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Microorganism as Biocontrol Agent Against *Glomerella cingulata* Affecting Tea (*Camellia sinensis* (L) *O. Kuntze*) in South India

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Abstract: Most of the reported antagonists bacteria and filamentous fungi naturally occur on rhizosphere soil or phylloplane layer. Brown blight disease, which is an important secondary foliar disease is caused by *Glomerella cingulata* (Gc). In the present study evaluation was done using selected strains of *Bacillus*, *Pseudomonas*, *Actinomycetes* and *Trichoderma* isolated from *Camellia sinensis* (tea) rhizosphere soils and phylloplane layer collected in different agro ecological zones of south India. Totally 165 bacteria, 15 *Actinomycetes* and 17 fungi isolates were isolated. Among the isolates, 3 isolates of *Bacillus* sp., one isolate of *Pseudomonas* sp. one isolate of *Actinomycetes* and seven isolates of *Trichoderma* showed maximum inhibition of the *G. cingulata.* Mainly *Bacillus* 2 *Bacillus* 3 showed 85.0 per cent and 82.5 per cent control respectively in dual culture method followed by *Actinomycetes*, where the growth inhibition was 75.0%. In cell free culture filtrate method, the maximum growth inhibition of Gc3 by BC3 (100%) and spore germination effect (10.49%) were observed in the first day. The growth inhibition was reduced in second and third day cell free culture filtrate of the bacteria. But no effect was found in the *Actinomycetes* cell free culture filtrate. The growth inhibition (64.68%) and cell free culture filtrate (74.28%) of *Trichoderma* (T4) have maximum inhibitory effect against the pathogen. The results showed a direct inhibition of the pathogenic fungus by these bacteria and fungi and were confirming to the synergistic effect of those beneficial microorganisms.

Key words: Antagonist · *G. cingulata* · *C. sinensis* · Synergistic · Rhizosphere · Phylloplane

INTRODUCTION

Brown blight is the one of the secondary foliar disease in tea caused by *Glomerella cingulata* which leads to reduction in the quality and quantity of tea production since it affects the leaves meant for beverage production [1]. Plant pathogens which include fungi are the most visible threats to food production. The modern methods to control pathogens focus on reduced fungicide use in different crops. Several physical and biological approaches have been evaluated as safer alternatives to the use of chemical fungicides. Biocontrol agents were effective, cheap, persistent and non-toxic to the workers. Development of fungicide resistance among the pathogens, ground water and foodstuff pollution and the development of oncogenic risks have further encouraged the exploitation of potential antagonistic microflora in disease management. Among the various antagonists used for the management of plant diseases bacteria and fungi play a vital role in disease control. Biocontrol agents may produce antibiotics and antifungal agents [2, 3]. The isolation of phylloplane microorganism helps in highly inhibiting the growth of the *G. cingulata* and other pathogen in tea [1, 4]. The rhizosphere micoflora also inhibit the growth of the various pathogens [5, 6]. It has been reported that *B. subtilis*, *P. chlororaphis* and endophytic *P. flurorescens* inhibited the mycelial growth of stem blight pathogen *Corynespora casiicola* under *in vitro*. [7] found that, among seven antagonists evaluated *T. viride* proved highly antagonistic against *C. gloeosporioides*. The antagonistic ability of *T. viride, T. harzianum, T. logidrachytum, Aspergillus niger, P. florescence* and *B. subtilis* were tried *in vitro* against *C. gloeosporioides* causing leaf spot in turmeric by dual

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culture technique. All the bioagents proved inhibitory to the growth of pathogen. In particular, the use of microbial antagonists for the control of diseases has been extensively investigated. The present investigation is an attempt to confirm the biocontrol potential of selected strains in controlling the growth of *G. cingulata*, under *in vitro* conditions.

MATERIALS AND METHODS

Sample Collection: The soil and leaf samples were collected from different tea gardens of southern India. The samples were brought to the laboratory and kept in the refrigerator and analysed in order to determine the number of bacteria, *Actinomycetes* and fungi active against the test pathogen. The soil samples were serially diluted and plated on nutrient agar and *Actinomycetes* isolation agar. The *Trichoderma* was isolated from the phylloplane layer of tea leaf by leaf washing technique [8] using modified *Trichoderma* specific medium [9] incubated at room temperature for 1-3 days. The biochemical tests for the identification of bacteria were carried out as described in Bergey's manual of systematic bacteriology [10].

Isolation of *Glomerella cingulata*: Field surveys were done in tea growing districts of south India viz., Anamallais, Central Travancore, The Nilgris, Wayanad and Koppa for the collection of various isolates of brown blight disease pathogen *Glomerella cingulata*. The infected leaves were collected and washed gently in distilled water and then dried by placing them in between folds of filter papers. The isolation of the respective pathogen was carried out *in vitro* first using water agar and then PDA. A total of three strains were isolated namely Gc2, Gc3 and Gc4. The colony of the sporulating state was purified by single spore isolation and those of non-sporulating fungi by hyphal tip method. Three isolates were compared with type strain, *Glomerella cingulata* (Gc1) procured from MTCC.

Effect of Cell Free Culture Filtrates on the Growth of *Glomerella cingulata* and Spore Germination Effect: The bacterial and fungal isolates of *Bacillus*1, *Bacillus*2, *Bacillus*3, *Pseudomonas*, *Actinomycetes* and *Trichoderma* were isolated from tea growing areas of south India. Studies were conducted on the inhibitory effect of cell free culture filtrates of the microbial isolates on the germination of *Glomerella cingulata*. Bacterial and *Actinomycetes* strains were incubated on nutrient broth medium for 24, 48 and 72 hours. *Trichoderma* was incubated for 3 days in potato dextrose broth. After the incubation period, the broth was filtered through 0.2 micron bacteriological filter under vacuum. 5% of filtrates were mixed with the sterilized PDA media in warm condition in Petri plates. Then the 5mm of pathogen mycelium block was placed on the Petri plate. 5mm of mycelia block without culture filtrate plate served as a control. To check the spore germination inhibition effect, the filtrates thus obtained were taken in cavity glass slides, which were placed in a humidity chamber at 100% relative humidity. The spores collected were introduced into the filtrates taken in a cavity glass slide. Spore germination was observed up to 72 hours.

Interaction between Selected Strains of Bacteria: The antagonistic interactions among the selected strains were carried out *in vitro*. The assays were conducted on nutrient agar plates spread with each of the bacterial strains by sterilized cotton buds. These plates were then patch inoculated equidistantly with four other bacterial strains and incubated at 28 ±1°C. After 24 hours, the plates were observed for the zone of clearance around the patched colonies that indicated incompatibility between the two strains.

***In vitro* Antagonistic Properties of Bacteria and *Actinomycetes*:** Antagonistic properties of isolates were tested against *Glomerella cingulata* on plates having equal proportion of nutrient agar medium and PDA by dual culture technique [11]. Agar blocks (7 days old, 5 mm dia.) containing 7-day-old mycelia were placed in centre of a Petri plate and inoculated with loopful culture (24 hours old) of bacterial strain, streaked 2 cm apart from the fungus. In another set of inhibition assay carried out, seven days old mycelium was ground in sterile pestle and mortar and the culture was spread on the PDA plates, inoculated with loopful culture (24 hours old) of bacterial strain and spotted on the plate. These plates were incubated at 28 ± 1°C. Plates inoculated with only fungal agar blocks served as control. Growth inhibition was calculated by measuring the zone of inhibition.

Dual Culture Method for *Trichoderma*: In order to study the hyperparasitism, the pathogen and antagonist were inoculated in PDA plates on diametrically opposite points. Due to the pathogen's slow growing nature the antagonists were inoculated only after the pathogen

colony reached considerable growth (3 days). Linear growth of the biocontrol agents colonizing either over or meeting each other was measured after 9 days of incubation. For testing antagonistic properties of *Trichoderma*, 6 mm discs of antagonist and *G. cingulata* cut from the edge of 7 days old culture were placed 3 cm apart on potato dextrose agar (PDA) plate. The Petri plates were incubated at 28 ± 1°C and periodical observations on the growth of the antagonist to colonize the pathogen were recorded. The untreated pathogen culture plate was maintained for comparison [12].

RESULTS AND DISCUSSION

Several bacteria were isolated from the disease free tea rhizosphere soil and *Trichoderma* was isolated from phylloplane layer of the different tea growing areas of south India. Totally 165 bacteria, 15 *Actinomycetes* and 17 *Trichoderma* sp. (Table 1a and 1b) were isolated. These isolates were screened for their antagonist effect on pathogen and five of them were selected for further study. The five isolates (abbreviated as BC1-BC5 and *Trichoderma* as T1-T7) were *Bacillus* sp. (three), *Pseudomonas* and *Actinomycetes*. They were identified by morphological and biological characters (Table 2). The production of extra cellular enzymes by the above isolates was also studied. All the isolates except BC1 produced a clear zone on skim milk agar plate which indicated production of protease enzyme by the biocontrol agents, (Table 3). Among the bacterial strains tested for antagonism studies, maximum growth inhibition was observed by the strain BC2 (85.0%) followed by BC 4 (82.5%) and BC1 (80.0%) (Table 4). When cell free culture filtrate was tested, strain BC3 showed superior growth inhibition rate (100 %) and (94.40%) against Gc4 and Gc1 respectively. BC1 showed highest inhibition (87.5%) against Gc4. The BC2 inhibition against Gc2 was 86.98% in first day filtrate whereas BC4 second day filtrate was effective only against Gc4 (85.60%) and third day filtrate of all biocontrol agents were less effective compared to the first day and second day filtrates. In phylloplane microorganism, WPB 104 produced zone of inhibition and cell free culture filtrate, which highly inhibited the growth of *Glomerella cingulata* and *Pestolotiopsis* [4]. [13] also reported that *Pseudomonas* has strong antifungal property against the pathogen. The overall observations indicated that the one day incubated cell free culture filtrate was the most effective followed by second and third day incubated cell free culture filtrate (Table 5). [14] reported the production of *Rhizoctonia solani* cell wall degrading chitinase in *Pseudomonas fluorescens*. *Bacillus subtilis* BN1 isolated from rhizospheric soil have best antifungal and antagonist property against the *Macrophomina phaseolina* which causes the root rot disease in *Pinus roxburghii* [15]. The cell free culture filtrates of *Bacillus subtilis* also inhibit the growth of other phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani* as reported by [15]. These could be the reasons for effective inhibition of the pathogen. The inhibition of *Glomerella cingulata* spores germination was very effective by BC1, BC2, BC3 and BC4. Among the isolates BC3 showed the highest inhibition of the spore germination followed by BC2, BC1 and BC4 respectively (Table 6). [16] reported the potential of tea phylloplane microorganism cell free culture filtrate in controlling *Exobasedium vexans* spore germination. The synthesis of inhibitory compounds such as non-volatile compounds and toxic metabolites are synthesized by biocontrol agents [17, 16]. In Actinomycetes, five days cell free culture extract has been proved non effective

Table 1(a): Total number of isolated microorganism.

Microorganism	Total no. of isolation	Effective biocontrol	Sources of isolation
Bacteria	165	4	Soil
Actinomycetes	15	1	Soil
Fungi	7	1	Phylloplane

Table 1(b): Phylloplane Fungi

Trichoderma isolates	Isolated from
T1	Anamallais
T2	The Nilgris
T3	The Nilgris
T4	UPASI- TRF Farm (Anamallais)
T5	Central Travancore
T6	Koppa (Karnataka)
T7	Gudalur

Table 2: Morphological, Physiological and Biochemical characterizations of selected. biocontrol

Characteristics	*Bacillus* sp.,1	*Bacillus* sp.,2	*Bacillus* sp.,3	*Pseudomonas* sp.	*Actinomycetes* sp.
Gram's stains	+	+	+	-	+
Motility	+	+	+	-	-
Endospore	+	+	+	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Urease	+	+	+	-	-
Citrate utilization	-	-	-	-	+
Methyl red test	+	+	+	-	+
VP	-	-	-	-	-

Table 3: Production of extra cellular enzyme studies of biocontrol agents.

Extra cellular enzymes	*Bacillus* sp. 1	*Bacillus* sp. 2	*Bacillus* sp. 3	*Pseudomonas* sp.	*Actinomycetes* sp.
Chitinase	-	-	-	-	-
Amylase	-	-	-	-	-
Cellulose	-	-	-	-	-
Protease	-	+	+	+	+

Table 4: Selective biocontrol agents against *G. cingulata.*

1	Bacteria	Place of isolated	Parts of isolates	Growth inhibition (%)	Code name
	Bacillus sp., 1	Anamalais	Soil	80.0	BC1
	Bacillus sp., 2	Vandiperiyar	Soil	85.0	BC2
	Bacillus sp., 3	Coonoor	Soil	82.5	BC3
	Pseudomonas sp., 1	Munnar	Soil	77.5	BC4
2	Fungi				
	Trichoderma sp.,	South India	Phylloplane	67.14	T4
3	*Actinomycetes*				
	Culture no.24	Munnar	Soil	75.0	BC5

Table 5: Growth inhibition of *Glomerella cingulata* in different biocontrol agents cell free culture extract

Growth inhibition (%)						
Pathogen code	Different days incubated filtrate	BC1	BC2	BC3	BC4	C.D.at P=0.05%
Gc1	24	76.22(8.5)	83.91(5.75)	94.40(2.0)	74.66(9.5)	(1.12)
	48	60.83(14)	38.33(18.5)	70.62(10.5)	73.33(10.0)	(1.46)
	72	32.86(24.0)	31.66(20.5)	26.57(26.25)	55.0(15.0)	(1.42)
Gc2	24	86.30(5.0)	86.98(4.75)	86.30(5.0)	81.48(7.5)	(0.65)
	48	63.01(13.5)	77.14(8.0)	76.61(8.5)	76.54(9.5)	(1.06)
	72	32.19(24.75)	71.23(10.5)	47.94(19.0)	50.0(12.0)	(1.31)
Gc3	24	77.14(8.0)	78.57(7.5)	85.71(5.0)	80.39(7.5)	(1.10)
	48	78.57(7.5)	72.14(15.0)	97.14(1.0)	76.47(9.0)	(1.17)
	72	69.28(10.75)	57.14(9.75)	58.57(14.5)	30.0(12)	(0.63)
Gc4	24	87.5(4.5)	86.11(5.0)	100(0)	82.5(7.0)	(0.64)
	48	84.02(5.75)	71.52(10.25)	81.25(9.75)	85.6(5.75)	(0.65)
	72	79.16(7.5)	72.91(9.75)	43.75(20.25)	45.50(11.5)	(0.80)

Values are indicated in parenthesis-Radial growth in mm*

Table 6: Spore germination (%) of *G. cingulata* in different day cell free culture filtrate

Biocontrol agents	24 hours	48 hours	72 hours
BC1	13.97 (19.0)	22.09 (19.0)	27.91 (24.0)
BC2	18.66 (25.0)	19.05 (16.0)	25.93 (14.0)
BC3	10.49 (15.0)	18.75 (15.0)	25.33 (19.0)
BC4	23.02 (32.0)	26.97 (24.0)	31.76 (25.0)
C.D at P=0.05%	2.09	2.65	2.12

* Values of parentheses indicate the number of germinated spores.

Table 7: Biocontrol studies (Growth of inhibition in %).

Trichoderma isolates	GC-1	GC-2	GC-3	GC-4
T1	60.56(28.0)	52.85(33.0)	53.62(32.0)	57.14(30.0)
T2	52.11(34.0)	57.14(30.0)	55.07(31.0)	55.71(31.0)
T3	59.15(29.0)	55.71(31.0)	56.52(30.0)	57.14(30.0)
T4	64.78(25.0)	62.85(26.0)	63.76(25.0)	67.14(23.0)
T5	60.56(28.0)	55.71(31.0)	56.52(30.0)	57.14(30.0)
T6	54.92(32.0)	57.14(30.0)	57.97(29.0)	55.71(31.0)
T7	61.97(27.0)	57.14(30.0)	55.07(31.0)	65.71(24.0)
C.D at p=0.05	1.23	1.40	2.23	1.40

9th day observation, T - *Trichoderma* sp. Values are indicated in parenthesis-Radial growth in mm*.

Table 8: Culture free extract (Growth of inhibition in %).

Trichoderma isolates	GC -1	GC-2	GC-3	GC-4
T1	49.65(18.0)	53.42(17.0)	51.42(17.0)	52.77(17.0)
T2	52.44(17.0)	50.68(18.0)	57.14(15.0)	55.55(16.0)
T3	55.24(16.0)	61.64(14.0)	57.14(15.0)	58.33(15.0)
T4	66.43(12.0)	72.60(10.0)	74.28(9.0)	72.22(10.0)
T5	58.04(15.0)	58.90(15.0)	60.0(14.0)	58.33(15.0)
T6	60.83(14.0)	69.86(11.0)	54.28(16.0)	61.11(14.0)
T7	55.24(16.0)	64.38(13.0)	54.28(16.0)	63.88(13.0)
C.D at =0.05	1.78	1.21	0.95	1.33

7th Day observation: T - *Trichoderma* sp. (Values are indicated in parenthesis-Radial growth in mm*.

Table 9: Interaction (synergism/antagonism) among selected biocontrol isolates of soil bacteria

	Bacillus sp.1	*Bacillus* sp.2	*Bacillus* sp.3	*Pseudomonas*	*Actinomycetes*
Bacillus sp.1	x	-	-	-	-
Bacillus sp.2	-	X	-	-	-
Bacillus sp.3	-	-	x	-	-
Pseudomonas	-	-	-	x	-
Actinomycetes	-	-	-	-	x

(-) indicates no antagonism; (x) indicates same organism.

Table 10: Interaction (synergism/antagonism) among selected biocontrol isolates of phylloplane fungi

	T1	T2	T3	T4	T5	T6	T7
T1	x	-	-	-	-	-	-
T2	-	x	-	-	-	-	-
T3	-	-	x	-	-	-	-
T4	-	-	-	x	-	-	-
T5	-	-	-	-	x	-	-
T6	-	-	-	-	-	x	-
T7	-	-	-	-	-	-	x

(-) indicates no antagonism; (x) indicates same organism; T - *Trichoderma* sp.

against the *G. cingulata*. But the zone of inhibition was the highest zone compared to all other isolates. In the seven different *Trichoderma* isolates used for the antagonistic activity against the pathogen, T4 was found to highly inhibit the growth of pathogen in both antagonistic and cell free culture extract (67.14 and 74.28%), followed by T7 65.71 % and 64.38 % (Table 7 & 8). α - 1, 3-glucanase and chitinase are supposed to be the major compounds for the antagonistic activity of *Trichoderma* spp., against plant pathogens (18&19). In the interaction between the antagonistic bacteria and fungi no clear zone was formed. The results indicated that all the selected strains of bacteria and fungi agents were separately compatible with each other (Table 9 & 10) and can be used for preparing an effective consortium. It is advisable to introduce consortia of different biocontrol agents which have various modes of action rather than introduce a single strain against blister blight disease in tea as reported by [16]. The biological control of bacteria and fungi may be due to the production of pathogenesis related secondary metabolites or may be through some toxic compounds produced by the biocontrol agents.

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