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In vitro evaluation of PSF against the causal agent of Brown root rot disease of Tea [*Camellia sinensis* (L.) O. Kuntze]

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The present study reveals the antagonistic potential of some PSF isolated from the tea soil of Rosekandy T.E. Southern Assam, which were tested against the brown root disease causing pathogen of tea (*Fomes lamoensis*). Five PSF viz. *Trichoderma harzianum, Aspergillus niger, Aspergillus flavus, Trichoderma asperellum* and *Penicillium funiculosum* were tested in vitro in dual culture by inoculating both the antagonist and the pathogen simultaneously (2mm apart). Studies on antagonism revealed that the different PSFs tested, occupied more area on PDA medium as compared to the pathogen, *F. lamoensis* by the 6th day of observation. Maximum percent of inhibition on the radial growth of the *F. lamoensis* was found with *Trichoderma harzianum* followed by*Aspergillus niger* and *Trichoderma asperellum*. The study presents the biocontrol potential of some naturally available PSF isolated from the tea soil, which may be tried as biocontrol agent against *F. lamoensis.*

Key words: Antagonistic potential, biocontrol, pathogen, percent of inhibition,PSF

INTRODUCTION

Tea is an evergreen plantation crop widely grown from tropical to temperate region in Asia and Africa. It has been closely associated with people's life as one of the best beverages. Tea is the most popular healthy beverage across the world, and is known widely for its antioxidant and medicinal properties. As tea is manufactured from young shoots of the tea bushes, foliar diseases are of great concern in the production of tea. The bushes of tea plants facilitates annual outbreak of the foliar diseases and their development during the growing season.

Brown root rot of tea, caused by *Fomes lamoensis* (Murr.)Sacc. and Trott. is one of the most important root diseases of tea in all tea growing region including Barak Valley of Assam. The disease is usually found in the tea bushes more than three years of age, but younger plants may also be attacked. The characteristic feature of Brown root rot disease is the presence of brown mycelium adhered with soil, sand and stone particles as encrustation, which cannot be easily removed or washed off. The name Brown root rot refers to a brown to black mycelia crust formed by the fungus on the surface of the infected roots and stem in advanced stage. The underside of the bark and the wood is marked by thin brown lines in a honeycomb pattern known as reticulations, which finally leads to the decay of the diseased root. The pathogen, in brown root rot gains entry through the roots or by infected materials coming into contact with healthy plants.

There are some reports, where some root pathogens have seen controlled with the help of which phosphate solubilizing strains in carnation, protected the plants systemically against *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi* . Inoculation of pepper with phosphate solubilizing bacteria significantly reduces the *Phytophthora* blight of peppers caused by *Phytophthora capsici* and increased the yield.

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In the present work an attempt has been made to observe the potential of the isolated phosphate solubilizing fungi against the brown root rot of tea causing pathogen *Fomes lamoensis in vitro*.

MATERIALS AND METHODS

Brown root rot causing organism infected material was collected from the root of the dead tea bushes from Rosekandy T.E. of Barak Valley (South Assam). Mercuric Chloride (0.1%) [or 0.35% sodium hypochlorite] was used for the surface sterilization of the diseased plant material and the pathogen was grown in PDA agar medium by inoculating a small mycelial mat scrapped from the diseased plant root.Potato Dextrose Agar (PDA) media was prepared in the laboratory of Agriculture and Microbial Ecology Laboratory of the Department of Ecology And Environmental Science, Assam University, Silchar. Media and necessary glassware were sterilized through autoclave.The test pathogen (*Fomes lamoensis*) was isolated from the infected roots of tea plants. Different phosphate solubilizing species isolated from the tea soil were maintained in pure culture in PDA slants. Colony interaction and hyphal interference between the pathogen and the test organisms were investigated. The potential antagonists and the test organisms were placed on the surface of a PDA agar medium containing Petri dishes opposite to each other at the periphery of the Petri dishes, 2 mm apart, wrapped in aluminium foil and kept for incubation separately at 25 ± 2^0C for 7days and antagonistic colony interaction were examined thereafter subsequently. The colony diameter was measured. It was maintained in triplicates. It is the most popular method of determining antagonistic activity. Control plates were kept simultaneously. The colony growth on both sides i.e. towards and opposing each other from loci was measured. The parameters used for the assessment of colony interaction were degree of inhibition or intermingled zone between both the interacting colonies.The organisms grew towards each other and one of the following reactions has been normally observed as follows: a) Mutual intermingling of the two organisms, (b) Inhibition of one organism on contact; the other organism continues to grow unchanged or at a reduced rate overgrowing the colony of the inhibited organism. (c) Mutual inhibition on contact; the space between the two colonies is small, but clearly marked, (d) Inhibition of one organism from a distance; the antagonist continues to grow through the resulting clear zone at an unchanged or reduced rate, (e) Mutual inhibition at a distance.Antagonistic effect of PSF against the Brown root rot pathogen of tea *Fomes lamoensis*was determined by employing dual culture technique. The measurement of growth inhibition zone was done by using the formula:

The inhibition of radial growth was calculated by using the formula :

$$\% \text{ inhibition} = 100 \times \frac{r1 - r2}{r1}$$

where r1= radial growth of the pathogen in control r2= radial growth of *Fomes lamoensis* in dual inoculation/culture

In case of liquid medium, potato dextrose broth (PDB) was prepared and distributed in 250 ml conical flasks and autoclaved. To each flask, 2mm of sterile pathogen (*Fomes lamoensis*) was added and inoculated with 2 mm size mycelia of the different test fungi (i.e. *Aspergillus flavus, A. niger, Penicillium funiculosum, Trichodema asperellum* and *T. harzianum*) and incubated at 30 ±20°C for 10 days after which mycelial fresh and dry weight was taken. Three different sets of observation were prepared.

RESULTS AND DISCUSSION

The colony interaction of the five phosphate solubilising fungi isolated from the tea soil of Rosekandy T.E (South Assam). i.e. *Aspergillus niger, Aspergillus flavus, Penicillium funiculosum, Trichoderma asperellum and Trichoderma harzianum* was recorded (Fig. 1A-I). These PSFs were maintained and screened against *F. lamoensis* for mycelial inhibition by *in vitro* dual culture technique and this was performed using PDA culture media. It was observed that *Aspergillus niger*, *Aspergillus flavus* and*Penicillium funiculosum* showed Bii type of interaction in which the test fungus under observation (i.e*., F. lamoensis*) had ceased to grow and was overgrown by the antagonistic colony of *Aspergillus niger, Aspergillus flavus* and *P. funiculosum* (i.e., antagonist), when grown with *Fomes lamoensis* in PDA plates respectively (Fig.1-F) while *Trichoderma asperellum* and *Trichoderma harzianum* showed light inhibition zone when(C type of interaction) grown with *Fomes lamoensis* in PDA media and are presented in Table 1 and Fig.1(h-l) The diameter of the radial growth of *Fomes*

Table 1 : Colony interaction between the phosphate solubilising microorganisms and *Fomes lamoensis*

PSM	COLONY INTERACTION
Aspergillus niger Van Tieghem	Bii
Aspergillus flavus Link	Bii
Trichoderma harzianum Rifai.	C
Penicillium funiculosum Thom.	Bii
Trichoderma asperellum Pers. Fr. Samuels Lieckfeldt and Nirenberg	C

A: Mutual intermingling growth, Bi:Overgrowth by antagonists, Bii:Intermingling growth in which the test fungus under observation (i.e., *F. lamoensis*) has ceased growth and is overgrown by another colony (i.e.,antagonist)., C: light inhibition, D: Not detected

Table 2 : *In vitro* antagonism of PSMs. against *Fomes lamoensis*

Interacting microorganisms	2nd day of observation		4th day of observation		6th day of observation	
	Diameter of radial growth (mm.)	Percent Inhibition of radial growth	Diameter of radial growth (mm.)	Percent Inhibition of radial growth	Diameter of radial growth (mm.)	Percent Inhibitionof radial growth
Fomes lamoensis	16.00±0.10	0.00±0.00	46.00±0.05	0.00±0.00	54.70±0.20	0.00±0.00
Fomes lamoensis + *Trichoderma harzianum*	12.00±0.00	25.00 ±0.08	14.00±0.29	69.56 ±0.22	14.00±0.10	74.40 ±0.30
Fomes lamoensis + *Aspergillus niger*	14.00±0.00	12.50±0.28	18.30±0.02	60.21± 0.18	19.00 ±0.00	65.26± 0.26
Fomes lamoensis + *Aspergillus flavus*	14.00 ±0.00	12.50±0.00	23.30 ±0.26	49.34±0.02	23.30±0.02	57.40±0.30
Fomes lamoensis + *Trichioderma asperellum*	14.00± 0.05	12.50±0.00	20.00±0.52	56.52±0.09	20.00 ±0.07	63.43±0.11
Fomes lamoensis + *Penicillium funiculosum*	13.00±0.04	18.75±0.14	21.30±0.05	53.69±0.08	21.60±0.15	60.51± 0.05
LSD (1%)	0.2163	0.5805	0.4428	0.5560	0.5027	0.9217
LSD (5%)	0.1541	0.4135	0.3154	0.3961	0.3582	0.6566
F-value	926.004	3854.78	1770.28	36176.72	15783.27	15878.67

*Calculation done as per Fokkema (1973)

of *F. lamaoensis*+ *T. asperellum* was 20.00 mm. *F. lamoensis*+ *A. niger* and *F. lamoensis*+*T. harzianum* recorded a radial growth of 18.30 mm and 14.00 mm respectively in the 4th day after incubation and are presented in the Table 2. Similarly, in the 6th day after incubation, the radial growth was recorded and *F. lamoensis*alone showed a radial growth of 54.70 mm, followed by *F. lamoensis*+ *A. flavus, F. lamoensis*+*P. funiculosum* and *F. lamoensis*+ *T. asperellum* with a radial growth value of 23.30 mm, 21.60 mm and 20.00 mm respectively. *F. lamoensis* + *A. niger* and *F. lamoensis* + *T. harzianum* recorded a value of 19.00 mm and 14.00 mm respectively. It can be observed from the Table 2, that the radial growth in the 6th day after incubation, in all the antagonist treatments in combination with *F. lamoensis* has ceased to grow after a certain stage of interaction with the root pathogen. Studies on antagonism revealed that the different PSFs tested, occupied more area on PDA medium as compared to the pathogen, *F. lamoensis* by the 6th day of observation. In the diameter of the radial growth, the percent inhibition in the 2nd day was found to be highest in *F. lamoensis*+ *T harzianum* (25%), followed by *F. lamoensis*+ *Penicillium funiculosum* (18.75%) and all others such as *F. lamoensis*+ *A. niger, F. lamoensis*+ *A. flavus* and *F. lamoensis* + *T. asperellum* showed the % inhibition of 12.5% each respectively. In the 4th day after incubation, the percent inhibition was again maximum in *F.*

lamoensis alone and the radial growth of the tested antagonists have been recorded in the 2nd day, 4th day and 6th day after incubation respectively. In the 2nd day after incubation, *F. lamoensis* recorded a radial growth of 16.00 mm, followed by *F. lamoensis*+ *A. niger, F. lamoensis*+ *A. flavus* and *F. lamoensis*+ *T. asperellum* with a radial growth of 14.00 mm in each cases and are presented in Table 2. The radial growth of *F. lamoensis*+*P. funiculosum* and *F. lamoensis*+*T. harzianum* recorded a value of 13.00 mm and 12.00 mm respectively (Fig. 1 E, F and I). In the 4th day after inoculation/incubation, the radial growth of *F. lamoensis* alone showed a value of 46.00 mm, followed by *F. lamoensis*+ *A. flavus* with a value of 23.30 mm. The radial growth of *F. lamoensis*+*P. funiculosum* was 21.30 mm and that

Table 3 : Mycelial growth of *Fomes lamoensis* and different PSF antagonists in liquid medium, (PD broth)

Interacting microorganisms	Mycelial fresh weight (mg.)
Fomes lamoensis	524.00± 0.06
Fomes lamoensis +Trichoderma harzianum	260.00± 0.13
Fomes lamoensis +Aspergillus niger	310.00±0.28
Fomes lamoensis +Aspergillus flavus	210.00±0.47
Fomes lamoensis +Trichioderma asperellum	287.00±0.27
Fomes lamoensis +Penicillium funiculosum	101.00±0.15
LSD (1%)	1.1613
LSD (5%)	2.3283
F-value	183.495

Table 4 : The dry weight of mycelial growth of *Fomes lamoensis* and different PSF antagonists

Interacting microorganisms	Mycelial dry weight (mg.)
Fomes lamoensis	23.93 ±0.11
Fomes lamoensis +Trichoderma harzianum	19.74±0.57
Fomes lamoensis +Aspergillus niger	26.47±0.57
Fomes lamoensis +Aspergillus flavus	19.09±0.33
Fomes lamoensis +Trichioderma asperellum	22.60±0.88
Fomes lamoensis +Penicillium funiculosum	16.46±0.40
LSD (1%)	2.3283
LSD (5%)	1.6588
F-value	73738.95

lamoensis+ *T. harzianum* (69.56%), followed by *F. lamoensis* + *A. niger* (60.21%), *F. lamoensis* + *T. asperellum*(56.52%) and *F. lamoensis* + *P. funiculosum* (53.69%) and the minimum in *F. lamoensis* + *A. flavus* (49.34%). In the 6th day observation, the maximum inhibition of the radial growth was found in *F. lamoensis* + *T. harzianum* (74.40%), followed by *A. niger* (65.26%), *F. lamoensis* + *T. asperellum* (63.43%), *F. lamoensis* + *P. funiculosum* (60.51%) respectively. The minimum inhibition of the radial growth was found in *F. lamoensis* + *A. flavus* (57.40%).It was observed during the time intervals that, the maximum percent inhibition of the radial growth was observed in *F. lamoensis*+ *T. harzianum* and the minimum in *F. lamoensis*+ *P. funiculosum*. It was observed that in all the PSFs their inhibition potential against the Brown root rot disease pathogen *F. lamaoensis* increasesd with the time intervals. The mycelial fresh and dry weight of all the PSFs and the test pathogen *F. lamaoensis* was recorded. The maximum fresh weight was observed in *F. lamaoensis* (524mg), followed by *F. lamoensis*+ *Aspergillus niger* (310 mg), *F. lamoensis*+*T. asperellum* (287 mg), *F. lamoensis* + *T. harzianum* (260 mg), and *F. lamoensis* + *A. flavus* (210 mg) respectively and the minimum mycelia fresh weight was observed in *F. lamoensis* + *P. funiculosum* (101 mg) and these results are presented in Table 3 . After the oven dry, the dry weight of all the PSF isolates alone with *F. lamaoensis* was recorded and the maximum weight was recorded in case of *F. lamoensis* + *Aspergillus niger* with a value of 26.47mg, followed by *F. lamoensis* alone with a value of 23.93 mg, *F. lamoensis* +*T. asperellum* (22.60 mg), *F. lamoensis* + *T. harzianum* (19.74 mg), *F. lamoensis* + *A. flavus* (19.09 mg) and *F. lamoensis* + *P. funiculosum* (16.46 mg) respectively and are presented in Table 4.

T. harzianum isolates reduced the colony growth of *C. capsici* when grown in dual culture. Members of *Trichoderma* spp. are known to be active hyperparasites of several fungi, and hence have been variously used as biocontrol agents. In studying the ability of antagonists to inhibit development of

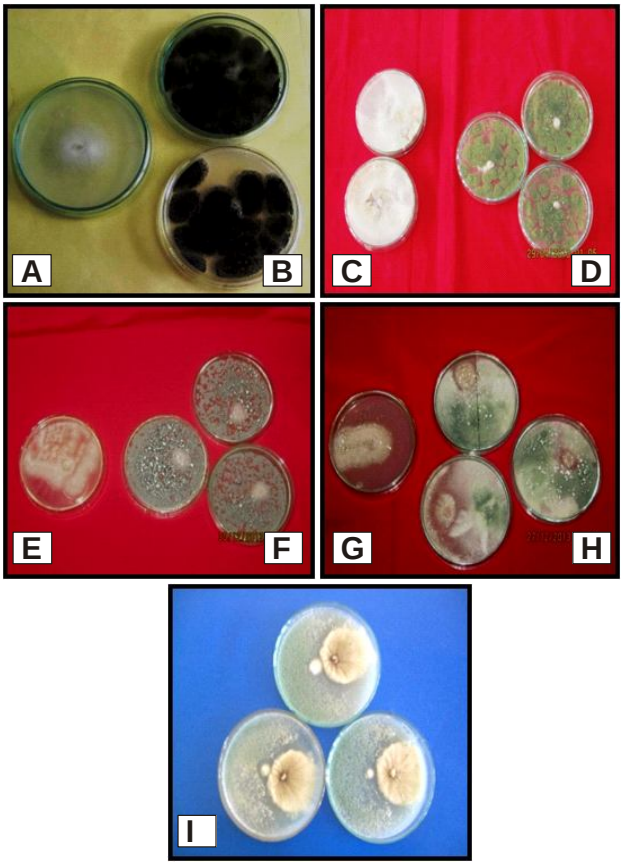

Fig 1: *In vitro* antagonistic interaction of *Fomes lamoensis* with different PSFs (6th day after inoculation) in dual culture.

A=*F. lamoensis* , **B**= *F. lamoensis+A. niger*, **C**=*F. lamoensis,* **D**= *F. lamoensis+A. flavus,* **E**=*F. lamoensis,* **F**=*F. lamoensis+P.funiculosum* , **G**=*F. lamoensis,* **H=***F.lamoensis+T. asperellum,* **I**=*Trichoderma harzianum*

the pathogenic fungi, the antagonists and the test fungi are often grown in dual cultures and the interactions are observed. Five possible modes of interacting colony growth as follows: (a) mutually intermingling growth where both the fungi grew into one another without any macroscopic signs of interaction, (b) intermingling growth where the fungus being observed is growing into the opposite fungus either above or below its colony, (c) intermingling growth where the fungus under observation has ceased to grow and is being overgrown by another colony (d) slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line of about 1-2 mm between the colonies is clearly visible and (e) mutual inhibition at a distance of >2 mm. In this study, each of the *T. harzianum*isolates assessed suppressed the growth of *Phomes lamoensis* eventually growing over it. In a similar experiment, Devaki *et al*. (1992) showed that *T. harzianum* suppressed the growth of *Pythium aphanidermatum* and *P. myriotylum* killing their mycelium within three days of inoculation as the test organisms were not recovered in the area of the culture grown over by the antagonist. The antagonist is known to control plant diseases by antagonizing plant pathogens through mycoparasitism, by producing metabolites such as Beta 1-3 and 1-4glucanases, directly competing with the pathogenic strain and inducing host resistance (Dennis and Webster, 1971a, b; Campbell, 1989; Epavier and Alabouvette, 1994; Lorito *et al*. 1996; Duiff *et al*. 1998). The *Trichoderma* isolates were all fast growing and on coming close to *F. lamoensis* the mycelia of *Trichoderma* initially grew around the *F. lamoensis*, then over it, and eventually overgrew it (Fig.1H, I). Inhibition zone was observed between *Trichoderma harzianum* isolates and *F. lamoensis*. Effect of the*Trichoderma* isolates on the radial growth of the pathogen colony, % inhibition of *F. lamoensis* from the area of interaction with *Trichoderma* and other *Aspergillus* sp. and *Penicillium* sp. are presented in Table 2. All the *T. harzianum* isolates tested significantly (Pd≤0.05) reduced the colony radius of *F. lamoensis* as compared to the control. Percent inhibition was highest for the isolate *Trichoderma harzianum* in 2nd day, 4th day and 6th days observations, respectively with a percentage inhibition value of 25%, 69.56% and 74.40% respectively though not significantly different from isolates and *F. lamoensis* was recovered from the interaction area in all the treatments assessed.*In vitro* screening of organisms is a valuable tool to select the potential strains of bio control agents (Dutta and Deb, 1988; Cirvilleri *et al*. 1999; Cook, 1993). In the biological control of plant pathogens using fungal, antagonists are biological agents with the potential to interfere in the life process of the plant pathogens. Antagonists may be applied to the soil to a) destroy the pathogen inoculum, b) prevent recolonization of the treated soil by a pathogen or c) protect germinating seeds and roots from infection etc. Antagonists are biological agents with potential to interfere in the life process of the plant pathogens. The fungal antagonists are known to be the most effective in the biological seed treatments to control the seed pathogens (i.e. *Chaetomium* sp. *Penicillium* sp., and *Trichoderma* sp). Several species of *Trichoderma* have gained the maximum popularity due to their highly antagonistic potential against several soil borne plant pathogens. The method of application of antagonists may be either direct inoculation to the soil or by stimulating the antagonists with the help of organic/inorganic soil amendments. *Trichoderma* spp. is 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 the root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients. (Islam *et al*. 2013).From the *in vitro* antibiosis study of the PSF isolates it was observed that they have good inhibitory effect on the tea root pathogen *F. lamoensis*. Depletion of nutrients, space, release of inhibitory substances/ antibiosis by the fungi are known to play a major role in antagonism and these factors are known to be mainly governed by the physico-chemical nature of the environment (Burgess and Griffin, 1967). Devi *et al.* (2013) reported from *in vitro* study that a positive antagonistic effect of *A. flavus*,*A. niger* and *T. harzianum* was observed which inhibited the growth and overgrew on the brown root rot causing organism of tea (*F. lamoensis*). *T. harzianum* also restricted the growth of the pathogen. *T. atroviride* and *Trichoderma* sp. which showed mutual intermingling of growth. *T. viride* showed intermingling of growth in which the pathogen ceased to grow and was overgrown by the antagonist. Plant dis-

eases play a direct role in the destruction of natural resources in agriculture. In particular, pathogens cause considerable loss, fungi being the most aggressive. Chemical compounds have been used to control the plant diseases (chemical control), but abuse in their employment has favored the development of pathogens resistant to fungicides. By contrast, the use of microorganisms that antagonize plant pathogens (biological control) is risk-free, when it results in enhancement of resident antagonists. Biological control of fungal plant pathogens appears as an attractive and realistic approach, and numerous microorganisms have been identified as biocontrol agents. A considerable role in limiting the population of these pathogenic fungi inhabiting the aboveground parts of the plants is played by antagonistic microorganisms. Such properties are first of all exposed by the fungi *Trichoderma* and *Gliocladium.*

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