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Research Article

Control of black rot disease of tea, *Camellia sinensis* (L.) O. Kuntze with mycoflora isolated from tea environment and phyllosphere

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ABSTRACT: The potential of some aeromycoflora and the tea phyllosphere micro organisms to control black rot disease of tea (causal organism – *Corticium theae* Bernard) was evaluated. Fungal microorganisms isolated from the tea plantation environment and phyllosphere of 11 clones of tea were evaluated. The fungal genera most frequently trapped from the environment of tea plantation were *Aspergillus flavus, Aspergillus niger, Curvularia* sp., *Penicillium* sp. and *Trichoderma atroviride*. The most frequently recovered mycoflora from the tea phyllosphere are *Aspergillus flavus, Aspergillus niger, Penicillium* sp., *Trichoderma atroviride* and *Trichoderma citrinoviride*. Experiment was carried out to assess the possible use of these micro organisms as biocontrol agents against the black rot disease of tea causing organism i.e. *Corticium theae* under *in vitro* and field conditions. The aqueous solution of the antagonists which showed maximum inhibition of the pathogen *in vitro* was applied under field conditions as foliar spray. The percentage symptom and senility index was found to be lowest in the plots sprayed with *A. niger* followed by *T. atroviride* and *T. citrinoviride*, respectively.

KEY WORDS: Aeromycoflora, antagonists, *Corticium theae,* phyllosphere

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INTRODUCTION

The microbial diversity of phyllosphere communities is influenced by plant age, species, micro- and macro-habitat, changes to environmental regimes and position of leaf on the plant (Kinkel 1997; Talley *et al*., 2002; Behrendt *et al*., 2004). Plant genera growing in close proximity have their own characteristic mycota (Kinkel, 1997) which is conditioned by the nature of the plant exudates, microclimate and by other members of the mycota (Goodman *et al*., 1986; Lucas and Knights, 1987; Osono and Mori, 2004).

Population of saprophytic micro-organisms in soil, leaf surface and air-borne propagules has been studied by different workers (Last, 1955; Ruinen, 1961; Dixit and Gupta, 1980; Satpute *et al.,* 1987). The report of the intensive investigations on leaf surface mycoflora has been reported by Last and Deighton (1965). The importance of studies on air spora over crop field to understand the dissemination and spread of microbes, especially the pathogen in the atmosphere have been emphasized by many workers (Pady *et al.,* 1965; Kaiser and Lukezie, 1966; Schnek, 1968; Tilak and Babu, 1981). Bordoloi and Baruah (1967) studied and reported the distribution of mycoflora in tea plantation, soil and air.

A number of studies have identified the ecological relationships between microbes and host plants (Baker and Cook, 1974). Knowledge of the occurrence of air-borne pathogens is helpful in controlling the disease. Some aerobiological studies conducted in India during early decades revealed the qualitative and quantitative features of air flora in different parts of the country (Rajan *et al.,* 1952; Lakhan pal and Nair, 1958; Shivpuri *et al.,* 1960; Bhati and Gaur, 1979; Satpute *et al.,* 1987). The possibility that tea may serve as vehicle for pathogen has been reported earlier (Ekanayaka *et al.,* 1987).

The mycoflora present in the air, phyllosphere and soil of the tea plantations may be interlinked and they may play important positive and or negative role in relation to disease development/control. Toxin producing organisms, if any, can be regarded under the negative role; on the other hand biological control measures of some specific tea diseases may be possible by using some of

the mycoflora trapped from the atmosphere of tea plantations (i.e. from the air and phyllosphere). No systematic study has been made on this aspect till date, especially under the agro-climatic conditions of Cachar district, Assam. Therefore, in the present work an attempt has been made to investigate the same.

MATERIALS AND METHODS

Study Area

Rosekandy Tea Estate is situated in the Barak valley which is surrounded by N. C. hills and Jaintia hills in the North, in the east by the state of Manipur, in the south by Mizoram and in the west by the state of Tripura and Sylhet district of Bangladesh (altitude of 26–30 m above main sea level and 24°8'N latitude and 29°15' E longitude.

Media for isolation of culturable fungi from phyllosphere and air

Rose Bengal agar media for the isolation of aeromicrobes and for the isolation of microbes from the phyllosphere, Czapek Dox Agar media (Tsao, 1964) were used**.**

Isolation of culturable fungi from tea phyllospheres

Eleven numbers of Tocklai (Tocklai Experimental Station, Jorhat, Assam) released tea varieties were selected for the experiment. For the isolation of leaf surface mycoflora the modified leaf washing technique of Dickinson (1971) was adopted for phyllosphere study. The tea varieties selected were TV-1, TV-9, TV-20, TV-23, TV-26, TV-27, TV-29, TV-30, S-3 A-3, Heelika and Paanitola.

The leaves collected for the isolation of phyllospheric micro organisms were of the same age/ flush. Discs of 4 mm diameter were cut randomly from five leaves of the same variety with a sterile cork borer. Fifty discs were placed in 250 ml conical flask containing 100 ml sterile distilled water and shaken for 20 minutes to get a homogenous suspension of the fungal propagules. From this, 1 ml suspension per plate (9 cm diameter) was poured in Petri plates. Czapek's Dox agar medium was poured into them and mixed thoroughly. Total mycobial population per square cm of leaf surface for each variety of tea was calculated separately using the following formula, after seven days of incubation. The experiment was repeated thrice.

$$\text{Total no. of fungi} = \frac{\text{Total no. of fungi in 1 ml x 100}}{\text{Total area of 50 discs x 2}}$$

Isolation of culturable fungi from air

Two methods were adopted for the isolation of aeromycoflora in the tea environment. Gravity Petri plate exposure method was followed by simply exposing the Petri plates containing media at 165 to 180 cm height in the tea field. Another way of trapping the air microbes was by using the two stage Andersen sampler. The two stage Andersen air sampler is a form of cascade impactor in which a two stage model provides a cut-off between respirable and non-respirable particles. The plates have progressively smaller holes from the upper most plate. Air was drawn through the sampler at 28.3 litres / min and air-borne bio – particles were deposited on the plates containing Rose Bengal Agar Medium, according to their aero – dynamic size. During the process, spores got impacted into sterile medium, which were kept for incubation at a temperature of 25°C ± 2°C (for 5–7 days). The sampler is run by AC current.

The total number of colonies isolated was correlated to the nearest count with the help of the correction factor table given by Andersen (1958) and the count was expressed as colony forming units per cubic meter of air (CFU/m^3). The correction factor was calculated as per the formula given below:

$$\text{Correction factor } (CFU/m^3) = \frac{(x + y) \times 1000}{28.5 \times 10}$$

Where x = total number of colonies in the top.

y = total number of colonies in the bottom.

Determination of fungal population

Populations of fungal microbes were determined by counting the number of colonies which appeared on the plates during incubation.

Identification of the isolated microorganism

After the isolation, the fungi were subcultured on potato dextrose agar (PDA) slants and identified consulting the literature (Raper, K. B. and Fennel, 1973; Gilman, 1956; Barnett and Hunter, 1972; Nagamani *et al*., 2002).

Antagonism studies

To ascertain whether antagonism existed between the test fungi and the pathogens, dual culture method (Wood, 1951) was employed. A 4 mm disc of the antagonistic fungi from 7 days old culture plate was placed in the petridishes containing sterile PDA medium at 2 cm apart from the pathogen. Three replicates were

prepared for each fungus. Respective controls were also made without the test fungi. All the plates were separately incubated at 25 ± 1°C for 7 days and the antagonistic colony interaction were examined thereafter. The kind and degree of antagonism was determined according to the classification of Skidmore and Dickinson (1976).

The colony growing 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 colonies. The inhibition of radial growth was calculated by using the formula of Fokkema (1973):

$$\text{Inhibition } \% = \frac{100 \times r_1 - r_2}{r_1}$$

r_1= radial growth of the pathogen in control

r_2= radial growth of *Corticium theae* in dual inoculation.

Field experiment

A field experiment was conducted to assess the efficacy of antagonistic microorganisms against black rot disease of tea in a randomized block design with six treatments and three replications. Each replicate consists of five tea bushes each; in one treatment fifteen bushes were taken under observation for each treatment. The treatment consisted of five microorganisms and an unsprayed control. The micro-organisms were sprayed on the heavily disease infested plots. The spray was repeated for three times at two weeks interval, while the control was sprayed only with water.

Field disease assessment

The experimental plants were examined for disease symptom and senility index. The tea bush plucking table was divided into four equal parts and values were assigned to each, proceeding from the infected part of the plucking table. Symptom expression in one-fourth of the plucking table was given the value 1; if half of the table was affected then the value 2 was given; if three quarter of the plucking table of the bush was affected value 3 was given, and if the symptoms were found throughout the plucking table or the plants showing symptoms of total defoliation/ death due to black rot disease the value 4 was given. A modified symptom and senility index described earlier by Dutta, (1981) was used for calculating for each group of plants in a single treatment as a percentage figure.

$$\text{Symptom \& Senility index} = \frac{\text{Sum of the individual rating value} \times 100}{4 \times \text{no of plants assessed}}$$

RESULTS AND DISCUSSION

The results of phyllosphere and aeromycoflora survey showed that the tea garden atmosphere was always with abundant with fungal spores. A total of 8 exposures were carried out by using Andersen air sampler**.** The total number of trapped micro-organisms ranged from 88.33 to 413.42 (Table 1). *Aspergillus flavus*, *Aspergillus niger*, *Curvularia lunata*, *Penicillium* sp. and *Trichoderma atroviride* were found to be dominant in the atmosphere of tea garden. Moderate population was shown by *Alternaria humicola*, *Fusarium* sp., *Penicillium rubrum* and least population of *Aspergillus aureus*, *Helminthosporium* sp., *Penicillium* sp. and *Aspergillus* sp. were recorded (Table 2). The total no of microbes ranges from 33.09 to 1259.8 in the phyllosphere. Maximum microbes were recovered from TV 27 clone, while the minimum was recovered from S 3 A 3 (Table 3). *A. niger* was found to be dominant in all the clones of tea followed by *A. flavus* while the least dominance was exhibited by *A. nidulans*, *Cladosporium* sp and *Trichoderma citrinoviride*. The antagonistic fungus grew over the colony of *C. theae* and completely inhibited its growth. The interaction was rated as Bii. *A. niger and T. atroviride* inhibited the growth of *C. theae* by 74.26% and 72.05% respectively (Table 5). The mycelial growth measurement of *C. theae* and the nine antagonists against each other on PDA on the seventh day after inoculation and percent inhibition of *C. theae* are summarized in Table 6. It can be seen that percentage symptom and senility index were found to be minimum in the black rot infested plots sprayed with aqueous extract of *A. niger* followed by *T. atroviride* and *T. citrinoviride* and maximum symptom and senility percentage was exhibited by the plots sprayed with *A. flavus* as compared to control.

Satyanarayana (1968) reported the presence of spores of *Corticium* and *Cephaleuros* in tea aerosphere. Maximum number of red rust spores was encountered during April and May and it was observed to reach its peak in the month of May. The population of leaf surface propagules has also drawn considerable attention. It is also known that these organisms play significant

Table 1: Number of microorganisms trapped in aerobiological survey done with 2- stage Anderssen sampler

Observation (no of Petri plates)	No of colonies		CFU / m^3
	Top	Bottom	
1	17	8	88.33
2	24	21	159.01
3	89	28	413.42
4	21	19	141.34
5	14	5	67.13
6	9	5	49.46
7	24	22	162.54
8	21	28	173.14

Table 2: List of the organisms trapped from the aerobiological survey

Name of the trapped organisms	Relative abundance	Diversity index
Aspergillus flavus	+++	
Aspergillus niger	+++	
Aspergillus fumigatus	++	
Aspergillus sp.	+	
Curvularia lunata	+++	
Alternaria humicola	++	2.42549
Fusarium sp.	++	
Penicillium rubrum	++	
Penicillium sp. (green)	+++	
Penicillium sp. (yellow)	+	
Trichoderma atroviride	+++	
Trichoderma citrinoviride	++	
Helminthosporium sp.	+	

+ – small population, ++ – moderate population, +++ – large population

Table 3: Phyllosphere mycoflora (per cm^2 of leaf) in different varieties of tea

Variety*	**Phyllosphere mycoflora (per cm)
TV 1	79.54
TV 9	61.07
TV20	75.28
TV 23	696.02
TV 26	567.77
TV 27	1259.8
TV 29	113.63
TV 30	113.63
S 3 A 3	33.09
Heelika	828.26
Paanitola	994.31

* TRA released clones
** Mean of five replicates

Table 4: ***In vitro*** **colony interaction of the antagonists with the test fungus (*Corticium theae*)**

Name of the antagonist	Type of colony interaction
Trichoderma atroviride	Bi
Trichoderma citrinoviride	Bi
Penicillium sp (greyish green colony)	A
Penicillium sp (fluorescent green)	D
Aspergillus niger	C
Aspergillus flavus	Bii
Aspergillus fumigatus	Bii
Curvularia sp.	A
Fusarium sp.	D

A: Mutual intermingling growth, **Bi**: Overgrowth by antagonism, **Bii**: Intermingling growth in which the test fungus under observation has ceased growth and is overgrown by another colony, **C**: Light inhibition, **D**: Not detected
*Type of colony interaction as per Skidmore and Dickinson, 1976

role in the resistant mechanism of plants from air borne plant pathogens. The reports of the intensive investigations on the leaf surface mycoflora are given by Last and Deighton (1965). A significant inhibitory activity was observed for *A. niger* and *T. viride* isolated from the phylloplane of rubber plant against *Corynespora cassiicola,* causal organism of *Corynespora* leaf fall disease of rubber (Evueh *et al*., 2011). Interestingly, the important issue that must be noticed in the present work is the effectiveness of *A. niger*, which appears to be the most effective antagonist in reducing the black rot disease in tea under *in vitro* and field conditions in barak valley, South Assam.

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Table 5: *In vitro* antagonism of fungal spp. against *Corticium theae*

Sl. No	Test mycoflora	Control (mm)	Interaction (mm)	% growth inhibition of *Corticium theae*
1.	*Trichoderma atroviride Karsten*	81.6 (±0.49)	22.8 (±0.18)	72.05
2.	*Trichoderma citrinoviride*	81.6 (±0.49)	23.5 (±0.91)	71.20
3.	*Penicillium* sp (greyish green colony)	81.6 (±0.49)	25.3 (±1.20)	68.99
4.	*Penicillium* sp (fluorescent green)	81.6 (±0.49)	60.3 (±2.10)	26.47
5.	*Aspergillus niger*	81.6 (±0.49)	21.00 (±0.57)	74.26
6.	*Aspergillus flavus*	81.6 (±0.49)	28.5 (±0.75)	65.07
7.	*Aspergillus fumigatus*	81.6 (±0.49)	24.00 (±1.65)	60.58
8.	*Curvularia* sp	81.6 (±0.49)	30.00 (±0.04)	63.23
9.	*Fusarium* sp	81.6 (±0.49)	28.8 (±0.88)	64.70
	CD at 1%			14.47
	CD ($p = 0.05$)			10.43

*Mean of three replications, the experiment is significant at 5 % level of significance
Calculation done as per Fokkema (1973)

Table 6: **Effect of foliar spray on the symptom and senility index of black rot disease of tea caused by *Corticium theae***

Treatments	Pre treatment	Percent symptoms during treatment			Post treatment
		1st	2nd	3rd	
Aspergillus niger	51.66	35	16.67	11.66	8.33
Aspergillus flavus	53.33	53.33	40	28.33	25
Penicillium sp. (greyish green colony)	56.66	45	43.33	31.66	20
Trichoderma atroviride Karsten	56.66	53.33	33.33	25	10
Trichoderma citrinoviride	55	53.33	45	35	10
Control	68.33	71.66	75	85	90
F – test	78.35	45.12	35.32	30.45	20.78

*mean of 15 plants. The experiment is significant at 5% level.

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