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Isolation and biocontrol potential of phylloplane *Trichoderma* against *Glomerella cingulata* in tea

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Three *Glomerella cingulata* and seven *Trichoderma* isolates were collected from tea phylloplane of different tea growing districts of southern India. Their cultural morphology and antagonistic potential against brown blight pathogen *Glomerella cingulata* were studied and results were documented. The growth rate of *Trichoderma* isolates was quite fast and their cultural morphology was alike on potato dextrose agar medium. Morphometric observation revealed that length of conidia was higher in isolate T6 (4.0) and length: breadth ratio of phialides highest in isolate T4 (7.9:3.1). The antagonistic potential of these isolates of *Trichoderma* sp., against *G. cingulata* pathogen showed that isolate T4 provided higher rate under *in-vitro* condition than the other *Trichoderma* isolates and the percentage of inhibition 67.14 % Gc4 followed by T7 (65.71 %) against Gc4. The volatile compounds of T4 inhibited the growth of Gc3 (50.0 %) followed by Gc1 (49.65 %). The non - volatile compound of *Trichoderma* (T4) cell free culture extract highly inhibited the growth of pathogen Gc3 (74.28 %) followed by Gc2 (72.60 %). The *Trichoderma* sp., antagonizes the pathogens by several mechanisms such as antibiosis, competition, mycoparasitism or other form of direct exploitation. This study revealed that, the *Trichoderma* strain (T4) was highly effective to control all isolates of *G. cingulata* based on results obtained.

Key words: *Glomerella cingulata*, biocontrol, antibiosis, antagonist, mycoparasitism, *Trichoderma*, tea.

Introduction

Tea is the most popular and inexpensive beverage crop. It has been cultivated in more than 50 countries. Tea being a perennial crop is prone to attack by many pests and diseases. The majority of the diseases in tea are of

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fungal origin. More than 400 pathogens causes various diseases in tea (Chen and Chen, 1990) viz., foliage, stem and root. Among the tea diseases, brown blight is a foliar disease caused by *Glomerella cingulata* (sexual) (STONEMAN) Spauld. et SCHRENK (*Colletotrichum gloeosporioides*-anomorph). Brown blight has been noticed in all southern tea districts. It's a weak parasite which is harmless unless it can gain entrance through a wound or into tissues. The two main reasons responsible for invasion of brown blight pathogen through leaf damage due to scorch and hail. Blister blight and the punctures of capsid bugs are also prevalent cause of brown blight infection (Eden, 1965). Severe infection cause defoliation, resulting in considerable damage (Rangaswami, 1972). Chemical control measures are effective in controlling tea diseases so far (Premkumar and Baby, 2005). Fungicides are not most desirable means of disease control and they are cost expensive, and causes serious environmental pollution and may induce pathogen resistance too (Conzalez and Collazo De Rivera, 1972; Ikediobi, 1985). In this context, biocontrol is a potential, alternative, and ecofriendly way to control the disease; one of the most interesting aspects of the science of the biological control is the study of the mechanisms employed by biocontrol agents to effect disease control (Howell, 2003). Several strains of the *Trichoderma* sp., are found to be effective biocontrol agents for the various plant pathogens (Amin *et al*. 2010) and they are characterized by rapid growth, abundant conidial formation and a high degree of ecological adaptability reported by Domsch *et al*. (1980), Papavizas (1985); Bissett (1991). Species of *Trichoderma* and *Gliocladium* are known to produce antibiotic substances. *Trichoderma* sp. is capable to induce metabolic changes in plants that increase resistance to a wide range of plant-pathogenic microorganisms and viruses (Harman *et al*. 2004). The mechanisms of mycoparasitism, antibiosis and competition afforded by *Trichoderma sp*., have been widely studied (Howell, 2003; Harman *et al*. 2004b). *Trichoderma* spp. can directly impact other fungi: after sensing a suitable fungal host, *Trichoderma* responds with the production of antibiotic compounds, formation of specialized structures, and degradation of the host's cell wall, followed by the assimilation of its cellular content, a process known as mycoparasitism (Chet and Chernin, 2002; Steyaert *et al*. 2003; Benitez *et al*. 2004). In fact, more than 100 different metabolites from *Trichoderma* sp., with known antimicrobial activities have been described so far, including antifungal cell-wall degrading enzymes, peptaibols and broad-spectrum antibiotics such as gliotoxin (Howell *et al*., 1993; Lorito *et al*., 1996; Sivasithamparam and Ghisalberti, 1998; Kim *et al*. 2002; Wiest *et al*. 2002; Pozo *et al*. 2004). Our emphasis in the present study is on the need for screening the isolates of *Trichoderma* having broad spectrum of antagonistic against foliar pathogen

Glomerella cingulata in order to bring efficient biocontrol *Trichoderma* against brown blight pathogen in tea.

Material and methods

Isolation of Glomerella cingulata

Field surveys were conducted 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* using water agar then PDA. 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 isolates by hyphal tip method. Three isolates were compared with type strain, *G. cingulata* (Gc1) procured from MTCC.

Isolation of Trichoderma

Seven Trichoderma labeled as T1, T2, T3, T4, T5, T6, and T7 were isolated from tea phylloplane collected from different tea gardens of southern India by leaf washing technique (Bhuvaneswari, 2006) using modified specific medium. The cultures of both *G. cingulata* and *Trichoderma* sp. were maintained on PDA slants at 4ºC for further use. Micrometric measurements of conidia and phialides were done by mounting 4 day old young culture in lacto phenol stain and observed under microscope. The length: breadth ratio of both conidia and phialides were recorded.

In vitro Screening of fungal Antagonists: (Dual culture method)

Seven isolates of *Trichoderma* sp. were screened against *G. cingulata* under *in-vitro* conditions. 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 meet each other the pathogens growth 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 ± 1ºC

and periodical observations on the growth of the antagonist to colonize the pathogen were recorded. The untreated pathogen culture plate was maintained for its comparison (Haung and Hoes, 1976).

Hyperparasitism

Hyphal interaction between the antagonist and the test pathogen was observed from dual culture. Mycelial mats were lifted gently from the zone of interaction in dual culture plates with the help of a needle and placed in a drop of lactophenol cotton blue on a microscopic slide and observed under microscope (Elad *et al*., 1983).

Antibiosis

Petri plates containing PDA medium overlaid with sterilized cellophane sheet were centrally inoculated with the antagonists. After 72 h, the cellophane sheet along with the fungal colony was removed and the plates were centrally inoculated with *Glomerella cingulata*. Pathogen grown on fresh PDA plates served as control (Denis and Webster, 1971a).

Effect of volatile compounds

Bottom portion of Petri plates only were taken and continued this study. The antagonists *Trichoderma* sp., of 3,5,7 days old age cultures were centrally inoculated by placing 6 mm disc of 3 days old culture on PDA plates individually and incubated at 28 ± 1ºC till the cultures were 3,5 and 7 days old. The upper plate was inoculated with test pathogen and they were made air tight by cellophane adhesive tape and incubated at 28 ± 1ºC. Observations were recorded by measuring the colony diameter of test pathogen.

Non-volatile Compounds

The effect of culture filtrate of *Trichoderma* sp. was studied following the method of Dennis and Webster (1971a) in order to study of *Trichoderma* isolates its antagonistic ability (6 mm disc) of 3 days old state was inoculated in potato dextrose broth and kept for incubation under shaking condition at 28 ± 1ºC for 10 days. The culture filtrate was obtained by passing the liquid culture through Whatman No.42 filter paper and the filtrate was collected in a sterilized vacuum flask. The culture filtrate of *Trichoderma* so obtained was then added to molten PDA to obtain final concentration of 10 % (v/v). The medium was poured into Petri plates (20 ml/plate) and plates were inoculated with 6mm disc

of test pathogens. PDA plates inoculated with *G. cingulata* but amended with sterile distilled water served as control. The plates were incubated at 28 ± 1ºC and observations on radial growth of pathogens were recorded periodically (Denis and Webster, 1971b).

Percent inhibition was calculated by using following formula;

$$\text{Inhibition (\%)} = \frac{\text{Colony diameter in untreated} - \text{Colony diameter in treatment}}{\text{Colony diameter in untreated}}$$

Antagonistic potential of Trichoderma isolates

Antagonistic potential of *Trichoderma* isolates were analyzed by different ratings. All the ratings were calculated after contact between pathogen and the antagonist using a modified Bell's scale method (Bell *et al*. 1982) scale (class 1-5) developed as follows : Class 1=the overgrowth): Class 2= the antagonist overgrew at least 2/3rd of pathogen surface (75% overgrowth); Class3= the antagonist colonized on half of the growth of the pathogen (50% overgrowth); Class 4= the pathogen and the antagonist locked at the point of contact; and Class 5= the pathogen overgrew the mycoparasite.

Results and discussions

The details of *Trichoderma* isolate T1, T2, T3, T4, T5, T6 and T7 with source of location is given in Table 1. The colony characteristics of colony morphology of seven *Trichoderma* sp., isolates was observed different time intervals (24 - 96 hrs.) which are tabulated in (Table 2). Colony morphology of *Trichoderma* isolates were identically similar to each other. Sporulation was started after 72 hrs of incubation at 28 ± 1ºC by all the isolates. Observations on colony characters showed no difference among the isolates studied. Micrometric measurement for isolates was revealed that maximum conidial length (4.0 µm) was registered by T4. The length and width of *Trichoderma* phialide was also measured and the higher phialide length was seen in T4 (7.9 µm) and the higher width is observed in T1 (3.1 µm). The smallest phialide was produced by T2 and T5 (2.6 µm) that were given in Table 3. This same trend of results was observed in the *Trichoderma harzianum* against the major fungal pathogen of beetle vine (Pandey *et al*. 2001). Such colony and morphometric characteristic were clearly resembled to that *Trichoderma* sp. The mycoparasitism grew towards host, ran parallel and coiled around host hyphae by mycoparasitism producing the haustoria knob like structure with penetration peg, penetrated the pathogen hyphae and finally the cytoplasm of pathogens

was lysed. Mycoparasitism includes both hyphal interaction and is the most vital mechanism of antagonism of fungal antagonist to give protection to the plants from the pathogen attack. Mycoparasitism as principle mechanism of biological control is favoured by many scientists (Elad *et al*. 1983). The radial growth rates of *Trichoderma* isolates were slightly different at the time of contact with the test pathogen (*G. cingulata*). The pathogen and antagonist grew until contacting them each other and the growth of pathogen got distributed as soon as get the contact with *Trichoderma*. The *Trichoderma* strains overgrew on the pathogen colony and complete invasion and sporulation occurred after four to six days. Isolate T4 grew very rapidly and produced higher inhibition as 67.14 % against Gc4 and followed by others isolates as followed Gc1 (64.78 %), Gc3 (63.76 %) and Gc2 (62.85 %) (Table 4, Plate 1).

The inhibitory effects of *T. virens* against *C. gloeosporioides* (Tasiwal *et al*. 2009) similarly, (Elad *et al*. 1983; Mukherjee and Sen, 1992) showed that while some pathogen yet there was a clear isolate to isolate variability in the degrees of parasitism. The inhibitory effects observed in this observation were mainly for competition for space and nutritional sources of the pathogen and antagonistic organism. Volatile compounds produced by *Trichoderma* sp., significantly inhibited the radial growth of all pathogens. Maximum growth inhibition given by Gc1 49.64 % and Gc2 50 % by T4 of five days old culture of *Trichoderma* sp., followed by T7 and other isolates (Table 5). Observations indicated that young cultures of antagonistic fungi produce more volatile compounds resulting in maximum inhibition of pathogen. Pant and Mukhopadhyay (2001) reported the volatile compounds produced from *Trichoderma* which also inhibited growth of various plant pathogenic fungi.

This may also be a reason for its antagonistic effect on *G. cingulata*. Culture free extract of *Trichoderma* sp., (T4) significantly inhibit the growth of the pathogens at 10 % concentrations and maximum inhibition given by T4 against Gc3 (74.28%) followed by T6 against Gc2 (69.86%) (Table 6). Antagonism of *Trichoderma* sp., cell free culture filtrate against various fungi mainly due to production at acetaldehyde and toxic compounds (Robinson and Park 1966 and Dennis and Webster 1971b). T4 was antagonistic to *G. cingulata* by totally overgrowing the pathogen within 5 to 6 days and were categorized in class-1 as per Bells Scale. Isolates T1, T2, T3, T5, T6 and T7 partially over day. In spite of attaining the point of contact on the third day the results were noted in (Table 7) showed that the isolates as starters were those that have the best antagonistic potential for widest those that have the best antagonistic potential for widest variety of plant pathogens. The trend of the results also indicated that there was not only variability amongst the isolates of *Trichoderma* sp., with differential degree of antagonism towards a single

pathogen of *C. gloeosporioides* (Tasiwal *et al*. 2009) Gc1, Gc2, Gc3 and Gc4. Where none of the isolates except T4 appeared to be common with the all Pathogens. But the antagonistic isolate T4 appeared to be a nearly assured and generalized choice in view of its wide spread effectivity against all the pathogen screened. It is well known that there is sufficient selectivity of isolates of *Trichoderma* sp., in their antagonistic efficiency towards a particular pathogen (Papavizas and Lumsden, 1980; Cook and Baker, 1983). It can be concluded that there is a ample scope to control major tea diseases through the use of biocontrol agents under field condition as few antagonists obtained from the results showed high activity against brown blight pathogens under *in-vitro* condition. In the present study all the isolates of *Trichoderma* sp., were produce volatile and non- volatile antibiotic inhibitory to the growth of *G. cingulata*. While the evidence for the role of competition and parasitism has been convincing and evidence establishing the importance of antibiosis has been more elusive.

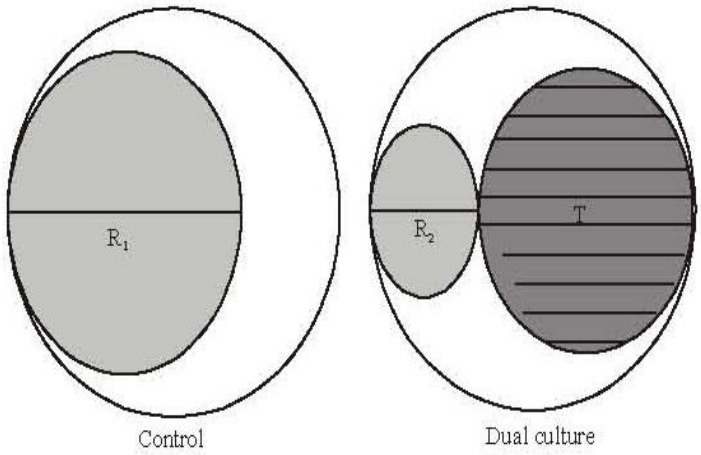

Fig. 1. Dual culture Technique R1 and R2; *Glomerella cingulata*; T- *Trichoderma* sp.

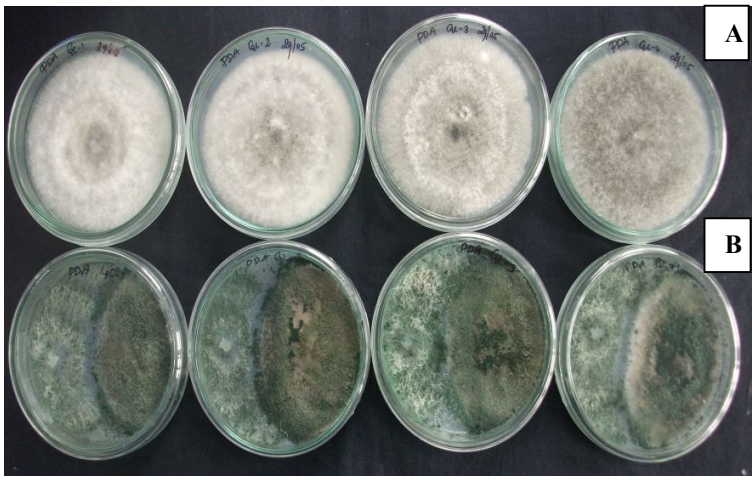

Fig. 2. Growth inhibition of *G. cingulata* by *Trichoderma* sp. (T4) (A) Plates of first row having *G. cingulata* free from *Trichoderma,* (B) Plats of second row having Trichoderma antagonistic the *G. cingulata.*

Table 1. Phylloplane Fungi

Trichoderma	**Isolates locations**
T1	Anamallais (Tamil Nadu)
T2	The Nilgris (Tamil Nadu)
T3	The Nilgris (Tamil Nadu)
T4	UPASI-TRF farm (Anamallais)
T5	Central Travancore (Kerala)
T6	Koppa (Karnataka)
T7	Gudalur (Tamil Nadu)

Table 2. Colony characters of seven isolates of *Trichoderma* sp.

Isolates	**24 hrs (1st day)**	**48 hrs (2nd day)**	**72 hrs (3rd day)**	**96 hrs (4th day)**
T1	Whitish mycelial growth	Thin Whitish mycelial growth	Dark greenish colour was found around the mycelial block	Old culture dark greenish colour and margin part of mycelial was greenish white
T2	A thin white mycelial growth	White mycelial growth was found	Light greenish coloured was found the old part of the fugal culture	Greenish White mycelium
T3	White mycelial growth around the mycelial disc	Raised white mycelial growth	Greenish mycelial mat was formed	Dark greenish mycelial growth around the mycelial block

T4	White mycelial growth	Same as 24 hrs but fluffy mycelial growth	The greenish mycelial growth was found and flattened mycelial growth around the mycelial block	Greenish White mycelium
T5	White mycelial growth near the mycelial disc	White mycelial growth was extended around the mycelial disc	Greenish coloured mycelium and fluffy	Old culture dark greenish colour and margin part of mycelial was greenish white
T6	Cottony mycelial growth around the mycelial disc	Same as 24 hrs	Greenish mycelial growth	Light green mycelial growth
T7	Whitish mycelial growth	Thin whitish Mycelial growth	Greenish mycelia growth and raised	Dense dark greenish in colour around the mycelial block and white colour mycelial growth in margin layer

Table 3. Micrometric measurements of Conidia and phialides

***Trichoderma* isolates**	**Conidia (µm)**	**Phialide (µm)**	
	L	L	B
T1	3.7	7.5	2.9
T2	3.5	7.4	2.5
T3	3.3	7.8	2.7
T4	3.8	7.9	3.1
T5	3.4	7.7	2.6
T6	4.0	7.8	2.7
T7	3.6	7.6	2.8
C.Dat P=0.05	0.12	0.25	0.30

Mean value of 10 observations L = Length, B = Breadth

Table 4. Biocontrol studies (Growth of inhibition in %)

Trichoderma isolates	GC-1	GC-2	GC-3	GC-4
T1	60.56(28.0)	52.86(33.0)	53.62(32.0)	57.57(29.7)
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)	62.86(26.0)	63.76(25.0)	67.14(23.0)
T5	60.56(28.0)	55.71(31.0)	56.52(30.0)	57.43(29.8)
T6	54.92(32.0)	58.57(29.0)	57.97(29.0)	55.71(31.0)
T7	61.97(27.0)	58.86(28.8)	55.07(31.0)	65.71(24.0)
C.D at P=0.05	1.23	1.75	2.23	1.46

(9^{th} day observation), values in the parenthesis indicated radial growth of pathogen

Table 5. Volatile compound studies (Growth of inhibition in %)

Trichoderma isolates	GC-1	GC-2	GC-3	GC-4
T1	36.64(22.3)	28.57(25.0)	42.5(27.0)	11.42(31.0)
T2	37.59(22.0)	30.0(24.5)	41.8(23.25)	8.5(32.0)
T3	40.43(21.0)	27.14(25.5)	47.5(21.0)	14.28(30.0)
T4	49.65(17.75)	34.29(23.0)	50.0(20.0)	28.57(25.0)
T5	38.06(21.5)	25.71(26.0)	30.0(28.0)	17.14(29.0)
T6	37.59(22.0)	24.29(26.5)	22.5(31.0)	14.28(30)
T7	48.23(18.25)	31.42(24.0)	25.0(30.0)	11.42(31.0)
C.D at P=0.05	1.36	1.69	0.89	1.61

(5^{th} Day observation), values in the parenthesis indicated radial growth of pathogen

Table 6. Cell free culture 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)	55.71(15.5)	63.88(13.0)
C.D at P=0.05	1.60	0.87	0.95	1.33

(7^{th} Day observation), values in the parenthesis indicated radial growth of pathogen

Table 7. Evaluation of *Trichoderma* isolates against *Glomerella cingulata* using dual culture, using Bell's scale

***Trichoderma* isolates**	**Gc1**	**Gc2**	**Gc3**	**Gc4**
T1	2	2	2	2
T2	2	2	2	1
T3	1	1	1	2
T4	1	1	1	1
T5	1	2	2	1
T6	2	1	1	1
T7	2	2	2	1

9^{th} Day observation

Conclusion

It can be concluded that the *Trichoderma* sp., isolates reduced the growth of all the isolates of *Glomerella cingulata* significantly and therefore, can be incorporated into integrated disease management for controlling brown blight disease in tea. The degree of antagonism varied between and within species of *Trichoderma* sp., against the plant pathogens.

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