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# Analysis of strain variation in *Colletotrichum gloeosporioides,* a phytopathogenic fungus of Mangrove plants of Sundarbans (Eastern India)

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Rural poor and marginalized people of mangrove forests of Sundarbans depend on mangrove plants for primary health problems. They harbour a wide array of novel phytoconstituents which are potential sources of future drugs against ancient and emergent diseases. This treasure house of mangroves is now the target of various fungal attacks which may create conservation problem in future and may affect the quality of drug. During the survey of foliicolous fungi of some ethnobotanically important medicinal mangrove plants of Indian Sundarbans a number of interesting potential fungi were isolated and recorded from infected leaves. Phytopathogenic fungus *Colletotrichum gloeosporioides* was found to grow abundantly in a wide range of mangrove hosts in different localities of Sundarbans. This has created an interest whether any strain variation exists in the aforesaid fungal species. Results indicate the existence of distinct strains within the isolates of aforesaid species.

**Key words:** Mangrove, foliicolous fungi, ethnobotany, *Colletotrichum gloeosporioides*

## INTRODUCTION

Mangroves are specialised forest ecosystem found at the land-sea interface of the tropical and subtropical regions of the world bordering the sheltered sea coasts and estuaries. These forest systems are dominated by the salt tolerant halophytic seed plants that ranged in size from tall trees to shrubs and being restricted to the intertidal belts are exposed to the high and low tides twice in 24 hours. This very vibrating ecosystem supports numerous terrestrial, benthic and aquatic organisms forming a complex association of species, exchanging materials and energy within the system and between the systems, and the adjoining coastal waters.

The Sundarbans are the largest mangrove forest in the world, covering about one million hectares of the Ganges-Brahmaputra delta in India and Bangladesh. Flora includes total of 69 mangrove species distributed in 49 genera and 35 families. Unlike most mangroves in other parts of the world, the Sundarban flora is dominated by the families Sterculiaceae and Euphorbiaceae. The vegetation is characterized by the Sundari (*Heritiera fomes* Buch.-Ham.) trees from which the name of the forest is derived. It is associated with other trees such as Gewa (*Excoecaria agallocha* L.), Goran (*Ceriops decandra* (Griff.) Ding Hou) and Keora (*Sonneratia apetala* (L.) Engl.). All most all the plants reported so far from this region are economically and commercially important. Various parts of the plant have been traditionally used in indigenous medicine by the local communities of the mangroves across tropical Asia and Oceania. The Sundarbans play a significant role in the regional economy of the Lower Gangetic Delta, and the national economy of Bangladesh (Getzner and Islam, 2013).

Mangroves constitute a dynamic ecosystem with a wonderful consortium of different life forms including plants, animals and microbes of both terrestrial and aquatic habitats. Several mycologists have shown interest in the exploration of fungi in tropical mangroves. Although a number of fungal species have been reported by a number of workers from Indian mangroves (Alias *et al.*, 2010; Bhimba *et al.*, 2011; Khan and Manimohan, 2011; Li *et al*., 2011; Pal, 2012; 2014; 2017; Sarma, 2012; Borse *et al*,.2013; Suciatmih and Rahmansyah, 2013) very little is known about fungal diversity and their parasitic relationship with

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mangrove plants of Sundarbans. Sundarbans has a humid, tropical maritime. The average maximum and minimum temp. 29° C (June-July) and 20° C (Dec.-Jan.) respectively and the humidity varies between 70-80%. Leaf-infecting fungi of Sundarbans play a significant role in litter decomposition apart from their involvement in various diseases of mangrove plants. Despite the fact, a large majority of them remains yet to be identified. This communication is intended to focus the extent of diversity, their host specificity, local distribution, pathogenicity and strain variation among the isolates of *Colletotrichum gloeosporioides* (Penzig) Penzig & Sacc.

## MATERIALS AND METHODS

### *Isolation of fungus from infected plant parts*

Both healthy and infected leaves were collected at random from living trees, shrubs and herbs of Sundarbans forest (Bhagbatpur, Mayadwip, Dulibhasani, Pirkhali, Prentice Island, Netidhopani, Bak-khali, Chulkati, Dhonchi, Saznakhali, Kalas Island, Luthian Island etc) in different seasons placed in polythene bags and brought to the laboratory for examination. The organisms were isolated from infected leaves following the standard method (0.1% $HgCl_2$ and sterile distilled water), identified and maintained in Potato-dextrose-agar medium at 25± 1°C. Dried infected plants were finally mounted on herbarium sheets.

### *Maintenance of stock cultures*

Fungal cultures were finally stored under three different conditions. Two sets of cultures were maintained at $5^0$ C and $20^0$ C respectively. The third set of culture was preserved in sterilized liquid paraffin and kept at $25^0$C. Subculture was accomplished at a regular interval of time.

### *Assessment of mycelia growth on solid medium*

For the preparation of inocula desired fungus was grown in a Petri dish (100 mm diam.) containing 0.5% Dextrose Agar medium. Usually a block (4 mm diam.) of agar containing mycelia was cut out with the help of a sterilized cork borer from the advancing zone of the mycelial mat (4-day-old culture) and transferred to PDA medium in a Petri dish (100 mm diam.), incubated at 26-$28^0$ C and under diffused light. The growth characteristics were noted after 5 and 30 days of inoculation. Rayner's (1970) colour chart was used for description of colour of mycelial mats.

### *Assessment of mycelial growth in liquid medium*

To study the mycelial growth in a liquid medium, an agar block (4 mm diam.) containing 4-day-old mycelia was transferred to Erlenmeyer flask containing the desired liquid medium (50 ml/250 ml flask) and incubated for 7 days at 26-$28^0$ C except otherwise stated. At the end of the experimental period, the mycelia were collected, transferred to aluminium foil cups of known weights, dried at $60^0$ C for 96 hr, cooled in a desiccator and weighed. The weight of mycelia recorded was the average of 3 replicates. The efficacy of fungicides was evaluated against fungal strains using poisoned food method.

### Pairing Technique

Inocula were prepared as described earlier and paired in possible combinations in Potato Dextrose Agar (PDA) medium within the Petridishes (100mm diam.) and incubated in temperature 26º – 28ºC for 14 days under diffused light. Reactions were noted after 14 days.

## RESULTS AND DISCUSSION

Detail list of some important medicinal mangrove plants of Sundarbans with their medicinal property have been published earlier ( Pal, 2017). In spite of their origin, natural drugs should not be viewed as simple tools of folk medicine since they are a class of pharmaceutical products and should meet the requirements of quality, safety and efficacy.

The increasing popularity of natural drugs made their use to become a public health problem, due to the lack of surveillance of use, efficacy, toxicity and quality of these natural products. Adverse long term herbal use, adulteration with toxic compound and contamination pathogenic microbial or natural toxins like mycotoxins have been reported for herbal products and medicinal plants. Most of the fungi isolated and identified from medicinal mangrove plants of Sundarbans have the ability to produce mycotoxins. If the raw material (i.e. leaf etc.) contains mycoflora, there always remains a chance that the final herbal product will also be

contaminated. Considering the worldwide increased use of herbal products, the risk of purchase and use of natural products contaminated with mycoflora, it is essential to set appropriate standards for toxigenic fungi in crude herbal drugs and medicinal plants in order to reduce the risks for consumers'.

In course of this study, a variety of fungi were isolated from infected leaves of mangrove plants of Sundarban. List of those isolated fungi and their host plants have been documented ( Pal, 2017). The listed fungi represent 3 classes, viz. Pyrenomycetes (1 species), Coelomycetes (12 species) and Hyphomycetes (13 species). The most prevalent genera are *Pestalotiopsis* (8), *Curvularia* (4), *Alternaria* (4), *Cladosporium* (3) and *Colletotrichum* (1). Total 26 species of fungi belonging to 11 genera were isolated from infected leaves from 50 hosts of mangrove plants of Sundarbans, out of which 21 species of fungi have been described in details (Pal, 2017). The spectrum of fungal diversity in Sundarbans appears to be very wide and hence frequent exploration is needed. It is not unreasonable to assume that diversity in parasitic fungi is related to parasitism since increase in diversity leads to enhance parasitism. It is of common occurrence in the field that a fungal parasite having different strains may attack a wide range of host species. Genetic diversity in all types of organisms is increasing with time due to natural and/or unnatural causes. Besides, evolutionary changes also take place in both hosts and parasites which is probably necessary for balancing the changes in resistance of the host and virulence of the pathogen.

### *Studies on strain variations in Colletotrichum gloeosporioides isolated from mangrove plants*

*Colletotrichum gloeosporioides* was found to grow on 14 different host species ( *Acanthus ilicifolius, Aegialitis rotundifolia, A. alba* , *A. officinalis* , *Ceriops tagal* , *Derris scandens* , *D. trifoliata*, *E. agallocha* ,*Lumnitzera racemosa, Nypa fruticans, P. Paludosa, Rhizophora apiculata* , *R. mucronata* and *Sonneratia caseolaris*). Since the organism is able to parasitize a variety of hosts it was considered worthwhile to study whether any strain variation exists within the species. This fungus was isolated from leaves of 14 different host species but after comparing the growth characteristics, 10 were selected for further study . A number of experiments were carried out with a view to confirm whether strain variation exists within the species. Apart from growth characteristics, their interactions, growth responses to amino acids, amide and fungicides were also studied.

### *Growth characteristics of different isolates of C . gloeosporioides*

Ten isolates of *C gloeosporioides* were grown in Petri dishes containing PDA medium and incubated at 26°C - 28°C under diffused light of the culture room . Growth characteristics were noted after 5 days and 30 days respectively . Results (Table 1 ) revealed that among the ten isolates, three ( D,I and J ) showed maximum growth **(**64 mm dia in 5 days), while isolate E exhibited minimum growth (29 mm diam.) in a similar period. Other isolates however, showed moderate growth ( 50 – 58 mm dia). In all cases mycelial mats covered the entire Petri dish within 30 days, except the isolate H, which exhibited 86 mm dia. The texture of mycelial mats of most of the isolates was found to be felty to subfelty. But isolates A and J showed lacunose and floccose respectively. The texture of all the cultures changed after 30 days of incubation. Except A and I, other isolates showed buff colour. It was interesting to note that out of 10 isolates, 4 (E, H, I and J) sporulated within 5 days but others did not sporulate even after 30 days of incubation on PDA. It appeared from growth characteristics that strain variation exists among the isolates of *C. gloeosporioides* obtained from 10 different host species. Therefore, it was decided to pair the 10 isolates in all possible combinations to study the pairing reaction between isolates.

### *Pairing between isolates of C. gloeosporioides*

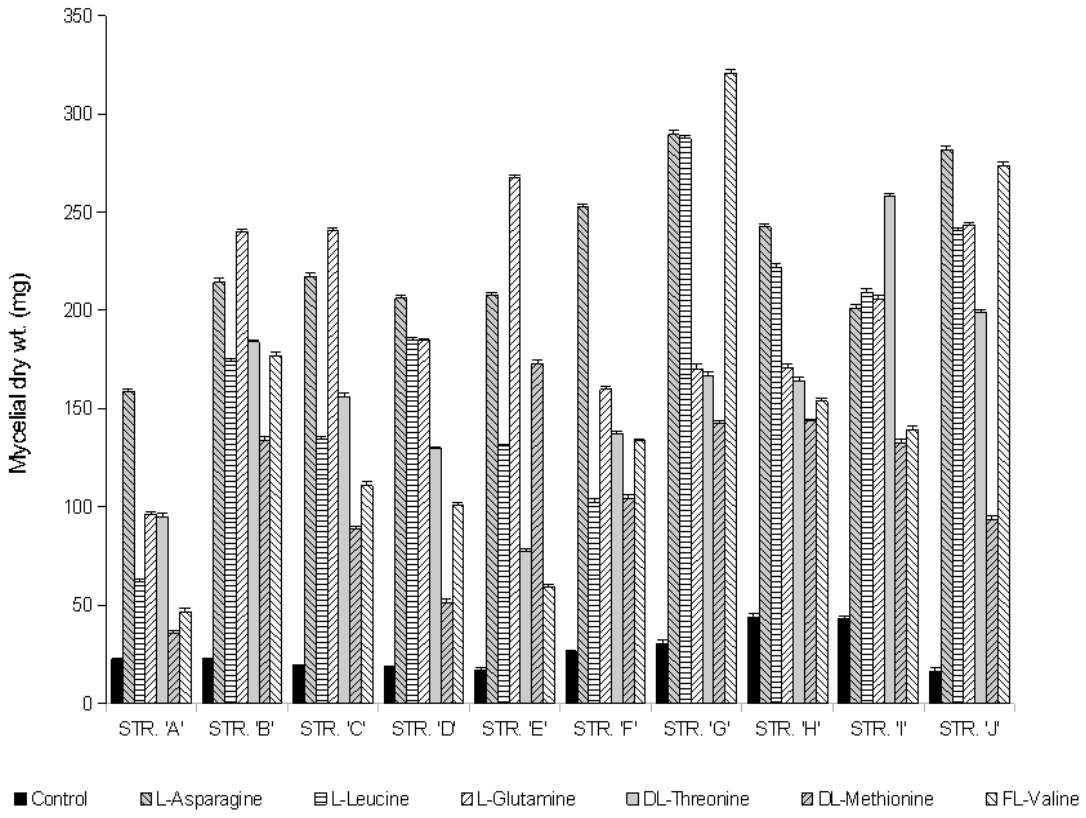
A pairing experiment was carried out with a view to study the reaction between different isolates of *C. gloeosporioides*. The isolates were paired in all possible combinations in Petri dishes (100 mm diam.) containing PDA and incubated for 14 days at $26^0$-$28^0$ C under diffused light of the laboratory. In case of control, pairing was done between the two subcultures of the same isolate. The paired cultures were examined after 14 days of incubation and the results are summarized in Table 2. The results of pairing experiment reveal 4 types of reactions viz., (i) homogeneous, (ii) line of contact, (iii) space of aversion and (iv) overgrowth. In most

**Table 1.** Comparison of growth characteristics of different isolates of *Colletotrichum gloeosporioides* on PDA medium

| Host | Code of isolates of *C.gloeosporioides* | Av. Diam. of mycelial mat (mm) (5 days)* | Texture | | Colour | | Sporulation | |
|---|---|---|---|---|---|---|---|---|
| | | | 5 days | 30 days | 5 days | 30 days | 5 days | 30 days |
| *Aegialitis rotundifolia* | A | 50.33±0.33 | Lacunose | Thin floccose growth around the inoculum and appressed throughout the periphery | White | Grey | – | – |
| *Rhizophora apiculata* | B | 58.00±0.57 | Felty to subfelty with thick mycelial mat around the inoculum | Downy around the inoculum and felty towards periphery | Buff | Buff | – | – |
| *Acanthus ilicifolius* | C | 52.33±0.33 | Downy around the inoculum and felty towards periphery | Downy (3 -4 rings around the inoculum) | White | White | – | – |
| *Sonneratia caseolaris* | D | 63.66±0.88 | Felty around the inoculums and subfelty towards periphery | Felty to subfelty | Buff | Buff | – | – |
| *Phoenix paludosa* | E | 29.00±0.57 | Felty | Felty | Buff | Buff | + | + |
| *Derris trifoliata* | F | 53.66±0.33 | Subfelty | Downy | Buff | Buff | – | _ |
| *Aegialitis alba* | G | 53.33±0.33 | Felty with depressed ring around the inoculum | Downy | Buff | Buff | – | – |
| *Ceriops tagal* | H | 49.33±0.33 | Felty to subfelty | Downy | Rosy buff | Buff | + | + |
| *Lumnitzera racemosa* | I | 64.00±0.57 | Felty | Downy | White | Buff | + | + |
| *Exoecaria agallocha* | J | 64.66±0.33 | Floccose around the inoculums, downy towards the periphery | Floccose | Buff | Buff | + | + |

*In all cases mycelial mat covered the entire Petri dish (100 mm in diam.) within 30 days of incubation except the isolate 'H' where it was found to be 86 mm.

Temperature – 26$^0$-28$^0$C; Initial pH – 5.5; Average of 3 replicates/treatment; + = Spore present; – = spore absent



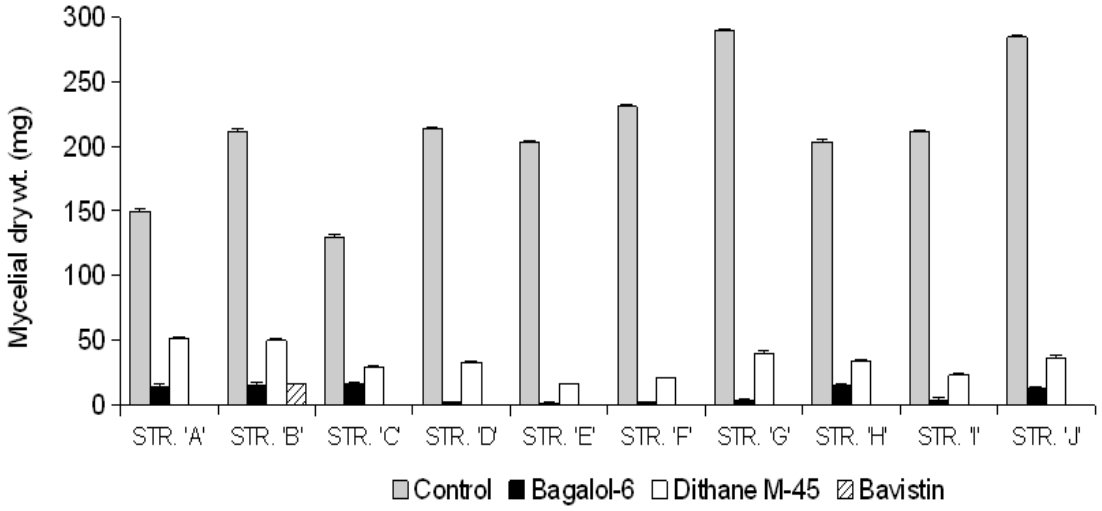

Growth responses of different strains of *C. gloeosporioides* to amino acids and amide

**Fig. : 1**

**Table 2.** Pairing reactions between isolates of C*olletotrichum gloeosporioides*

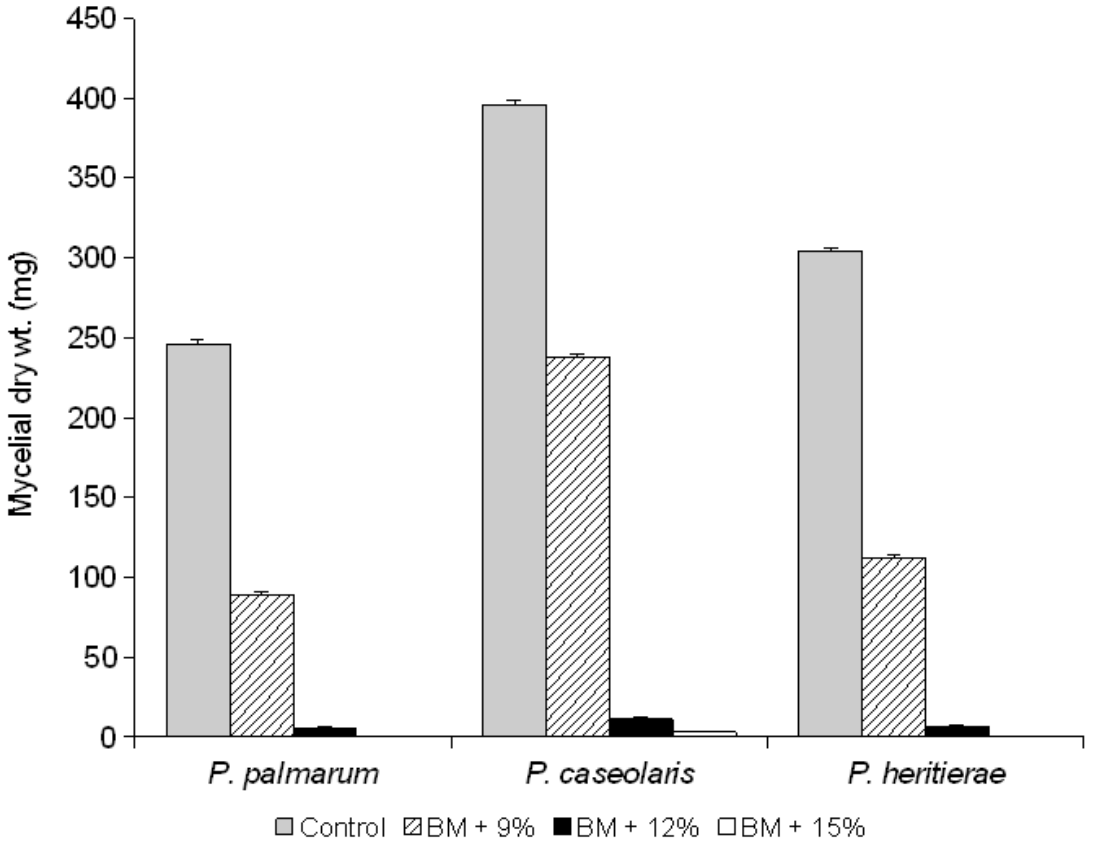
| Host | Code of isolates of *C. gloeosporioides* | Combinations | Reactions |
|---|---|---|---|
| *Aegialitis rotundifolia* | A | A x A | Homogeneous |
| *Rhizophora apiculata* | B | B x B | Homogeneous |
| | | B x A | Space of aversion |
| *Acanthus ilicifolius* | C | C x C | Homogeneous |
| | | C x A | Space of aversion |
| | | C x B | Line of contact |
| *Sonneratia caseolaris* | D | D x D | Homogeneous |
| | | D x A | Space of aversion |
| | | D x B | Space of aversion |
| | | D x C | Space of aversion |
| *Phoenix paludosa* | E | E x E | Homogeneous |
| | | E x A | Space of aversion |
| | | E x B | Line of contact |
| | | E x C | Space of aversion |
| | | E x D | Space of aversion |
| *Derris trifoliata* | F | F x F | Homogeneous |
| | | F x A | Space of aversion |
| | | F x B | Line of contact |
| | | F x C | Line of contact |
| | | F x D | Space of aversion |
| | | F x E | Space of aversion |
| *Aegialitis alba* | G | G x G | Homogeneous |
| | | G x A | Space of aversion |
| | | G x B | Space of aversion |
| | | G x C | Space of aversion |
| | | G x D | Line of contact |
| | | G x E | Overgrowth |
| | | G x F | Overgrowth |
| *Ceriops tagal* | H | H x H | Homogeneous |
| | | H x A | Space of aversion |
| | | H x B | Line of contact |
| | | H x C | Line of contact |
| | | H x D | Space of aversion |
| | | H x E | Space of aversion |
| | | H x F | Line of contact |
| | | H x G | Space of aversion |
| *Lumnitzera racemosa* | I | I x I | Homogeneous |
| | | I x A | Space of aversion |
| | | I x B | Line of contact |
| | | I x C | Line of contact |
| | | I x D | Line of contact |
| | | I x E | Line of contact |
| | | I x F | Line of contact |
| | | I x G | Space of aversion |
| | | I x H | Space of aversion |
| *Exoecaria agallocha* | J | J x J | Homogeneous |
| | | J x A | Space of aversion |
| | | J x B | Space of aversion |
| | | J x C | Line of contact |
| | | J x D | Line of contact |
| | | J x E | Overgrowth |
| | | J x F | Line of contact |
| | | J x G | Line of contact |
| | | J x H | Line of contact |
| | | J x I | Line of contact |

Light – diffused light; Temperature – $26^0$-$28^0$C; Diameter of inoculums – 4 mm;
Incubation period – 14 days; 3 replicates/ treatment.




**Effect of different fungicides on mycelial growth of different strains of *C. gloeosporioides***

**Fig.: 2**




**Growth responses of some fungi to different concentrations of NaCl**

**Fig. : 3**

cases either line of contact or space of aversion was noticed. Out of 55 pairings only 3 (G x E, G X F and J x E) showed overgrowth. Homogeneous reactions (interacting mycelia intermingled freely) were noted only when the mycelia from the same culture were paired. The results of pairing experiments also confirmed that strain variation exists among the isolates of *C. gloeosporioides*.

### *Growth response of different strains of C. gloeosporioides to various amino acids and amide.*

The results of previous experiments suggest that strain variation exists in *C. gloeospo rioides*. To study the growth responses of these strains to different amino acids and amide (L-Asparagine, L-Leucine, L-Glutamine, DL-Threonine, DL-Methionine, DL-Valine) an experiment was carried out *in vitro* under identical conditions. Utilization of amino acids could be regarded as one of the important characteristics in determining the strain of a species. In this experiment, a basal medium (Glucose, 10 g; $KH_2PO_4$, 1 g; $MgSO_4$. $7H_2O$, 0.5 g; Thiamine hydrochloride, 500 $\mu$g; 1 litre distilled water) was supplemented separately with desired amino acids. The amount of nitrogen present in 0.2% Asparagine was taken as a standard and the initial pH was adjusted to 5.5 by using either N/10 HCl or N/10 NaOH. The results are presented in Figure 1. Among the amino acids tested L-Asaparagine was preferred by 5 strains (A, D, F, H and J), while 3 strains (B, C and E) favoured L-Glutamine. For strain G and I, DL-Valine and DL-Threonine respectively were most favourable for growth. All the strains, however, sporulated in presence of L-Asparagine although the intensity of sporulation varied considerably with the strains. The strain E showed little sporulation in Control medium (without any amino acids or amide). It is necessary to mention here that all the strains exhibited poor growth (16-44 mg) in basal (control) medium.

### *Effect of fungicides on mycelial growth of different strains of C. gloeosporioides*

Three different fungicides viz., Bavistin (a.i. 50%), Bagalol-6 (a.i. 50%) and Dithane M-45 (a.i. 75%) were tested against the strains of *C. gloeosporioides*. The flask containing sterilized medium (Glucose, 15 g; L-Asparagine, 2 g; $KH_2PO_4$, 1 g; $MgSO_4$. $7H_2O$, 0.5 g; 1 litre distilled water) were supplemented with desired amount of fungicide. A control set was maintained without any fungicide. Each flask containing 50 ml medium was inoculated as described earlier and incubated for 7 days at $26^0$-$28^0$C. At the end of incubation period mycelia were collected, dried and weighed. Results are given in Figure 2. The non-systemic fungicides (Bagalol-6 and Dithane M-45) were found to be less inhibitory than the systematic one (Bavistin) at 10 $\mu$g $ml^{-1}$ level. Bavistin (10 $\mu$g $ml^{-1}$) appeared to be most effective in controlling the growth of all the strains except B which showed poor growth (16 mg dry wt. of mycelia) even after 7 days of incubation. Between Bagalol-6 and Dithane M-45, Bagalol-6 was more effective for all strains tested. Among the strains, C was apparently less sensitive to Bagalol-6. Similarly, strain A, B and G were less sensitive than other strains to Dithane M-45. Strains G and C showed maximum (289 mg) and minimum (130 mg) growth respectively in control medium.

Variability and pathogenicity of isolates of *C. gloeosporioides* from *Hevea brasiliensis* (Willd. ex Juss.) Muell. Arg. were studied. Twenty seven isolates of this fungus differed greatly in appearance and intensity of sporulation. Later, physiological and physiological variations between two isolates (1 and 5) of the same fungus from rubber plant (*H. brasiliensis*) were also recorded. It was concluded that both the isolates secreted $\alpha$-1, 4-glucanase and â-glucosidase when carboxymethyl cellulose was used as the main source of carbon in the liquid medium. The activities of these cellulolytic enzymes were greater in isolate 1 than in isolate 5. The morphology of the two isolates was similar but differed in colour and length of conidium. Isolate 1 sporulated profusely compared to isolate 5. In the present study, pairing experiments were carried out in all possible combinations among the 10 isolates from different hosts. The strains were differentiated on the basis of their distinctive growth characters, pairing reactions, growth responses to different amino acids and amides and tolerance to different fungicides. The results confirm the existence of 10 distinct strains within *C. gloeosporioides*. This is not unnatural since previous workers have also reported strain variation in this fungus. It is not known why this pathogen prefers a wide range of mangrove hosts in Sunderbans. Cross inoculation tests on different mangrove hosts in future will determine the host specificity of different strains of *C. gloeosporioides*, if any.

***Studies on salt tolerance of selected foliar fungi isolated from mangrove plants***

The results of experiments presented strongly indicated that strain variation exists in *C. gloeosporioides*. Apart from strain variation, salt tolerance of some foliar fungi isolated from mangrove plants were also studied *in vitro*. Because it was presumed that the isolated microorganisms could be more tolerant to hypersaline environment like their host species and if so, the salt tolerant gene could be exploited in future. In view of the above statements it was considered worthwhile to test the salt tolerance of organisms. Initially, a pilot experiment was designed using 27 foliar fungi including *C. gloeosporioides* to study their growth responses to 6% NaCL supplemented with a nutrient medium (glucose, 15 g; asparagine 2 g; $KH_2PO_4$, 1 g; $MgSO_4$. $7H_2O$, 0.5 g; distilled water, 1 litre). A control set was maintained without NaCl.. pH was also adjusted to 5.5 by using N/10 NaOH. Each flask containing 50 ml medium was inoculated as described earlier and incubated for 8 days at $30^0$-$32^0$C. Only organisms which showed less than 30% reduction in growth in 6% NaCl were selected for testing at a higher level of NaCl. In this experiment growth responses of three selected fungi viz., *F. solani, P. caseolaris* and *P. heritierae* to different concentrations of NaCl (9%, 12%, 15% and 18%) were tested under identical conditions. It is evident from the results (Figure 3) that *P. caseolaris* is most tolerant to NaCl. It grows even at 15% NaCl, whereas total inhibition of growth was noted for *F. solani* and *P. heritierae*. It is necessary to mention here that *P. caseolaris* which was found to be most tolerant to NaCl among 27 foliar fungi could not grow in the medium when concentration of NaCl was increased to 18% level.

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