the pathogen was carried out *in vitro* using water agar medium. The mycelial stage of the sporulating fungi was purified by single spore isolation method. The fungus was maintained in potato dextrose agar (PDA) slants at 4 ^{o}C and revived at monthly interval.

Molecular identification of wood rot pathogen

The molecular identification of indigenous wood rot pathogen (AHWRST-5, AHPM-4 and WHCS) was carried out using molecular tool. The method employed was DNA isolation, PCR amplification, sequencing and phylogenetic analysis. PCR was performed with primer pairs targeted to the 28s rRNA gene. The PCR positive samples were identified by DNA sequencing of the internal transcribed spacer (ITS) region of the rRNA gene. The sequences were then analysed using basic local alignment search tool (BLAST) software available in NCBI (http://www.ncbi.nlm.nih.gov/blast). Three isolates of wood rot pathogen were sequenced and deposited in NCBI (US) and EMBL (Japan) repository.

Isolation of bio-control agents

Rhizosphere soil samples at 0-9 inch depth were collected from different tea growing districts, Anamallais, The Nilgiris, Central Travancore, High Range, Wayanad and Koppa for isolation of bio-control agents (*Bacillus* sp., *Pseudomonas* sp. and *Trichoderma* sp.) which could be region specific and native isolates. The biocontrol agents were isolated by standard serial dilution plating techniques, sub-cultured, brought to pure culture and stored in agar slants at 4 ^{o}C for further studies. Isolated strains were identified using standard bacteriological biochemical and molecular techniques. In case of fungal biocontrol agent *T. viride* used in the present study was obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh – 160036, India.

Screening of biocontrol agents for antagonism

The bacterial and fungal isolates were screened for their antagonistic potential against *Hypoxylon* spp. following dual culture technique. Antagonistic potential of bacterial strains was ensured based on the inhibition zone formed around the bacterial colonies by inhibiting the pathogen's growth. In the case of *Trichoderma* spp. *in vitro* screening was carried out by placing a mycelial plug of 4 days old culture of both pathogen and the antagonist. The time for the first contact between antagonist and pathogen, and the advancement of the antagonist on the pathogen colony was noted and the efficient strains were short listed. An untreated control plate was maintained for comparison. After screening, proven biocontrol agents were confirmed through molecular identification; the sequences were submitted to NCBI and published through EMBL.

Evaluation of chemical fungicides

Four systemic fungicides (Hexaconazole 0.01%, Tebuconazole 0.05%, Tridemorph 0.5, and Benomyl 0.1%) and a contact fungicide (copper oxychloride 50WP 1.0%) were selected and screened against wood rot fungal pathogen (*Hypoxylon* spp. and *Hypoxylon serpens*) *in vitro* using food poisoning technique. Suspensions of

Table 1. Source of collection and identification of wood rot pathogen isolates

Strain code	Source	NCBIAccession Number	Identified as
AHWRST-5	Stanmore Estate, Anamallais	JQ063454	*Hypoxylon serpens*
AHPM-4	Pannimade Estate, Anamallais	JQ063453	*Hypoxylon* sp.
WHCS	Chulika Solamalies, Wayanad	JQ362418	*Hypoxylon* sp.

fungicide were prepared in sterile distilled water and appropriate quantity of the stock solutions were mixed with molten, cooled PDA medium, so as to obtain the required concentration, and dispensed uniformly into Petri plates. Then inoculum of the pathogen were cut using sterile cork borer (7 mm) and inoculated into the solid media. A control plate devoid of the chemical fungicide was taken for comparison. The growth of the pathogen was measured at after 10 days of incubation.

Evaluation of plant extracts

Plant aqueous extracts of *Azardirachta indica,* acetone extracts of *Pongamia pinnata*, *Cinnamom, Artemisia nilagirica*, *Lantana camera, Ageratum conyzoides* and a bryophyte, *Heteroscyphus argutus* were freshly prepared using sterile distilled water and acetone and made upto 20 per cent concentration. The aqueous and acetone extract of respective plants were filtered with sterile membrane filter unit and was used for conducting the bio-efficacy studies. The PDB medium was mixed with respective botanical preparation at 10% individually. The fungal pathogen (*Hypoxylon* sp.) disc was transferred to PDB medium and incubated at room temperature. After 10 days of incubation, fungal mycelial mat was filtered with help of Whatman No. 1 filter paper and the dry weight of fungal mycelium was recorded. Also the inhibition of radial growth was measured along with control plate according to Sunder *et al*., 1995 using the formula

$$\text{Per cent inhibition (PI) \%} = ((X - Y)/X) \times 100$$

Where, X is fungal growth in control plate and Y is growth in treated plate.

Effect of bio-fungicides against wood rot pathogen

The commercial liquid bio-fungicides 'Expel' (combination of Canolar extract and Tea tree oil) was procured from Advance pesticides Pvt. Ltd, Nashik, Maharashtra, India and Disrupt (*Stemona sessilifolia* plant extract) was obtained from Gassin Pierre Pvt Ltd, Kolkata. The respective bio-fungicides were tested against wood rot pathogen at various concentrations *viz*, 1000, 2000, 3000, 4000 and 5000 ppm using food poisoning technique *in vitro.* After 10 days of incubation, growth of fungal mycelium was recorded.

Results and discussion

The colony and spore morphology of the isolates were studied on PDA medium and compared with *H. serpens* (MTCC type strain 2338). The identification of three isolates of wood rot pathogen was confirmed as *H. serpens* (AHWRST-5) collected from Stanmore estate, *Hypoxylon* spp. (AHPM-4) from Pannimadu estate of Anamallais and *Hypoxylon* spp. (WHCS) from Chulika Solamalies of Wayanad respectively

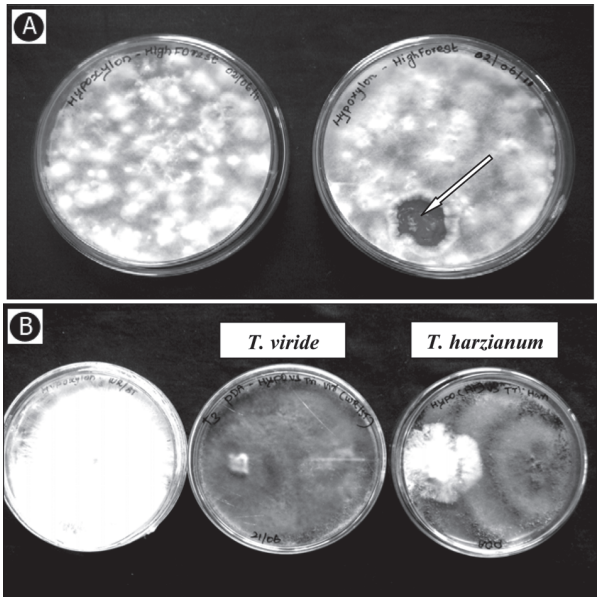

Fig. 1. Inhibition pattern of bacterial and fungal bio-control agents on *Hypoxylon* spp. (A) Pseudomonas sp. inhibiting the growth of *Hypoxylon* spp. (B) *T. viride* and *T. harzianum* inhibiting the growth of *Hypoxylon* spp.

(Table 1) on the basis of molecular identification (5.8S ribosomal RNA gene). The sequences were later submitted to NCBI. These *Hypoxylon* spp. were partially sequenced and are available in public domain as Taxon ID 301117 and EMBL Bank ID JQ063454 for *H. serpens*, Taxon ID 1131860 and EMBL Bank ID JQ063453 and JQ362418 for *Hypoxylon* sp.

In this study, a total of 300 bacterial strains were isolated from different agro climatic zones of southern India. Among them, 12 strains were efficiently antagonistic against wood rot pathogen. Five strains of *Pseudomonas* sp. and five strains of *Bacillus* sp. showed higher antagonistic potential against this pathogen. The antagonistic potential of the bacterial colonies was graded according to the

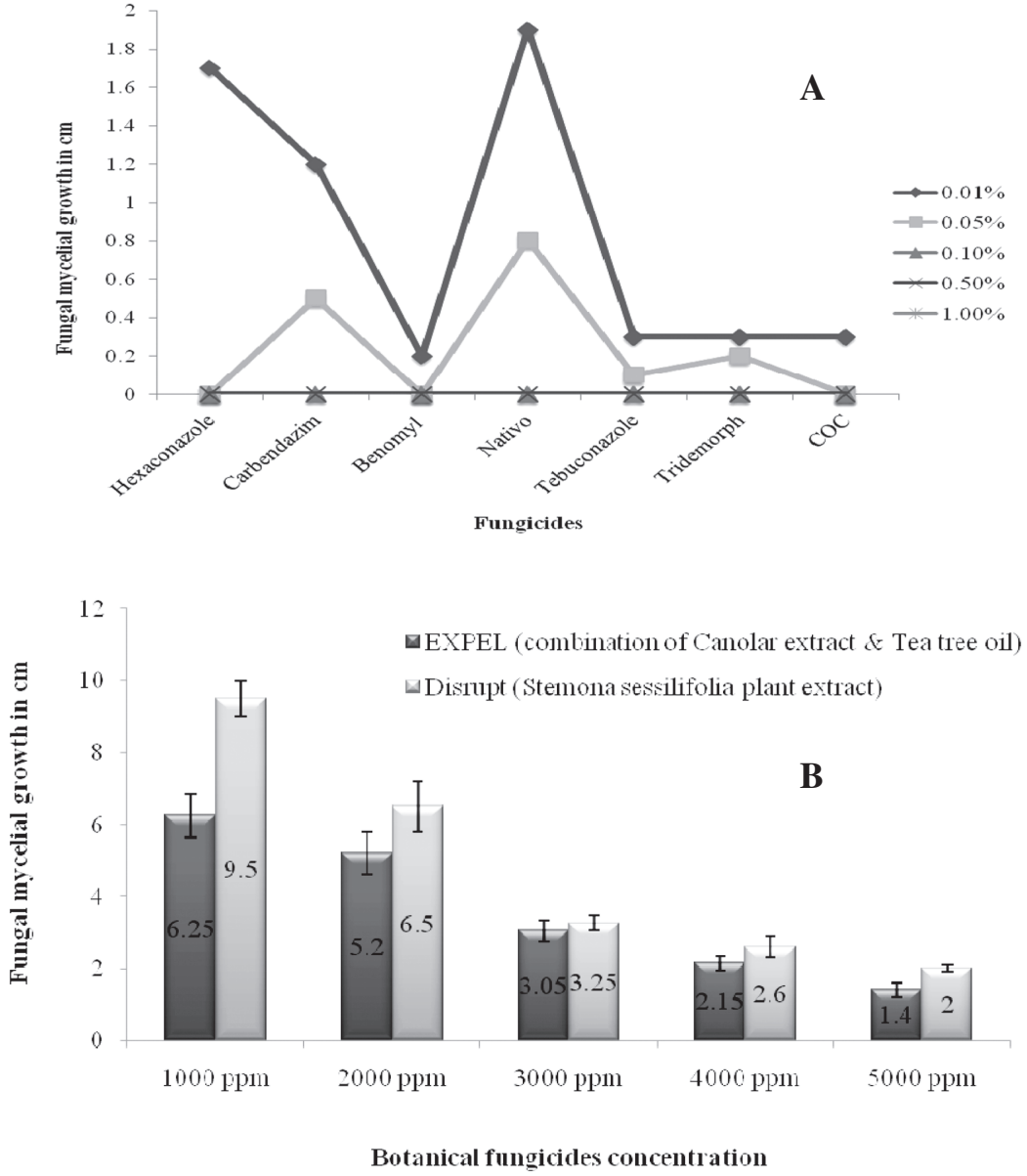

Fig. 2. Screening of (A) chemical and (B) bio-fungicides against wood rot pathogen under *in vitro* condition

Table 2. Source of collection of bio-control agents screening agents against *Hypoxylon* spp. under *in vitro* condition

Tea districts	Number of bacterial isolates	Number of *Trichoderma* isolates	Inhibition zone produced by antagonists	
			Bacterial antagonists	Fungal antagonists
Anamallais	10	5	1(1-2 cm)	0
Central Travancore	100	10	1 (<1 cm)	-
High Range	50	5	2	1
Wayanad	50	5	1	-
The Nilgiris	25	5	1	1
Koppa	25	5	0	1
Total	350	35	0	3

Values in parenthesis indicate diameter of inhibition zone produced by bacterial antagonists.

Table 3. Molecular characterization of bio-control agents and screening against *Hypoxylon* spp.

Strain Number	Biocontrol agents	Isolated from	Gen Bank Accession Number (NCBI)	Percentage of inhibition(%)
HBCWR-3	*Bacillus* sp.	Tea plant, Wayanad, Kerala	JN616372	85
WR5-4	*Pseudomonas* sp.	Infected tea stem, Karnataka	JQ319656	80
WR46-2	*Bacillus* sp.	Soil, Anamalais, TN	JN616373	75
TVWR	*T. viride*	obtained from MTCC	-	80
THWR	*T. harzianum*	Soil, Anamalais, TN	-	45

diameter of the inhibition zone formed around the bacterial colonies (Fig. 1a). In the case of *Trichoderma* sp, of the total 35 strains isolated, 15 strains showed higher antagonistic potential against *Hypoxylon* sp. (Table 2). *T. viride* procured from MTCC showed maximum inhibition against wood rot pathogen when compared to the native isolates of *T. harzianum* (Fig. 1b). Similar findings were reported earlier by Ponmurugan and Baby (2003) where *T. harizianum* and *G. virens* suppressed the growth and reproduction of tea pathogens.

The percentage inhibition by five selected strains of *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* sp. against wood rot pathogen were calculated according to Vincent's formula. The percentage of inhibition was high for *Bacillus* sp. (85% and 75%), followed by *Pseudomonas* spp. (70%) and 80 per cent for *T. viride* against *Hypoxylon* spp. Efficient and specific biocontrol strains of bacteria and fungi against wood rot pathogen were identified and designated as WR46-2 & HBCWR-3 for *Bacillus* sp. and WR5-4 for *Pseudomonas* sp. The organisms were molecularly characterised using 16s ribosomal RNA gene, the sequences were published in NCBI (Table 3).

Selective surgery and protection of wounds by applying copper oxychloride paste was the common practice followed for the control of stem diseases in India (Premkumar *et al*., 2009). In an earlier study, benlate, bravocarb and kocide completely inhibited the growth of wood rot fungus *in vitro*. A field trial on controlling *H. serpens* revealed that, regular and surgical pruning were beneficial for the control of this

Table 4. Bioefficacy of botanical fungicides (10%) against *Hypoxylon* spp.

S. No.	Plant	Family	Growth inhibition (%)
1	*Azardirachta indica*	Meliaceae	50 ± 28.96^{c}
2	*Pongamia pinnata*	Fabaceae	30 ± 17.40^{de}
3	*Cinnamomum xanthocarpum*	Lauraceae	33 ± 19.09^{d}
4	*Artemisia nilagirica*	Astereceae	70 ± 40.43^{a}
5	*Lantana camara*	Verbenaceae	25 ± 14.58^{ef}
6	*Ageratum conyzoides*	Astereceae	20 ± 11.63^{f}
7	*Heteroscyphus argutus*	Bryophyte	60 ± 34.72^{b}
	CD at 5 %		7.4

Values are mean ± SE of 3 replication followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Table 5. Effect of chemical and bio-fungicides against *Hypoxylon* spp. under *in vitro*

Dosages	Bio-fungicides		Dosages	Chemical fungicides					
	Expel	Disrupt		Hexaconazole	Carbendazim	Benomyl	Tebuconazole	Tridemorph	Copper oxychloride
(ppm)	*Hypoxylon* spp. growth in cm		(%)	*Hypoxylon* spp. growth in cm					
1000	6.3±0.6	9.5±0.5	0.01	1.7 ± 0.2	1.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
2000	5.2±0.6	6.5±0.7	0.05	0.0 ± 0.0	0.5 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0
3000	3.1±0.3	3.3±0.2	0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4000	2.2±0.2	2.6±0.3	0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5000	1.4±0.2	2.0±0.1	1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

The values are mean ± S.E. in terms of *Hypoxylon* spp. growth in diameter (cm) of three replicates

disease in Kenya. The use of benomyl integration with surgical high prune was the best option for checking the spread of the disease (Onsando, 1986; Onsando and Langet, 1989). In the present study, the systemic fungicides, benomyl, tebuconazole, tridemorph and contact fungicide (copper oxychloride) at 0.05 per cent inhibited the growth of pathogen. Benomyl 50% WP even at the lowest concentration (0.01%) completely inhibited the growth of the fungus followed by copper oxychloride (Fig. 2).

Various plant extracts and efficient biocontrol agents are being involved to minimize the disease pressure. In the present study, aqueous extracts of *Azadirachta indica,* acetone extract of *Artemisia nilagirica*, *Heteroscyphus argutus*, *Lantana camara* and *Pongamia pinnata* plants and biofungicide (Expel) were tested against *H. serpens in vitro*. Results revealed that 10 per cent extract of *A. nilagirica* showed 70 per cent growth inhibition of *H. serpens.* Leaf powder of *A. nilagirica* even at the lower dose (5.1%) retarded the growth of wood rot fungus followed by *H. argutus* (60%), *A.indica* (50%), *L. camara* (45%) and *P. pinnata* (40%). The leaf extracts of *C. xanthocarpum* (33%) and *A. conyzoides* at 10% were ineffective against the wood rot pathogen (Table 4). Kordali *et al.,* (2005) reported that *Artemisia* spp. showed antifungal and antioxidant activities. Similar trend was noted when using *A. indica* against *H. serpens* pathogen *in vitro.* The present study noted that, *A. nilagirica* extract was effective at all concentrations (0.2, 0.4, 0.6, 0.8 and 1.0%) in controlling spore production and spore germination. A commercial liquid bio-fungicide (Expel) was also found effective at all concentrations against *Hypoxylon* spp. *in vitro* (Table 5 & Fig. 2).

Conclusion

From this study, it has been concluded that *Bacillus* spp. *Pseudomonas* spp. and *Trichoderma viride* (MTCC) exhibited superior antagonistic potential against wood rot pathogen in tea. The chemical fungicides benomyl 50WP, copper oxychloride, a botanical fungicide 'Expel' and extracts of *A. nilagirica, H. argutus*, *A. indica* provided good control against *Hypoxylon* spp.

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