Research Article

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Isolation and identification of bacterial strains from different tea growing areas against *Macrophoma* sp. in southern India tea plantation

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Abstract

Branch canker is an important secondary stem disease in tea caused by *Macrophoma theicola*. Three different *Macrophoma* spp. were identified from three tea growing regions of southern India and their identity was confirmed by 18S rRNA method. Bacterial biocontrol isolated from four different locations of south India were also identified through 16S rRNA method. The bacterial strains were identified and evaluated for their antagonistic potential for the control of branch canker. Dual plate technique revealed that among the four bacterial strains, *Bacillus amyloliquefaciens* (78.9±0.4) showed maximum level of antagonism against all the three strains of *Macrophoma* spp. followed by *B. subtilis* (72.9±0.6) and *B. licheniformis* (64.2±0.7). In culture filtrate studies also, *B. amyloliquefaciens* (80.2) was found to possess highest antagonistic activity against the test pathogen, followed by *B. subtilis* (74.8) and *Pseudomonas fluorescens* (69.0). Among these, one potent strain of *B. amyloliquefaciens* was taken for GC-MS study and their bioactive compounds were identified as 1H-indene, 1-methylene, 3-hexadecene (Z), benzene acetic acid, phenol, and caffeine. These compounds could be responsible for antifungal activity against branch canker pathogen.

Keywords: Antagonistic potential, bacterial strain, GC-MS, *Macrophoma* spp., nucleotide sequences

Introduction

India produces about 1,239 million kg of tea from 5,79,353 ha and in South India 221 million kg tea is grown in 1,19,740 ha in the Western Ghats (Anonymous, 2016). All parts of the tea bush *viz*., foliage, stem and roots are susceptible to diseases. Around 10 to 15 per cent of crop loss is due to diseases and pests (Chen and Chen, 1990). Stem diseases like branch canker (*Macrophoma theicola*), wood rot (*Hypoxylon serpens*), collar canker (*Phomopsis theae*) and thorny stem blight (*Tunstallia aculeata*) are predominant in southern India (Agnihothrudu, 1964). Among the leaf diseases, grey blight, blister blight and brown blight are common in south India. Stem diseases are the reason for stagnate crop production and sometimes kill the tea bushes (Arulpragasam, 1992). Pruning operation carried out in tea bushes once in four years increases the risk of stem diseases since it exposes the wood tissues to saprophytic and parasitic fungi. The fungal pathogen can easily enter through prune cuts or tissues damaged by sun-scorch. The pruning cuts also provide an ideal surface for germination of spores (Oteino, 1997). In Taiwan, it has been observed that *M. theicola* cause twig die-back disease in mature tea plant (Arulpragasam, 1992). *Macrophoma* sp. fructifications occur in large numbers all along the dead branches and cause very minute cracks on the surface of the bark (Shanmuganathan and Bopearatchy, 1972). Vidhyapallavi *et al.* (2011) reported that *Pseudomonas* sp. showed highest antagonism against the pathogen causing grey blight disease, *Pestolatiopsis* sp., under *in vitro* conditions. Similarly *Trichoderma* sp. also has

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very high antagonistic activity against *Hypoxylon serpens* which causes wood rot disease in tea plant (Nepolean *et al.,* 2014). Many microorganisms have been reported as having antagonistic potential activity against the plant pathogens (Lemanceau and Alabouvette, 1993). The objectives of the present study were to isolate, characterize and to examine potential biocontrol agents against branch canker pathogen *Macrophoma* spp.

Materials and methods

Isolation and identification of pathogen

The canker infected tea wood specimens were collected from different tea growing regional centers of UPASI Tea Research Foundation Experimental Farm (Valparai), UPASI Tea Research Foundation Regional Center located in Koppa (Karnataka) and Vandiperiar (Kerala). The study was conducted in UPASI Tea Research Institute, Valparai, Tamil Nadu, India. The branch canker pathogens were identified based on the spore morphology and culture characters as outlined by Petch (1923) and Barnett and Hunter (1998). Further, identification of the three different strains of *Macrophoma theicola* isolated from the stem specimens were confirmed through genomic DNA amplification by18S rRNA sequence analysis (Toju *et al*., 2012). The three branch canker pathogen strains were given the code name as NBCC2-7, VPM and UPA-62. These nucleotide sequence data were deposited to NCBI Gene Bank nucleotide databases (Accession number JQ362417 for NBCC2-7, KP004441 for VPM and KP179221 for UPA-62).

Isolation and identification of bacterial strains

The soil samples were collected from different tea growing regions of southern India. Samples were serially diluted and plated on nutrient agar (MacFaddin, 2000) and King's B medium (King *et al*., 1954). Four different bacterial isolates were identified using various standard biochemical tests (oxidase, catalase, gram staining and methyl red) described by Bergey's Manual of systematic Bacteriology (Claus and Berkeley, 1986; Stolp and Gadkari, 1981). The phenotypic characters were identified by using the methods described by Gordon *et al.* (1973). The isolation of genomic DNA and PCR amplification of 16S rRNA gene were carried out as per the method described by Marchesi *et al.* (1998).

Bacterial nucleotide accession numbers

The four bacterial strains were given the code named and their sequence data determined and deposited in the NCBI gene bank data base. The NCBI accession numbers given to these strains are KM853034 for WP1O4 (*Bacillus amyloliquefaciens*), KM527836 for CS2 (*B. subtilis*), KM527837 for TRB (*Pseudomonas fluorescens*) and KM527838 for AWRH-40B (*B. licheniformis*).

Phylogenetic analysis

The sequences of 16S rRNA genes were compared with the sequences available from GenBank using the BLASTN programme and were aligned using CLUSTAL W software (Thompson *et al.,* 1994). Distances were calculated based on Kimura's two-parameter correction (Kimura, 1980). Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987).

Dual plate technique

The branch canker pathogen inhibition capacity of bacterial biocontrol strains were determined following the method out lined by Huang and Hoes (1976). The 6 mm disc of seven days old culture of pathogen was placed at the centre of petri plate containing PDA medium. The bacterial strains were streaked at two opposing sides and their plates were incubated for six days at 25 to 30 ºC. The growth diameter was measured and compared with control growth. Bacterial strains were replaced by sterile nutrient broth medium. The antagonistic activity was measured using Bell' scale method (Bell, 1982). The results were expressed as the mean of the percentage of inhibitions according to the formula, PI = C-T/C×100, where, C is fungal radial growth of the pathogen in control and T, radial growth of the pathogen and bacterial strain.

Effect of cell free culture filtrates

The bacterial culture was studied by Dennis and Webster method (1971). The bacterial strain was inoculated in 100 mL sterile nutrient broth in 250 mL conical flasks on rotatory shaker and incubated

at 25 to 30 ºC. The filtrate was centrifuged for 15 min at 10,000 rpm. The supernatant was filtered using by Millipore membrane filter paper (0.22 µm). The bacterial culture filtrate was added to 100 mL molten nutrient agar (NA) to obtain final concentration of 4 per cent. The culture was poured into Petri plates (20 mL plate^{-1}) and inoculated with 6 mm disc of test pathogen. NA plates were inoculated with test pathogen and mixed with sterile distilled water to serve as control. The bacterial cultures of different durations, *i.e.*, 24, 48 and 72 h and their inoculated plates were incubated for six days at 25 to 30 ºC. The percentage of inhibitions was calculated by the above formula.

GC-MS analysis in bacterial culture

Bacterial culture supernatant was collected after centrifugation in 125 mL separating funnel and extracted with 50 mL of ethyl acetate. After partition, the extract was evaporated to near dryness at 60-65 ºC using rotary vacuum evaporator. The final residue was analyzed in GC-MS as per the following conditions: Column: HP-5MS (5% Dipheny l/95% Dimethyl poly siloxane), 30 × 0.25 mm × 0.25 µm df, Equipment: GC-7890A (Agilent), Carrier gas: 1mL/min-splitless, Detector: Mass detector - 5975C (Agilent), Software: Chem station, injection volume: 1µL (Mareeswaran, 2017)

Results and discussion

Identification of *Macrophoma* spp.

The branch canker pathogens isolated from the diseased stem specimens collected from three different tea growing areas of southern India were identified. Their spore morphology was characterized according to descriptions by Petch (1923). Based on the spore morphology, three different strains of *Macrophoma* spp. was identified and were code named as NBCC-2, VPM and UPA-62 (Table 1). The wood rot pathogens (AHWRST-5, AHPM-4 and WHCS) were isolated from different tea growing areas and identified by using molecular tools.

Biochemical characterization of bacterial strains

The bacterial strains isolated from the soils collected from three different tea growing regions were characterized morphologically and biochemically as per procedure given in Bergey's Manual of systematic Bacteriology (Claus and Berkeley, 1986; Stolp and Gadkari, 1981). The morphological and biochemical characterization of these bacterial strains revealed the presence of three *Bacillus* spp. and one *Pseudomonas* sp. The three *Bacillus* spp. were grown on nutrient agar at 25 to 30 °C and were identified as gram positive when analyzed. The *Pseudomonas* sp. was grown on King's B medium at 25 to 30 °C and was identified as gram negative. All the *Bacillus* spp. strains and the single *Pseudomonas* sp. answered positive for catalase and oxidase. Methyl red test showed positive for the three *Bacillus* spp. and negative for *Pseudomonas* sp. (Table 2). These results are in conformity with the findings of Kuberan *et al.* (2014).

Molecular accession number of bacterial strains

Molecular identification of the three *Bacillus* spp. and the single *Pseudomonas* sp. were confirmed as *B. subtilis* (KM527836), *B. licheniformis* (KM527838), *B. amyloliquefaciens* (KM853034) and *Pseudomonas fluorescens* (KM527837) through 16S rRNA sequence technique (Table 3). The different bacterial strains such as *Bacillus* sp. (HBCWR-3), *Pseudomonas* sp. (WR5-4) and *Bacillus* sp. (WR46-2) were identified by using 16S rRNA

Table 2. The biochemical characterization of bacterial strains

Test	*Bacillus* spp. (3 strains)	*Pseudomonas* sp. (1 strain)
Gram's stains	+	-
Catalase	+	+
Oxidase	+	+
Methyl red	+	-

(+) Positive, (-) Negative

Table 1. Isolation of branch canker pathogen from different tea growing areas of southern India

Strain code no.	Tea growing areas	Branch canker pathogen identified as	Gen Bank accession number
NBCC2-7	Koppa	*Macrophoma* sp.	JQ362417
VPM	Vandiperiyar	*Macrophoma* sp.	KP004441
UPA-62	UPASI-TRF, Farm	*Macrophoma theicola*	KP179222

Table 3. Isolation of bacterial strains from different tea growing areas of southern India

Strain code no.	Tea areas	Bacterial strains identified as	Gen Bank accession number
TRB	Koppa	*Pseudomonas fluorescens*	KM527837
AWRH-40B	Wayanad	*Bacillus licheniformis*	KM527838
WP-104	UPASI-TRF, Farm	*Bacillus amyloliquefaciens*	KM853034
CS-2	Coonnoor	*Bacillus subtilis*	KM527836

gene and the sequences were submitted in NCBI (Nepolean *et al*., 2014).

Phylogenetic tree analysis

An identification of bacterial nucleotide sequence was carried out by similarity search with the help of BLAST search method (Altschul *et al.*, 1990) and phylogenetic tree was constructed by using neighbor-joining method (Saitou and Nei, 1987). The bacterial nucleotide sequences data revealed 99% similarity with other bacterial nucleotide sequences of NCBI data bases (Fig 1).

B. amyloliquefaciens as a potential biocontrol agent has been proved by several authors (Yuan *et al*., 2012; Nam *et al*., 2009) against the pathogen of *Fusarium oxysporum f.* sp. *cubense.* In the present study, *B. subtilis* inhibited the growth of *Macrophoma* spp. by 70 to 72 per cent followed by *B. licheniformis* (62 to 64%). *Bacillus* spp are considered to be having biologically active compounds and their involvement in microbial disease were given by Asaka and Shoda (1996) and Emmert and Handelsman (1999). The antagonistic potential of the *B. licheniformis* strain

Fig. 1. Phylogenetic tree constructed based on neighbor-joining method

Effect of dual plate studies

In vitro screening of bacterial biocontrol strains against branch canker pathogen under dual plate technique showed that the *B. amyloliquefaciens* gave the maximum growth inhibition for all the three branch canker pathogens (Table 4). The percentage of inhibition of *B. amyloliquefaciens* (75 to 78%) was the highest followed by *B. subtilis* (70 to 72%), *B. licheniformis* (62 to 64%) and *Pseudomonas fluorescens* (31 to 36%). The efficiency of has also been proved by Kamil *et al.* (2007) against damping disease. Mitoi *et al*. (2012) reported the antagonistic potential of *B. licheniformis* against the pathogen *Alternaria alternata*. Though several workers have reported that *Pseudomonas* sp. gave superior antagonism against plant pathogens (Chakraborthy *et al.,* 1994), in the present study, *P. fluorescens* gave the lowest growth inhibition of 31 to 36 per cent against all the three *Macrophoma* spp. (Table 4).

Table 4. ***In vitro*** **screening of bacterial biocontrol against branch canker pathogen under dual plate technique**

Bacterial isolates	Accession Number (NCBI)	Growth inhibition (%)		
		Macrophoma sp. (JQ362417)	*Macrophoma* sp. (KP004441)	*Macrophoma theicola* (KP179222)
Bacillus amyloliquefaciens (WP104)	KM853034	78.9±0.4^{a}	76.8±0.9^{a}	75.5±0.9^{a}
Bacillus subtilis (CS2)	KM527836	70.1±0.5^{b}	72.9±0.6^{b}	71.4±0.7^{b}
Pseudomonas fluorescens (TRB)	KM527837	36.3±1.3^{d}	33.4±0.9^{d}	31.1±0.9^{d}
Bacillus licheniformis (AWRH-40B)	KM527838	64.2±0.7^{c}	63.3±0.9^{c}	62.2±0.9^{c}
C.D. at P = 0.05		2.4	2.5	2.5

Values are mean ± SE of five replication of three repeated experiments. Mean in the same column followed by the same letter are not significantly different at 0.05 % level as determined by DMRT

Effect of culture filtrate studies

Free culture study revealed that *B. amyloliquefaciens* exhibited highest antagonistic potential against the pathogen *Macrophoma* spp. (Table 5). This is in agreement with the result of Yuan *et al.* (2011) on the antagonistic potential of *B. amyloliquefaciens* against the pathogen of *Fusarium oxysporum f.* sp. *cubens.* The bacterial strains *B. subtilis* showed satisfactory level of antagonism against the test pathogen (Table 5). Douville and Bolland (1992) and Romero *et al.* (2007) reported the antagonistic potential of the same bacterial strain. In the present investigation, *P. fluorescens* and *B. licheniformis* gave only minimum inhibitory effect against *Macrophoma* spp. (Table 5). The antagonistic potential of *P. fluorescens* against certain root rot disease has been reported by Vanitha and Ramjegathesh (2014) and several scientists have reported that *B. licheniformis* gave superior antagonistic potential against *Botrytis cinerea* and *Phytophthora capsici* (Moon *et al.,* 2002; Lee *et al.,* 2006; Lim and Kim, 2010).

Effect of GC-MS studies

The potential bacterial strain of *B. amyloliquefaciens* was subjected to GC-MS analysis to identify the compounds responsible for its antagonistic potential. Analytical research revealed that nine compounds were detected which are known having antifungal-antioxidant-antimicrobial and antibacterial activity (Table 6). Among the nine compounds detected from the bacterial culture filtrate supernatant of *B. amyloliquefaciens,* compounds such as 1H-indene, 1-methylene, dichloroacetic acid, 4-hexadecy ester, 3-hexadecene (Z), benzeneacetic acid, phenol, and caffeine were higher in percentage peak area of GC-MS spectrum (Fig. 2). Elwenees and Derbalah (2011) reported that the compound group of hexadecanoic has got antifungal activity against powdery mildew pathogen. Goncalves *et al.* (2003) has also reported antifungal activity of benzoic acid. Similarly, Fernando *et al.* (2005) reported the antifungal activity of benothiazole. The antagonistic potential of phenols and benzene compounds has been studied and proven to be useful against the pathogen of *Fusarium oxysporum* (Botta *et al.*, 2005;

Table 5. ***In vitro*** **screening of bacterial biocontrol against branch canker pathogen (cell free culture filtrate)**

Bacterial culture filtrate (4%)	Growth inhibition (%)								
	24 hrs			48 hrs			72 hrs		
Code	NBCC2-7	VPM	UPA-62	NBCC2-7	VPM	UPA-62	NBCC2-7	VPM	UPA-62
WP1O4	80.2^{a}	78.5^{a}	79.9^{a}	78.0^{a}	79.4a	78.6^{a}	76.0^{a}	80.0^{a}	78.6^{a}
CS-2	70.0^{b}	68.0^{b}	68.6^{b}	73.6^{b}	74.8^{b}	73.7^{b}	61.3^{b}	69.5^{b}	63.9^{c}
TRB	45.2^{d}	49.7^{c}	45.9^{c}	69.0^{c}	61.2^{c}	67.0^{c}	25.4^{c}	31.7^{d}	29.4^{d}
AWRH-40B	17.2^{c}	29.5^{d}	25.2^{d}	37.1^{d}	40.9^{d}	38.8^{d}	62.9^{b}	67.1^{c}	65.9^{b}
CD@P=0.05	8.4	4.0	7.5	3.9	2.1	3.5	2.5	1.7	1.9

Values are mean of five replications of three repeated experiments. Mean in the same column followed by the same letter are not significantly different at 0.05% level as determined by DMRT.

Table 6. Components identified in the sample of bacterial culture filtrate (WP1O4 - *Bacillus amyloliquefaciens*) by GC-MS

No.	RT	Name of the compound	Molecular formula	Molecular weight	Peak area (%)
1.	3.547	1H-Indene, 1-methylene	$C_{10}H_8$	128	5.2
2.	5.332	Dichloroacetic acid,4-hexadecy ester	$C_{14}H_{26}C_{l2}O_2$	296	7.8
3.	6.470	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	576	9.4
4.	6.703	Phenol, 2,4-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	209	9.8
5.	7.572	3-Hexadecene (Z)	$C_{16}H_{32}$	224	11.0
6.	8.435	Benzeneacetic acid, α,3,4-tris [(trimethylsilyl) oxy]-, trimethylsilyl ester	$C_{20}H_{40}O_5Si_4$	472	12.3
7.	9.717	Benzenemethanol, α-(1-aminoethyl)	$C_9H_{13}NO$	151	14.2
8.	9.950	4-Trifluoroacetoxytridecane	$C_{15}H_{27}F_3O_2$	296	14.5
9.	10.876	Caffeine	$C_8H_{10}N_4O_2$	194	15.9

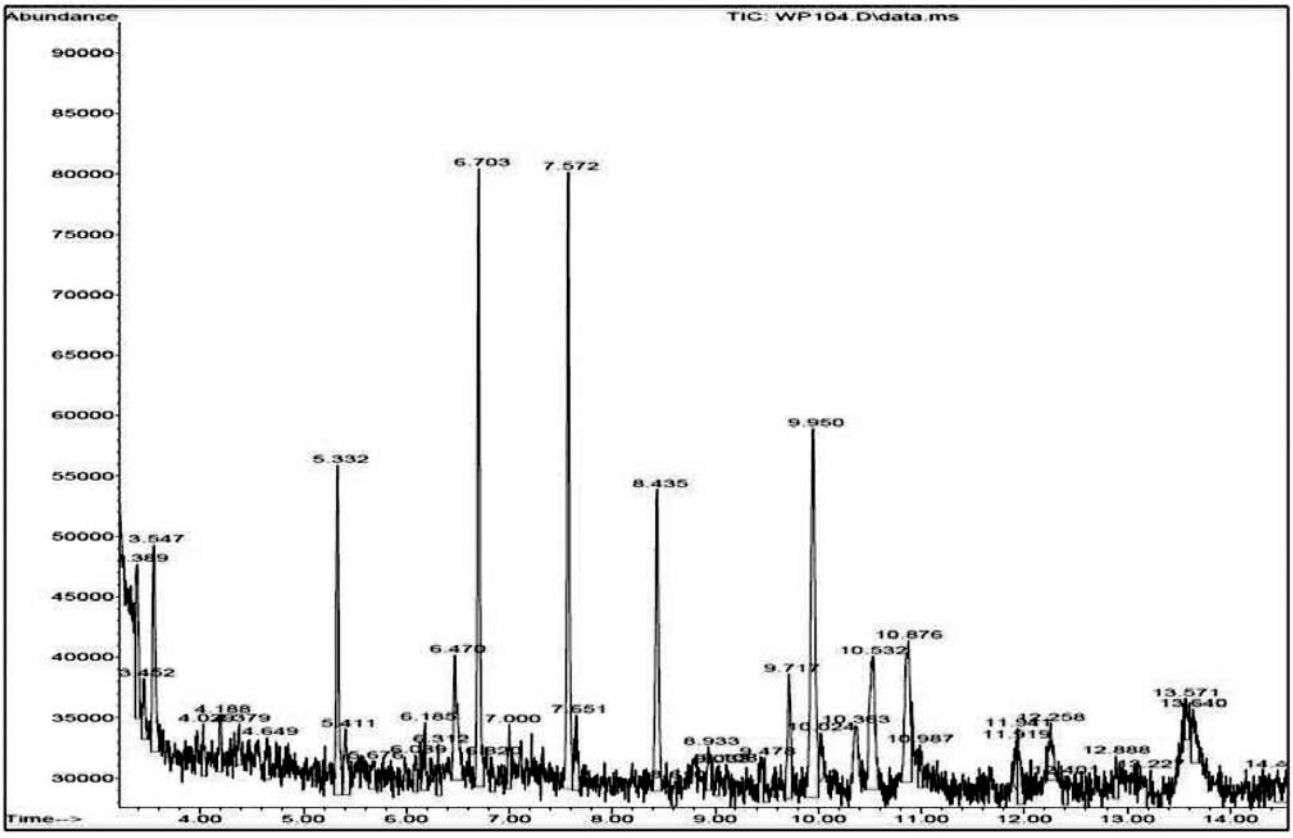

Fig. 2. GC-MS spectrum of WP1O4 (*Bacillus amyloliquefaciens*)

Yuan *et al.*, 2012). Therefore, it can be concluded that, the antifungal activity of *B. amyloliquefaciens* against the pathogen *Macrophoma* spp. could be due to this compounds.

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