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Control of Black rot disease of tea {*Camellia sinensis* (L.) O Kuntze} with the mycoflora isolated from tea environment and phyllosphere

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The potential of some aeromycoflora and the tea phyllosphere microorganisms 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 of tea clones 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 microorganisms 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 *Aspergillus niger* followed by *Trichoderma atroviride* and *Trichoderma citrinoviride*, respectively.

Key words : Aeromycoflora, antagonistic, biological control, *Corticium theae,* phyllosphere, and tea clonal variety

INTRODUCTION

Air is the carrier but not the medium of growth for microbes. Studies on aeromicrobial populations are essential for understanding the survival of microbes in the soil, leaf surface and air. The presence of spores of specific plant pathogenic fungi near crop fields may suggest when measures should be taken against that particular disease (i.e. disease forecasting).

The phyllosphere may be defined as that part of the leaf serving as the interface between the plant organ and the environment. Although the phyllosphere has been referred to as a relatively "hostile environment", a number of macro and microorganisms successfully exploit this niche; thus, it serves as a microcosm for a complex series of multitrophic interactions (Jose *et al.* 2009).

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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 & Knights, 1987; Osono & Mori, 2004).

Population of saprophytic microorganisms 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 important studies of air spora over crop field to understand the dissemination and spread of microbes especially the pathogens ones in the atmosphere have been emphasized by many

workers (Pady *et al.*,1965; Kaiser and Lukezie, 1966; Schnek, 1968; Datar and Mayee, 1981; Tilak and Babu, 1981). Bordoloi and Baruah (1967) have 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 in the last decades have revealed the qualitative and quantitative features of air flora in different parts of the country (Rajan *et al.*,1952; Lakhanpal 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 or 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. The area has an altitude of 26- 30 m above main sea level and falls under 24°8'N latitude and 29°15' E longitude. Total grant area of this estate is 1702.01 hectare and area under tea is 574.70 hectare. The estate is located at a distance about 28 km from Silchar town. It is surrounded by about 8 to 10 revenue villages, cultivated fields and Chutla Bheel on all sides, respectively.

Media for isolation of aeromycoflora and microbes from phyllosphere

Rose bengal agar media (Raper and Thom,1949) 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. In the present investigation 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 microorganisms 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 replicates. 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. Observations were repeated at least once.Total no. of microbes = Total no. of microbes in 1ml X 100 / Total area of 50 discs X 2 (*Area of one disc = πr^2, where 'r' is the radius of discs in cm)

Isolation of culturable fungi from air

Two methods were adopted for the isolation of aeromycoflora in the tea environment. Gravity Petriplate exposure method was carried out by simply exposing the Petriplates containing media at human height in the tea field atmosphere. 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 two stage model providing a cut- off between respirable and non- respirable particles. The plates have progressively smaller holes from the upper most plate. Air is drawn through the sampler at 28.3 litres / min and air-borne bioparticles are deposited on the plates containing Rose Bengal Agar Medium, according to their aero-dynamic size. During the process, spores get impacted into sterile medium, which were kept for incubation at a temperature of 25 ^{0} ± 2 ^{0} 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 were expressed as colony forming unit per cubic meter of air (CFU/m^3). The correction factor was calculated as per the formula given below- Total numbers of fungal colonies X 1000/ Total volume of the air sampled X time, (CFU/ m3)= (x + y) X 1000/28.5 X 10 where x = total number of colonies in the top, y =total number of colonies in the bottom.

Determination of microbial population

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

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

Antagonism studies

To ascertain whether antagonism existed between the test fungi and the pathogens, dual culture method was employed. A 4 mm disc of the antagonistic fungi from 7 days old culture plate was placed in the Petridishes containing sterile Potato dextrose agar 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 ^{0} 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).

Dual culture method (Wood, 1951) was followed for examining the interactions. A 4 mm discs of the pathogen and test fungus in triplicate on PDA containing Patridishes were placed from their 7 days old cultures. The plates were then incubated for 7 days at 25±1°C. Control plates were kept simultaneously. The colony grew 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 by Fokkema (1973) :- % inhibition = 100 x 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 in the Section 91-B of Rosekandy Division of Rosekandy Tea Estate (24^{0}8' N; 29^{0}15' E) 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 consisted 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 microorganisms 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 are found throughout the plucking table or the plants are 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 : Symptom & Senility index= Sum of the individual rating value × 100 / 4 × no of plants assessed.

RESULTS AND DISCUSSION

The results of phyllosphere and aeromycoflora survey showed that the tea garden atmosphere was never free of fungal spores. A total of 8 exposures were carried out by using Andersen Air Sampler. The total number of trapped microorganisms ranged from 88.33 to 413.42 (Table 1). This suggests that large number of aeromycoflora can be trapped which can be further screened for the biological control studies of the tea diseases. *Aspergillus flavus*, *Aspergillus niger*, *Curvularia lunata*, *Penicillium* sp. and *Trichoderma atroviride* were found to be dominant in the atmosphere of tea garden.

Table 1 : Results of the aerobiological survey done with 2-stage Andersen sampler

Observation (no of Petriplates)	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	Their population status
Aspergillus flavus	+++
Aspergillus niger	+++
Aspergillus fumigatus	++
Aspergillus sp.	+
Curvularia lunata	+++
Alternaria humicola	++
Fusarium sp.	++
Penicillium rubrum	++
Penicillium sp.(green)	+++
Penicillium sp.(yellow)	+
Trichoderma atroviride karsten	+++
Trichoderma citrinoviride	++
Helminthosporium sp.	+

* + - small population, ++ -moderate population, +++ - large population

Table 3 : Total no of microbes with the respective clones of tea calculated as per the formula given by Dickinson (1971).

Clones of tea leaves*	Total no of microbes
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

The moderate population was shown by *Alternaria humicola*, *Fusarium* sp. *Penicillium rubrum* while *Aspergillus aureus*, *Helminthosporium* sp, *Penicillium* sp. and *Aspergillus* sp. exhibited least population (Table 2). The total no of microbes ranged from 33.09 to 1259.8 in the phyllosphere. Maximum microbes were recovered from TV 27 clonal variety of tea, while the minimum was recovered from S 3 A 3 (Table 3). *Aspergillus niger* was found to be dominant in all the clones of tea followed by *Aspergillus flavus* while the least dominance was exhibited by *Aspergillus nidulans*, *Cladosporium* sp. and *Trichoderma citrinoviride* (Table 4). The antagonistic fungus grew over the colony of *Corticium theae* and completely inhibited its growth. The interaction was rated as Bii. *Aspergillus niger* and *Trichoderma atroviride* inhibited the growth of *Corticium theae* by 74.26% and 72.05% respectively (Table 5). The mycelial growth measurement of *Corticium theae* and the nine antagonists against each other on Potato Dextrose Agar on the seventh day after inoculation and per cent inhibition of *C. theae* are summarized in Table 6.

Both antibiosis and parasitism play important role in biocontrol of plant diseases. There are two ways by which biocontrol agents can suppress the plant pathogen: (i) production of antibiotics or (ii) production of hydrolytic enzymes. Antagonistic microorganisms reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion. The exploitation of biocontrol agents for the management of plant diseases have achieved greater significance in recent times due to its readily available nature, antimicrobial activity, easy biodegradability, non-phy-

Table 4 : List of organisms isolated from the leaves of some clonal tea plants by leaf washing technique

Organism isolated	Clones of tea										
	TV1	TV9	TV20	TV23	TV26	TV27	TV29	TV30	S3A3	Heelika	Paanitola
Alternaria alternata	-	+	+	-	-	-	+	-	-	-	+
Aspergillus flavus	-	+	+	-	-	+	+	+	+	+	+
Aspergillus niger	+	-	+	+	+	+	+	+	+	+	+
Aspergillus nidulans	-	-	-	+	-	-	-	-	-	+	-
Curvularia sp.	-	+	-	+	-	-	-	-	+	-	+
Cladosporium sp.	+	-	-	-	-	-	+	+	-	-	-
Fusarium sp.	-	+	-	-	-	-	+	+	-	+	-
Mucor sp.	-	-	-	-	+	-	-	+	-	+	+
Penicillium sp.	+	+	-	-	+	+	+	-	-	+	+
Trichoderma atroviride	-	-	+	-	+	+	+	-	-	-	-
Trichoderma citrinoviride	-	-	-	+	+	-	-	-	-	-	

Table 5 : *In vitro* colony interaction of the antagonists with the test fungus (*Corticium theae*)

Name of the antagonist	Type of colony interaction
Trichoderma atroviride Karsten	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

totoxicity, besides inducing resistance to host. Satyanarayana (1968) studied the air spora of the tea fields at Jorhat using the modified Durhams spore trap. He reported about the spores of *Corticium* and *Cephaleuros* in air. 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 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 *Aspergillus 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.

In the present work a total of 15 fungal species have been isolated and identified from the air and phyllosphere of tea plantation area. Among them

Table 6 : *In vitro* antagonism of fungal spp. against. *Corticium theae*

Test mycoflora	Control (mm)	Interaction(mm)	% growth inhibition of *Corticium theae*
Trichoderma atroviride Karsten	81.6(±0.49)	22.8(±0.18)	72.05
Trichoderma citrinoviride	81.6(±0.49)	23.5(±0.91)	71.2
Penicillium sp. *(greyish green colony)*	81.6(±0.49)	25.3(±1.2)	68.99
Penicillium sp. *(fluorescent green)*	81.6(±0.49)	60.3(±2.1)	26.47
Aspergillus niger	81.6(±0.49)	21.00(±0.57)	74.26
Aspergillus flavus	81.6(±0.49)	28.5(±0.75)	65.07
Aspergillus fumigatus	81.6(±0.49)	24.00(±1.65)	70.58
Curvularia sp.	81.6(±0.49)	30.00(±0.04)	63.23
Fusarium sp.	81.6(±0.49)	28.8(±0.88)	64.7

*Calculation done as per Fokkema (1973)

Penicillium, Aspergillus, Fusarium and *Curvularia* are found to be dominated in all the conditions .Total fungal population in the phyllosphere of clonal variety was highest in TV- 27 followed by Paanitola and Heelika. Further investigations are required to be carried out in order to get an insight on the probability of the production of mycotoxins on the processed tea, if any and to reduce the possible contamination of the same with the toxin-producing micro-organisms in tea at large. The initial studies gave positive indication to this effect (Dutta *et al.*, 2008).

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