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In vitro study of antagonistic effect of *Trichoderma sp.*, on tea plant pathogen, *Phomopsis theae.*

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ABSTARCT

Antibiosis and parasitism play an important role in bio control of plant diseases. A large number of plant diseases are successfully controlled through bacterial and fungal antagonism. To solve, a fungal antagonist Trichoderma sp., was isolated (antagonist) from the soil sample to be used as a biocontrol agent. The study was to isolate tea plant pathogen; Phomopsis theae from infected tea stem while causes drastic effect in the social, economic aspects of the tea planters. Interaction of Pathogen with Antagonist by various culture methods to prove the biocontrol efficiency was studied by Dual culture method, antibiosis, extraction and bioassay method. The interaction of Antagonists Fungi with the pathogen on the tea plant was studied to know the success of field trials. In the present study, attempts were made to control Phomopsis canker with bio control agents Trichoderma sp under in vitro conditions.

Key words: Trichoderma sp, Phomopsis theae, Antagonistic activity, Tea plant.

INTRODUCTION

The *Trichoderma* Species are capable of production of β-xylosidase, α-glycosidase, β-glycosidase, cellobiohydrolase, trypsin-, chymotrypsin- and chymoelastase-like proteases and *N*-acetyl-β-glucosaminidase, which are extracellular enzymes important for the biocontrol activity. *Trichoderma spp*., are free-living fungi that are common in soil and root ecosystems.

They are highly interactive in root, soil and foliar environments. They produce or release a variety of compounds that induce localized or systemic resistance responses in plants. Trichoderma strains have long been recognized as biological agents, for the control of plant disease and for their ability to increase root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients. Antagonist microorganisms, such as *Trichoderma*, reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion.

The majority of diseases in tea are of fungal origin and bacteria and one each of virus and algae. In a recent monograph on tea diseases, [1] described nearly 400 pathogens. Irrespective of the pathogen and the parts affected, the disease symptoms manifest as debilitation, defoliation and sometimes death of bushes. Crop loss due to this disease is substantial as it leads to capital losses. No information is available on the response of tea plants to infection by *Phomopsis sp* canker pathogen. Tea is the most popular and inexpensive beverage produced from young leaves of the commercially cultivated tea plant.

The disease is of great importance as the area under replanting and new clearing is increasing in recent years. Despite its importance, effective control measures are not available other than pruning to healthy wood and application of copper fungicides on prune cuts. Control of soil borne diseases through antagonistic microorganisms is an effective means [2]. The bio control agents from plant protection species is the filamentous fungal genus *Trichoderma* which is of great economic importance as sources of enzymes and antibiotics; plant growth promoters; degraders of xenobiotics, and most importantly, as commercial biofungicides.

Collar canker disease caused by the fungus *Phomopsis theae* Petch is the most common stem disease in young tea. This disease is a serious problem in all tea growing areas of the world leading to replanting debacle [3,4].The disease has great economic importance of the area under replanting and new clearings with clonal tea are increasing in recent years. The disease is prevalent in young tea plantations planted with clonal tea and to a certain extent also in mature tea fields. There are two ways by which bio control agents can suppress the plant pathogen: (i) production of antibiotics or (ii) production of hydrolytic enzymes. A large number of plant diseases have been successfully controlled through bacterial and fungal antagonists [2].

In the present study, attempts were made to control *Phomopsis* canker with bio control agents *Trichoderma sp* under *in vitro* conditions.

MATERIALS AND METHODS

Collection of tea plant

The collections of tea plant from Ooty and Coonur, Tamilnadu, South India.

Microorganism

Isolation of *Trichoderma sp*

Soils were collected from 15 different tea cultivation areas under main farm Vandichollai, Coonur Tea Research Institute at 0-9 inches. Depth microorganisms were isolated by serial dilution technique. 1 gram soil was mixed into 9 ml of sterile distilled water then 1 ml of suspension was taken into another tube containing 9 ml of sterile distilled water. This serial dilution technique was continued up to 1: 10,000. From the final dilution (1: 10,000), 1 ml suspension was transferred to each of the five Petri dishes. 20 ml of melted potato dextrose agar medium was poured in each plate and mixed with the suspension by giving a gentle whirling motion to the plate and allowed them to incubate in room temperature. Sub culturing was performed and the culture of *Trichoderma* in pure form was maintained. Preserved and expressed on dry matter.

Isolation of *phomopsis theae*

The pathogen, *P. theae* was isolated from collar canker affected tea plants collected from different agro-climatic zones of south India regions. Diseased specimens were kept in moist chamber to develop fruiting bodies. Spore masses exuded from pycnidia were

transferred to 2% water agar medium amended with streptomycin sulphate (50 mg I''^{1}). Actively growing mycelial tips of the fungus were transferred to PDA medium and purified by repeated sub culturing and finally transferred to PDA slants. The cultures were sub cultured for every three months.

Interaction with Dual culture method

Potato dextrose agar (PDA) plates are inoculated with 5mm mycelial discs of *P. theae* as well as the antagonists on diametrically, opposite points. Since the pathogen under study is slow growing, antagonists are inoculated only after the pathogen colony grew to 20-30 mm in diameter. Radial growth of the pathogen and antagonists are measured at 24h intervals. Hyphal interaction is studied in dual culture by inoculating them in water agar plates over laid with sterile cellophane sheets. After 7 days pieces of cellophane sheets ($1cm^2$) are cut from the interacting zone, stained with lacto phenol cotton blue and observed under microscope.

Antibiosis between the tea pathogen of *Phomopsis theae* and biocontrol agent *Trichoderma*.

Petri dishes containing malt extract medium (MEA) were over laid with sterilized cellophane sheets. They centrally inoculated with 5mm mycelia discs of the antagonists. After 72 h the cellophane sheet along with the fungus are removed and the plates centrally inoculated with 5 mm mycelia discs of *P. theae*. Pathogen inoculated on fresh MEA plated served as control. Radial growth is measured every 24 h and per cent inhibition calculated.

$$I = \frac{a-b}{a}$$

I = Percentage Inhibition of Pathogen growth
a = Growth of Control (mm)
b = Growth of pathogen on culture filterate medium (mm).

Extraction and bioassay method

The antagonists are grown in potato dextrose broth (PDB) under shake condition. The culture filtrate of 7 day old cultures is filter and bioassay at 10 per cent level on PDA against *P. theae* and calculated the per cent inhibition on radial growth.

Interaction of Antagonists Fungi in tea plant

Tea stem bits of 70 * 10 mm size are taken in test tubes containing moist cotton at the bottom and steam sterilized. Spore suspensions of *P. theae* and fungal antagonists ($1.5*10^{-6}$ spores ml^{-1}) are prepared from PDA cultures and sprayed on these stem bits under aseptic condition. The spore suspension of antagonists is sprayed before inoculating with the pathogen, simultaneously with the pathogen and after the establishment of the pathogen. Interaction is also studied by inoculating *Trichoderma* on stem bits on 3, 5, 7 and 11 d after the inoculation of the pathogen. The tubes are incubated at 30 ± 2^{0} C and observed for the development of the pathogen.

RESULTS

Isolation of *Trichoderma*

Soils were collected from tea cultivation areas under the main farm of Vandichollai, Coonur. Tea Research Institute at 0-9 inches. Depth microorganisms were isolated by serial dilution technique. This serial dilution technique was continued up to 1: 10,000. From the final dilution (1: 10,000). The *Trichoderma* isolation in potato dextrose agar medium. Sub culturing was performed and the culture of *Trichoderma* in pure form was maintained and preserved.

Isolation of *Phomopsis theae*

The pathogen, *P. theae* was isolated from collar canker affected tea plants collected from different agro-climatic zones of south India regions. Diseased specimens were kept in moist chamber to develop fruiting bodies. Actively growing mycelia tips of the fungus were transferred to PDA medium and purified by repeated sub culturing and finally transferred to PDA slants. The cultures were sub cultured every three months.

Interaction by Dual culture method

In the dual culture experiment the pathogen and antagonists grew until they came in contact with each other. Further growth of the pathogen was inhibited, while the antagonists continued their growth and completely covered the pathogen in about seven days. *Trichoderma type 1* colonised the pathogen faster than *Trichoderma type2*. This clearly indicated the potential of fungal antagonists in parasitising the pathogen. Microscopic observation confirmed this. Colonization of the pathogen mycelium by the antagonists and lysis of *Phomopsis theae* mycelium were observed under microscope. Production of cell wall lytic enzyme is known in *Trichoderma type1* and *Trichoderma type2*.The result shown Table: 1 and 2)

Antibiosis between the tea pathogen *Phomopsis theae* and biocontrol agent *Trichoderma.*

Among the antagonists *Trichoderma* type1 registered higher antibiosis than *Trichoderma Type II*. Inhibition on the growth of the pathogen was 61%, 76% for *Trichoderma Type I* and *Trichoderma Type II* respectively. Inhibition of the growth of *P. theae* might be due to the diffusible metabolites secreted by the antagonists. The antagonists completely inhibited the mycelia growth of antibiotics which induced swelling and plasmolysis of the cells. The result shown Table: 3

Extraction and bioassay method

The growth of the pathogen was significantly inhibited in the medium amended with culture filtrates of the antagonists. The result indicated that PDB was *Trichoderma Type I* and *Type II* producing toxic metabolites in both the antagonists. The inhibition in growth was 61% and 76% *Trichoderma Type I* and *Type II* respectively. The observation is in agreement with reported the superiority of PDB in producing toxic metabolites by the antagonistic fungi. The result shown Table: 4

Interaction of Antagonists fungi in tea plant

The reproductive stage of the pathogen were inhibited at varying levels when the tea stem bits were inoculated with *Trichoderma type I* and *type II* various time lag on the establishment of the pathogen. Pycnidial formation was completely arrested when *Trichoderma* was inoculated 3 day after the inoculation of *Phomopsis theae* .But when the inoculation was done after 5, 7, and 9 day normal development of pycnidia took place with cirrhus exudes. The cirrhus exudes were subsequently colonized by Trichoderma which resulted in the suppression of the formation of fresh pycnidia. Interestingly, when the inoculation was made 11day after the inoculation of pathogen development of fresh pycnidia was observed after the first series. However the number was comparatively less than that control. In all cases *Trichoderma* was normally sporulated. This clearly showed the inhibitory effect of antagonists on the growth and reproduction of *Phomopsis theae*. The observations on the inhibitory effect have been reported in rice-sheath blight system. Thus the present investigation clearly showed the possibility of employing *Trichoderma* type I and *Type II* the control of *Phomopsis theae* under field conditions. The result shown Table: 5

DISCUSSION

Antibiosis and parasitism play an important role in biocontrol of plant diseases. A large number of plant diseases are successfully controlled through bacterial and fungal antagonism. The in vitro antagonism of *Trichoderma sp*., against root pathogens of tea was studied. The efficacy of *Trichoderma* bioformulations in controlling some of the primary and secondary root diseases has been reported. Collar canker disease caused by the fungus *Phomopsis theae* Petch is the most common stem disease in young tea. This disease is a serious problem in all tea growing areas of the world. There are two ways by which biocontrol agents can suppress the plant pathogen: (i) production of antibiotics or (ii) production of hydrolytic enzymes. A large number of plant diseases have been successfully controlled through bacterial and fungal antagonists. It has been reported that the interaction between *P. theae* and fungal antagonists such as *Trichoderma harzianum and Gliocladium virens* were studied in *vitro* through dual culture and antibiosis techniques, which revealed that pathogen growth was suppressed significantly [5]. The biocontrol agents from plant protection species is the filamentous fungal genus *Trichoderma* which is of great economic importance as sources of enzymes and antibiotics; plant growth promoters; degraders of xenobiotics, and most importantly, as commercial biofungicides. Antagonist microorganisms, such as *Trichoderma*, reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion [6].

Table: 1 Antagonistic effect of *Trichoderma Type 1* on *Phomopsis theae* by Interaction by Dual culture Method

Day after Inoculation	Hyperparasitism (Linear growth in mm)	
	Phomopsis theae	***Trichoderma Type1***
1	18	15
2	36	23
3	48	35
4	53	47
5	69	58
6	75	67
7	87	73
8	93	86

Table: 2 Antagonistic effect of *Trichoderma Type 2* on *Phomopsis theae* by Interaction by Dual culture Method

Day after Inoculation	Hyperparasitism (Linear growth in mm)	
	Phomopsis theae	***Tricoderma Type2***
1	18	19
2	37	28
3	45	35
4	53	48
5	65	59
6	74	68
7	87	78
8	95	85

Potato Dextrose Agar (PDA) media was prepared in the laboratory of Dr. G. R. Damodarn CAS Media and necessary glassware were sterilized in autoclave. Trichoderma were isolated by serial dilution technique briefly. Sub culturing was performed and the culture of *Trichoderma* in pure form was maintained and preserved. The pathogen *P. theae* was isolated from collar canker affected tea plants collected from different agro-climatic zones of Conoor region.

Table: 3 Antibiosis between the tea pathogen Phomopsis theae and biocontrol agent *Trichoderma sp*

No of Days	Radial growth of *P. theae* mm	
	Culture filtration of *Trichoderma Type 1*	Culture filtration of *Trichoderma Type2*
1	0.0 (100)	0.0 (100)
2	0.0 (100)	0.0 (100)
3	0.0 (100)	0.0 (100)
4	1.5 (95)	1.2 (93)
5	3.4 (81)	2.8 (89)
6	7.6 (71)	6.8 (84)
7	11.5 (65)	11.2 (80)
8	15.7 (61)	14.9 (76)
9	0.5	0.7

Table: 4 Effect of toxic metabolites produced by *Trichoderma Type 1* and *Type 2* on the radial growth of *Phomopsis* theae: Extraction and bioassay method

Day after Inoculation	Culture media	
	Potato Dextrose Broth (PDB)	
	Trichoderma Type 1	*Trichoderma Type2*
1	0.0 (100)	0.0 (100)
2	0.0 (100)	0.0 (100)
3	0.0 (100)	0.0 (100)
4	0.8 (97.4)	3.6 (90.6)
5	4.5 (89.5)	5.4 (82.5)
6	6.5 (79.0)	7.6 (78.5)
7	8.9 (75.3)	13.3 (67.5)
8	18.8 (58.3)	16.7 (62.3)
9	2.62	1.38

Table: 5 Interaction of fungal antagonists with *Phomopsis theae* on sterilized tea stem bits

Treatment	Time of inoculation of antagonists	Observation
Phomopsis theae + *Trichoderma Type 1*	Post-inoculation	Cirrus exuted from pycnidia. *Trichoderma Type 1* completely covered pathogen mycelium including cirrus
Phomopsis theae + *Trichoderma Type2*	Post-inoculation	Cirrus exuted from pycnidia. *Trichoderma Type2* completely covered pathogen mycelium including cirrus
Phomopsis theae + *Trichoderma Type 2*	Simultaneous	Pathogen failed to developed
Phomopsis theae + *Trichoderma Type 2*	Simultaneous	Pathogen failed to developed
Trichoderma Type 1 + *Phomopsis theae*	Pre-inoculation	Pathogen failed to developed
Trichoderma Type 1 + *Phomopsis theae*	Pre-inoculation	Pathogen failed to developed
Control	*Phomopsis theae* alone	Cirrus exuded from pycnidia with normal development of spores

CONCLUSION

To prove the success of the bio control agent area the pathogen various types of interaction studies were carried out and it was observed that percentage of inhibition of the pathogen by the antagonist is ranging from 58 % to 76% which proves it to be successful to some extend but direct and interaction with plant and the Pathogen indicates prevention is better than cure ie., the

application of bio control agent on tea plant is to be done on the susceptible period of infection or at the early stages.

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