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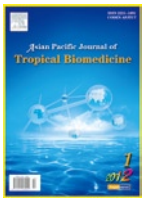

Document heading

In vitro studies of biocontrol agents and fungicides tolerance against grey blight disease in tea

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ABSTRACT

Objective: To isolate the biocontrol agents and their fungicides tolerance against grey blight disease in tea. **Methods:** Grey blight is the leaf disease of *Camellia sinensis* (*C. sinensis*) caused by fungus *Pestalotiopsis* sp. Grey blight pathogen was isolated from infected leaves of tea plant from different agro climatic zone of southern India. A total number of 320 bacterial strains were isolated from various soil samples, among these isolates, three *Bacillus* (MB1, MB2 and MB3), three *Pseudomonas* (MP1, MP2 and MP3) which showed higher antagonistic effect was taken for study. Production of cell wall lytic enzymes by the selected biocontrol isolates were studied by using mineral salt medium (MSM) with various carbon source (0.1%). All the selected isolates (MB1, MB2, MB3, MP1, MP2, and MP3) utilized all the substrates used. Fungicide tolerances of the selected bacterial strains were studied *in vitro*. The commonly used fungicides in tea plantation such as hexaconazole, carbendazim, mancozeb and copper oxy chloride (COC) were tested in three concentrations **Results:** The results clearly indicated that the isolated biocontrol agents were capable of producing cell wall lytic enzymes which is the main mechanism in bacterial biocontrol agents. All the six selected biocontrol strains showed growth tolerance in carbendazim followed by hexaconazole. No growth was observed in COC and mancozeb. **Conclusions:** From this study it was concluded that the isolated biocontrol were tolerant to commonly used fungicides carbendazim and hexaconazole against grey blight disease.

1. Introduction

Grey blight disease of tea caused by *Pestalotiopsis* sp. has been reported from all tea growing countries of the world. Though the disease appears on bare stalk and young shoots as well, the disease generally affects mature leaves[1]. After the extensive mechanized harvesting had been brought into practice, this disease gained more importance. The mechanized harvesting practice weakened the bushes and also provided sites for infection by this pathogen[2].

Tea being a leafy crop, any damage to the leaf will result in yield and also affect the quality of made tea. A large number of plant diseases have been controlled through bacterial and fungal antagonists. Interaction between bio control agents and plant pathogens has been studied extensively and the application of biocontrol agents to protect some commercially important crops is promising. Biological control using microorganisms can be economic, self perpetuating and usually free from residual side effects[3]. Fungicidal protection is the prime strategy in the control of plant diseases. In tea, maximum fungicide usage was for the control of leaf diseases such as blister blight, anthracnose and grey blight but continuous usage of chemical fungicide may lead to residual side effect and also causes disease resistant to the plants.

Reports evidencing the success of phylloplane bacteria as biocontrol against foliar diseases are available[4]. Hence in this present study, an attempt was made to screen the antagonistic activity of some of the soil isolates and fungicides tolerance against the grey blight diseases under *in vitro* condition.

2. Materials and methods

2.1. Isolation of pathogen

Pathogen was isolated from grey blight infected leaves of

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tea plant from different agro climatic zone of southern India, *viz.* Anamallais, Coonoor, Munnar and Wayanad. Infected leaves were washed in tap water and then with distilled water. The infected portions were cut into small pieces and surface sterilized with 0.1% mercuric chloride, repeatedly washed with sterile distilled water, blotted in sterile filter paper and then inoculated on water agar plates amended with streptomycin sulphate (50 mg/L) to prevent the growth of bacteria[5]. The plates were incubated for 3-5 d and the growing mycelial tips were aseptically transferred to PDA (Potato Dextrose Agar) medium for further purification. The pure culture was stored in PDA slants at 4 ℃.

2.2. Isolation of bacteria from soil

Bacterial cultures were isolated from various soil samples, collected at various elevations in different agro climatic regions. The serial dilution and plating method, was followed to isolate the *Bacillus*, *Pseudomonas* type colonies by using Nutrient Agar (NA) and Kings B(KB) medium. The colonies that grown were sub cultured and brought to purity and stored for further use.

2.3. Screening for antagonisms

The isolated bacterial colonies were screened for their Antagonism against *Pestalotiopsis* sp., following dual culture technique[6]. The mycelial plug of four day old, actively growing *Pestalotiopsis* sp. was grinded and spread uniformly in plates containing PDA medium with the help of a sterilized spatula. These plates were spot inoculated with 24 h culture of isolated bacterial strains.

Plates were incubated at (30 ± 2) ℃ for 3-5 d. Antagonism was graded by observing the zone of inhibition produced around the bacterial colonies. The level of antagonism was graded as no antagonism (-), those showing inhibition zone of less than 0.5cm (+) with 0.5-1.0 cm (++) those with 1.0 cm (+++).

2.4. Test of lytic enzymes

Mineral salt medium (MSM) with various carbon source (0.1%) was used in the study to detect the production of cell wall lytic enzymes by the selected isolates[7]. The substrates used as carbon source were chitin, casein, starch, carboxyl methyl cellulose (CMC) and Pectin. 0.1% concentration of these substrates was individually added in the MSM. The media were autoclaved (121 ℃ at 15 Lbs) and the molten cooled media were poured on sterile Petri dishes (9 cm). The selected bacterial isolates were streaked on the plates and incubated at room temperature (28±2) ℃ for 3 d and observed for their growth utilization of the substrates was designated by '+' and '-'.

2.5. Fungicide tolerance

Fungicide tolerance of the selected bacterial strains were studied *in vitro* by poison food technique[8]. The commonly used fungicides in tea plantation are taken in three dosages such as recommended dose (RD), higher dose (HR) and lower dose (LR) respectively. Hexaconazole (RD 2.85 ppm/100 mL, LD 1.42 ppm/100 mL and HD 4.27 ppm/100 mL), carbendazim (RD 500 ppm/100 mL, LD 250 ppm/100 mL and HD 1 000 ppm/100 mL. Mancozeb and copper oxy chloride (RD 3 000ppm/100 mL, LD 1 500 ppm/100 mL and HD 4 500 ppm/100 mL) were tested in three concentration.

Respective concentration of each fungicide was added to molten sterile nutrient agar media (NA)and poured onto separate sterile Petri dishes .The selected bacterial strains were streaked on to the respective plates at (28 ± 2) ℃ for 24-48 h and observed for their susceptibility/tolerance to the fungicides.

3. Results

Bacterial biocontrol agents are advantageous due to rapid growth, aggressive colonization, easy handling and better survival. A total number of 320 bacterial strains were isolated and brought to pure culture. Testing of the bacterial strains for antagonism by dual plate assay revealed that among the 320 strains, 56 strains exhibited a zone of inhibition of + category, 41 of ++ and 6 of +++ category, and the rest without any antagonistic potential. All the 6 +++ strains,3 strains identified as *Pseudomonas* sp. and the other 3 strains were identified as *Bacillus* sp. Table 1 outlines the list of soil bacteria isolated from various agro climatic zones and their antagonism.

Production of cell wall lytic enzymes by certain forms of bacteria is the basis of control of plant pathogens. In the lytic enzyme assay, all the selected isolates (MB1, MB2, MB3, MP1, MP2 and MP3) utilized all the substrates used. This clearly indicated that they are capable of producing cell wall lytic enzymes which is the main mechanism in

Table 1
List of soil bacteria isolated from various agro climatic zones and their antagonism.

Tea districts	Number of bacterial isolates	Diameter of inhibition			Total
		(<1 cm)	(1-2 cm)	(>2 cm)	
Central travancore	100	-	-	-	-
High range	100	16	41	6	63
Wayanad	120	40	-	-	40
Total	320	56	41	6	103

Table 2
Substrate utilization by selected bacterial strains.

Strain	Substrate (0.1%)				
	Chitin	Casein	Starch	CMC	Pectin
MB1	+	+	+	+	+
MB2	+	+	+	+	+
MB3	+	+	+	+	+
MP1	+	+	+	+	+
MP2	+	+	+	+	+
MP3	+	+	+	+	+

Table 3
Fungicides tolerance against grey blight pathogen (*Pestalotiopsis* sp.).

Strain	Hexaconazole			Carbendazim			Mancozeb			COC		
	LD	RD	HD	LD	RD	HD	LD	RD	HD	LD	RD	HD
MB1	−	−	−	+++	+++	+++	+	+	−	+	+	−
MB2	−	−	−	+++	+++	+++	+	+	−	+	+	−
MB3	−	−	−	+++	+++	+++	+	+	−	+	+	−
MP1	+++	+++	+++	+++	+++	+++	+	+	−	−	−	−
MP2	+++	+++	+++	+++	+++	+++	+	+	−	−	−	−
MP3	+++	+++	+++	+++	+++	+++	+	+	−	+	+	−

LD: Lower dose; RD: Recommended dose; HD: High dose; (+++): Good growth; (++): Moderate Growth; (+): Poor growth; (−): No growth.

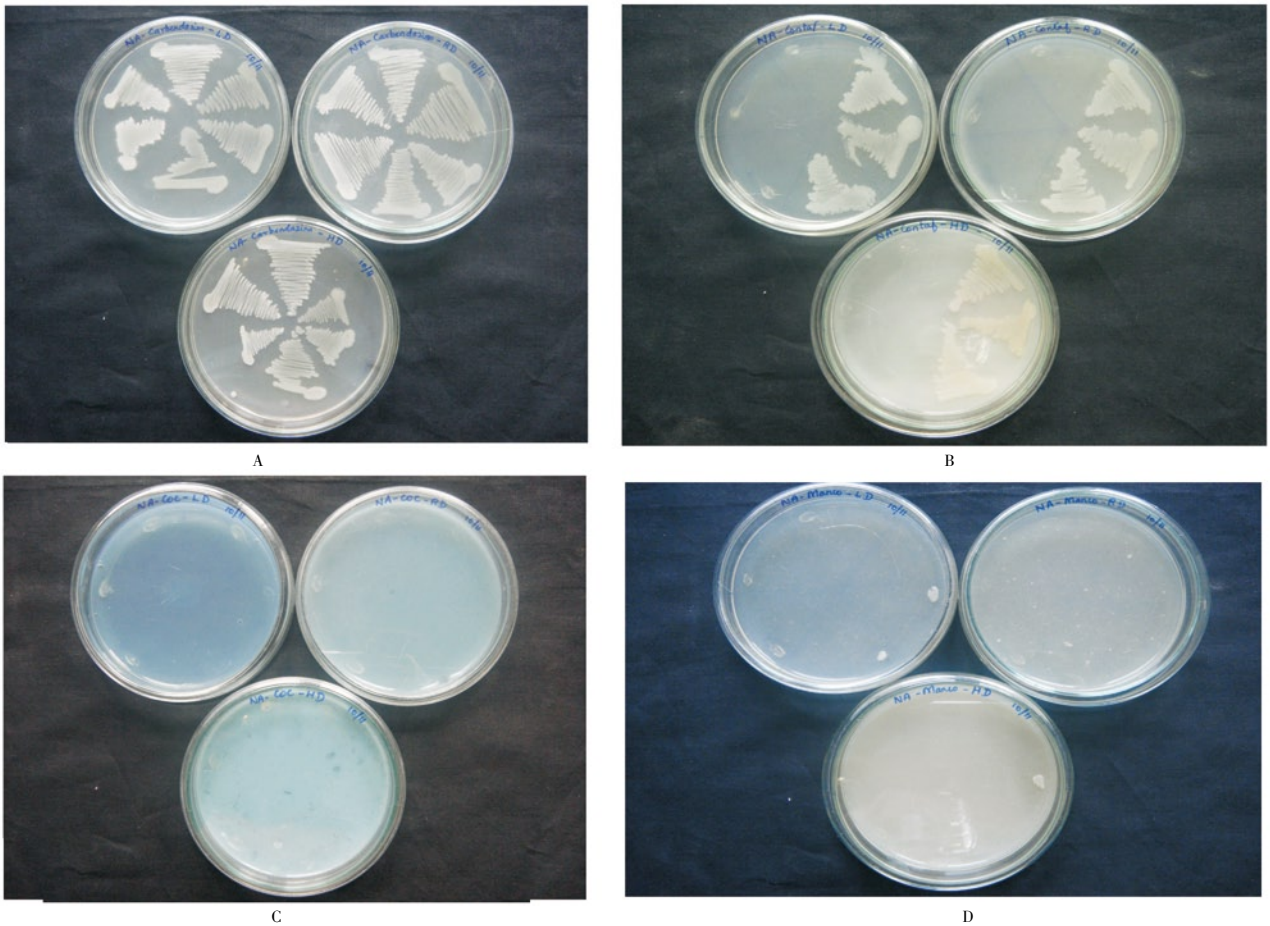

Figure 1. Fungicides tolerance of biocontrol agents in different concentration (A: carbendazim; B: hexaconazole; C: copper oxy chloride; D: mancozeb).

bacterial biocontrol agents. More over the lytic enzymes would suppress the growth of fungal pathogens at the spore germination level than in the advanced. Table 2, shows the growth of the selected bacterial strains on different substrates provided. A comparison was made between the 6 strains and the type cultures of *Bacillus subtilis* and *Pseudomonas fluorescence* (obtained from CAS in Botany, University of Madras).These six strains were further tested for their susceptibility/tolerance against commonly used fungicides *in vitro*.

The selected 6 strains were tested for their susceptibility/ tolerance against commonly used fungicides. Among the 6 strains, there was a wide variation in their growth in each of the doses of respective fungicides. In case of hexaconazole, MB1, MB2, MB3 could not survive even at the lower dosage, were as MP1, MP2, MP3, exhibited a growth of +++ category. In case of carbendazim, all the 6 strains had a +++ category growth in all dosages. In case of mancozeb, all the 6 strains exhibited + category growth in the lower dose (LD) and recommended dose (RD) and had no growth in high dose (HD).

Growth of the strains, varied in case of COC, as the strains MB1, MB2, MB3, MP3 had + growth in the lower dose and recommended dose and no growth in higher dose, where as MP1, MP2 strains had no growth in all the doses of COC (Table 3 & Figure 1). Experiments are underway to test the efficiency of these strains in the nursery and field conditions.

4. Discussion

In this present study, each three beneficial biocontrol strains *Pseudomonas* and *Bacillus* sp. showed higher antagonistic effect against the grey blight pathogen *in vitro*. Similar results were already reported by[9-11] for the control of various fungal pathogens for the management of several crop diseases. The study was continued by using various carbon sources (0.1%) to detect the production of cell wall lytic enzymes by the selected isolates. In the lytic enzyme assay, the strains MB1, MB1, MB3, MP1, MP2 and MP3 utilized all the substrates tested.

This indicated that they were capable of producing an array of lytic enzymes, which is the main mechanism of bacterial biocontrol agents. Moreover, these lytic enzymes would suppress the fungal growth[7]. The isolated bacterial strains were proved tolerant with selected fungicides (hexaconazole and carbendazim) *in vitro*. The same result was previously reported by Malathi[8]. Bacterial strains showed tolerance in hexaconazole and carbendazim.

Conflict of interest statement

We declare that we have no conflict of interest

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