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Influence of meteorological factors on atmospheric fungal spores over crop canopy of rice in West Bengal, India with reference to major fungal diseases

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Rice is a major cereal crop and staple food for the people of India. Fungal spores in the atmosphere are considered to be one of the most potent bio-pollutants, which are pathogenic to rice crops having direct impact on our food security. The aim of the present study was to assess the concentration of airborne fungal spores over a paddy field in relation to meteorological parameters and disease severity with an objective to prepare an efficient forecasting system for most important fungal diseases. Volumetric aero-mycological survey was carried out by Burkard personal sampler and Andersen sampler that were operated for three consecutive *kharif* (rainy) seasons (2015-2017) at weekly intervals over a rice field in West Bengal, India. The meteorological parameters during the investigation period were recorded at the nearest meteorological station. The concentrations of culturable and non-culturable spore types varied in different growth phases. The dominant genera trapped by Burkard sampler were *Cladosporium* sp. (9.97%), *Memnoneilla* sp (6.60%), *Trichoconis* sp (5.63%), *Stemphyllium* sp. (5.65%), smut spores (5.65%), *Helminthosporium* sp. (5.22%), *Trichoderma viride* (5.02%), *Pyricularia* sp. (4.75%). Among the 18 mould types recorded by Andersen sampler, *Aspergillus niger* (8.95 %) was the most dominant one, followed in the degree of prevalence by *Aspergillus clavatus* (6.98%), *Penicillium claviformae* (6.11%), *Trichoderma lignorum* (5.50%), *Penicillium expansum* (5.44%), *Alternaria alternata* (5.43%) and *Fusarium oxysporium* (4.33%). A statistical correlation was calculated among the fungal spore loads with the five meteorological parameters, which would be beneficial to predict the spore concentration with the respective meteorological parameters. The total spore count exhibited a negative correlation with maximum temperature, minimum temperature, relative humidity, rain fall and wind speed but positively correlated with age of the plant. The regression model based on the spore count and meteorological variables supplemented by host factors is found to be useful for prediction of rice diseases. Three major fungal diseases were considered with the concentration of the respective fungal pathogens. Relationship between the disease scales along with the growth phases were also recorded.

Keywords: Fungal spores, Meteorological variables, rice canopy, rice diseases.

INTRODUCTION

The atmosphere contains a heterogenous mixture of bioparticles such as fungal spore, pollen, insect parts, trichomes, etc., of which fungal spores constitute a significant fraction of airborne bioparticles, and their prevalence in air is almost 10^2 - 10^3 times higher than that of pollen grains (Jones and Harrison, 2004). Composition and concentration of airborne mycoflora depend on several factors including topography, time of day, meteorological parameters, type of vegetation, air pollution, agriculture, industrial and other human activities (Pepeljnjak and Sevic Klaric, 2003).

Airborne fungal spores have an important role in the spreading of many plant diseases causing serious agricultural loss, with occasional epidemics (Saha, 2016). Fungal spores in the atmosphere affect the grain production with direct impact on our food security (Tilman *et al.* 2002; Strange, 2005). Airborne fungal spores which cause plant diseases occur a few days earlier in the atmosphere than the appearance of the actual disease symptoms. Agriculture sciences have been trying to maximize the crop yield. But there is a need to protect the crop from their pathogens like fungi. Rice (*Oryza sativa*) is the main agricultural crop and also the prime staple food for the people of West Bengal. West Bengal ranks 1st in India in rice production, producing 14%

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of the total production in India (Anjaneyulu 2015; Jain 2018). It is grown over 58.30 million ha with an average yield of 2,256 kg/ha/annually. In the North 24 Parganas district, the total area under rice cultivation is about 273.6 thousand ha and its annual production is 2,628 kg/ha (Aktar, 2015).

Volumetric survey of air spora was first conducted in India at Vishakhapatanam (Chakraborty *et al.* 2003). This study was followed by Vittal and Krishnamoorty at Madras (Adhikari *et al.* 2004). Deka (2013) using volumetric sampler reported aero-mycoflora over three different rice fields for two consecutive years in Kamrup district, Assam. She produced the fungal spore calendar of that region and also correlated the fungal spore concentrations with the meteorological parameters. Volumetric assessments of airspora in agricultural fields have been conducted in West Bengal (Das and Gupta Bhattacharya 2007; Chakraborty *et al.* 2003; Uddin 2004), but did not focus on volumetric survey over rice field in West Bengal. To fill up this knowledge gap, present study was designed to investigate the occurrence of fungal spores in the air over rice field in West Bengal with reference to major fungal diseases of rice. The aero-mycological data over crop field are therefore indispensable to understand the disease epidemiology, control and establishing the forecasting probabilities (Saha, 2022).

The objective of the present work is to assess the concentration of fungal spore load with special reference to pathogenic fungal spores over the crop fields and their relation with disease incidence, severity, meteorological parameters and growth phases of the rice crop.

MATERIAL AND METHODS

Sampling site

Sampling of mycospores was carried out over a rice (cv. Swarna / MTU 7029) field for three consecutive *kharif* (rainy) seasons (June-December) from 2015 to 2017 at Barasat (22° 41' 3" N, 88° 26' 27" E) The sampling site was more or less square in size which measured 0.065 ha with an average altitude of 10 msl. There was a banana orchard 30m to the east and a mango orchard at 20 m distance on the south. The mixed vegetation of various grasses and herbs were also noticed around the rice field. Rice was grown for both kharif (July - December) and rabi (January - May) seasons. There was no crop grown in the plot during the intermediate period. No pesticide or fertilizer was used during the total survey period. Tractor was used for ploughing the experimental plots. During rainy season (*kharif*) irrigation was not needed as sufficient rainfall occurred during these seasons.

Sampling methods

For air monitoring, two samplers were used namely, Burkard personal one day volumetric sampler manufactured by Burkard Manufacturing Co. Ltd, Rickmansworth, UK and Andersen two-stage volumetric sampler manufactured by Thermo Andersen, Smyrna, USA (Andersen, 1958). Both the samplers were placed at a height of 1.5 m above the ground level. Sampling was carried out at weekly intervals.

Burkard personal sampler was used to assess culturable and non-culturable fungal spores. A special type of microslide smeared with glycerine jelly in the form of a thin film was used for trapping the fungal spores. The sampler was operated for 5 minutes. After each exposure, the slide was mounted with DPX as a mountant. Then the slide was observed under microscope. The conversion factor (20) was calculated following the method of The British Aerobiology Federation (British Aerobiology Federation 1995).

Andersen two-stage sampler was used to assess culturable fungal spore types at specific level. The sampler sucked 28.3 liter of air per minute impinged it successively on to the Petridishes containing Sabouraud's agar media. The sampler was operated for 5 minutes. The number of colonies recorded from two Petriplates were combined, averaged and converted into number of colony forming unit (CFU) per m^3 of air. The exposed plates were incubated for 1-3 days at 28±2 ^{0}C. The Conversion factor was calculated following the method of The British Aerobiology Federation (British Aerobiology Federation 1995).

Identification

Identification of airborne spores of fungi and fungal colonies was mainly based on the colour, size and shape of colony, other diagnostic morphological characteristics of fungal spores and fungal colonies observed under compound microscope (Jones and Harrison 2004). Identification was further confirmed with the help of standard manuals and published literature (Ellis, 1977; Ellis and Ellis, 1985).

Collection of Meteorological data

During the period of investigation, monthly records of total rainfall (mm), maximum and minimum temperature (^{0}C), relative humidity (%) and wind speed (mph) were obtained from Dum Dum Airport meteorological centre, about 5 km away from study area (Das and Gupta Bhattacharya, 2007).

Major fungal diseases

Three major fungal diseases were observed viz. blast (C.O. *Pyricularia oryzae*), brown spot (C.O. *Helminthosporium oryzae*) and false smut (C.O. *Ustilaginoidea virens*) during the three consecutive *kharif* seasons in the rice field. The favourable growth phases for these three diseases were recorded. The first day of disease incidence and the periods of maximum disease incidence was also recorded.

Pathogenic fungal spore concentration

As the *Ustilaginoidea virens* is non-culturable fungal type, so for the determination of pathogenic spore concentration viable spore trapping methods by use of Burkard personal volumetric sampler was considered. Three seasonal pathogenic spore concentration was calculated along with their percentage concentration to the total air spora studied.

Observation for disease severity

The disease severity was worked out at every subsequent seven-day intervals before harvesting of crop. The standard IRRI evaluation scale was used (IRRI 2002). Evaluation was done on randomly selected plants in each replicated block (total 16 hills/block). The following data were collected at seven-day intervals during the three consecutive *kharif* seasons from sixteen hills: For Blast and Brown leaf spot disease, the data were recorded for number of infected plants in hills, percentage of infected area on each leaf and sizes of infection spots.

For False smut disease, the following data were recorded:

1. Number of infected tillers in hills.
2. Smutted balls on panicle.

Scale for Blast disease of rice 0-9

Scale	Affected leaf area
0	No lesions observed
1	small brown speaks of pin point size
2	small roundish to slightly elongated about 1-2 mm in diameter
3	same as in scale 2, but on upper leaves also
4	less than 4%
5	4-10%
6	11-25%
7	26-50%
8	51-75 %
9	more than 75%

Scale for Brown spot of rice 1-9

Scale	Affected leaf area
1	No incidence
2	Less than 1%
3	1-3%
4	4-5%
5	11-15%
6	16-25%
7	26-50%
8	51-75%
9	76-100%

Scale for False smut disease of rice 1-9

Scale	**Infected florets**
0	No incidence
1	Less than 1%
3	1-5%
5	6-25%
7	26-50%
9	51-100%

Statistical analysis

Correlation between fungal spore count and meteorological factors such as maximum and minimum temperature (ÚC), rainfall (mm), relative humidity (%) and wind speed (mph) were calculated using Pearson's Product Moment method. Correlation matrix and regression equations were used to determine the effect of meteorological factors and age of the plant on the spore concentration. Adjusted p-values are reported considering multiple comparisons. To find the effects of different meteorological parameters on spore count, a generalized linear model (GLM) was also fitted with log link using R.3.1.2 software (www.cran.org). A statistical relationship was also made between the three major fungal diseases along with the crop age.

RESULTS AND DISCUSSION

Fungal spore diversity

During the entire sampling period (three consecutive *kharif* seasons from 2015 to 2017), a total of 22 culturable and non culturable (by Burkard personal volumetric sampler) and 18 culturable (by Andersen two stage volumetric sampler) fungal air spora were recorded. The anamorphic fungi were found to dominate the mold spectrum. *Cladosporium* sp. was the most dominant mold type with 9.97 % contribution to total mold count in Burkard sampler while it is 7.67% of total mould count in Andersen sampler. The next most prevalent mold type is *Aspergillus niger* (8.95 %). Other predominant mold types are *Aspergillus clavatus* (6.98%), *Memnoneilla* sp. (6.60%), *Penicillium claviformae* (6.11%), *Trichoconis* sp (5.63%), *Stemphyllium* sp. (5.65%), Smut spores (5.65%), *Trichoderma lignorum* (5.50%), *Penicillium expansum* (5.44%), *Alternaria alternata* (5.43%), *Helminthosporium* sp (5.22%), *Trichoderma viride* (5.02%), *Pyricularia* sp (4.75%) and *Fusarium oxysporium* (4.33%). Non-sporulating colonies contributed a significant fraction (4.57%) to total mold count. Other mold types were recorded in low concentration (seasonal percentage contribution < 4.00%) over the rice canopy (Figs. 1 and 2).

Considering the total spore or CFU count in air a peak was observed in October while lower mold count was recorded during June by both the sampler during three consecutive crop seasons (Fig.3).

Fungal spore calender

A fungal spore calender (2015-2017) was prepared on the basis of monthly (July-December) periodicities of various molds recorded by two samplers over rice field in kharif season at Barasat (Fig- 4). This calendar showed *Claviceps* sp., *Drechslera* sp. and *Helminthosporium oryzae* spore concentration increased as the rice crop developed from vegetative to fruiting stage. *Epicoccum* sp., *Periconia* sp., smut spore, *Fusarium moniliformae*, *Fusarium oxysporium*, *Nigrospora oryzae* spore concentrations fluctuated sporadically through the entire *kharif* season. While rest fungal spore concentrations showed more or less uniform occurrence through the entire crop season for three consecutive years.

Statistical analysis

Correlation matrix

The correlation matrices of spore count and CFU count per cubic meter of air using two samplers supplemented with meteorological variables have been depicted (Tables 1 and 2). In *kharif* season, at Barasat the spore concentration/m^3 of air was positively significant with age of the plant and negatively significant with minimum temperature and relative humidity (Table 1). That means as the age increases the spore count also increases significantly but when minimum temperature and RH increases the spore count decreases significantly. The relationships among these five meteorological factors also showed the positive or negative correlations among themselves; however, all the effects were not statistically significant (Table 1). For Andersen sampler, the CFU count was also positively correlated with age of the plant and negatively correlated with minimum temperature and RH (Table 2).

Regression Equation

To study the effect of different covariates viz., age of the plant and meteorological covarites on the

Table 1: Correlation matrix of spore count/ m³ of air with meteorological factors Sampler 1 (Burkard personal one day volumetric air sampler)

	Spore count	Age	MaxT	MinT	RH	Rain	Wind
Spore count	1	0.57*	-0.32	-0.52*	-0.38*	-0.3	-0.33
Age		1	-0.39	-0.59*	-0.47*	-0.05	-0.34
MaxT			1	0.78*	0.62*	0.06	-0.09
MinT				1	0.81*	0.28	0.18
RH					1	0.38*	0.23
Rain						1	0.09
Wind							1

*Significant correlation at 5% level of significance

Table 2: Correlation matrix of CFU count/ m³ of air with meteorological factors

	C.F.U. count	Age	MaxT	MinT	RH	Rain	Wind
C.F.U. count	1	0.53*	-0.32	-0.48*	-0.46*	-0.21	-0.23

*Significant correlation at 5% level of significance.

Table 3: Regression table.

Factor	Sampler -1			Sampler-2		
	Estimate	p-value	Comment	Estimate	p-value	Comment
Intercept	5.5883	<0.0001	Significant	5.5393	<0.0001	Significant
α_1	0.004	<0.0001	Significant	0.0024	<0.0001	Significant
α_2	-0.0114	0.0003	Significant	-0.0159	<0.0001	Significant
α_3	-0.0013	0.7028	Not significant	0.0001	0.9641	Not significant
α_4	-0.0028	<0.0001	Significant	-0.0048	0.001	Significant
α_5	-0.0124	0.0004	Significant	0.0079	0.0664	Not significant
α_6	-0.0122	<0.0001	Significant	0.0008	0.7571	Not significant

Table 4: First incidence and development of fungal diseases along with growth phases

Causal organism	Day of first incidence of spore in air (day)	Day of first onset of disease (day)	Day of highest concentration of spore/m³ in air (day)	Period of maximum incidence of disease (day)	Susceptible growth phases of the crop
Pyricularia oryzae	21-23	78-81	88-91	93-118	Panicle initiation to flowering
Helminthosporium oryzae	50-58	89-94	115-122	103-124	Flowering to early ripening
Ustilaginodea virens	121-127	136-141	153-159	148-168	Early ripening to pre-harvest

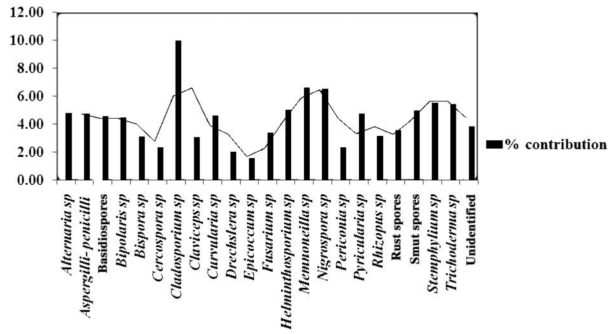

Fig. 1: Percentage contribution of individual culturable and non-culturable spore types in two consecutive kharif seasons.(By Burkard personal one day volumteric sampler)

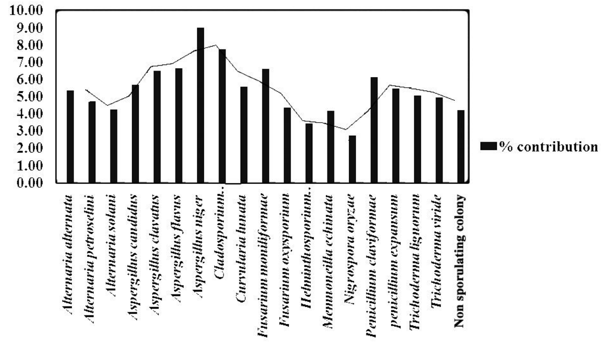

Fig. 2: Percentage contribution of individual culturable mold types in two consecutive kharif seasons.(By Andersen two stage volumetric sampler).

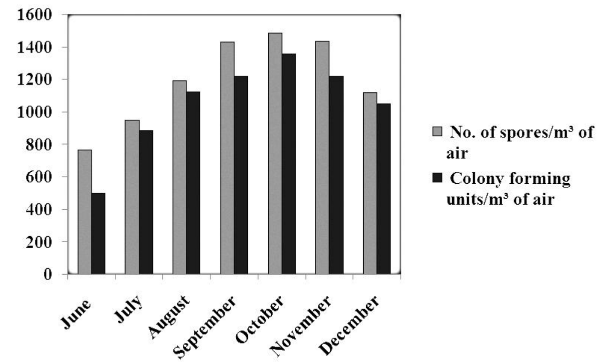

Fig. 3: Graphical representation of seasonal fluctuation of total spore/ CFU count by two volumtric sampler.

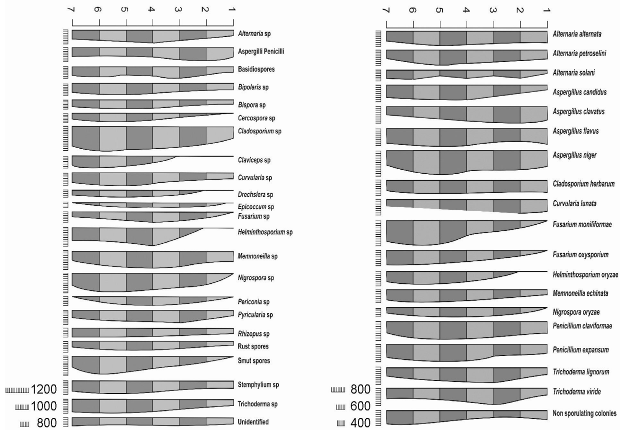

Fig. 4A: Fungal spore calenders representing 22 Culturable and non-culturable spore types along with unidentified spore types in average values, **4B:** Culturable mold calendar representing 18 mold types along with non-sporulating colonies for three consecutive *kharif* seasons.

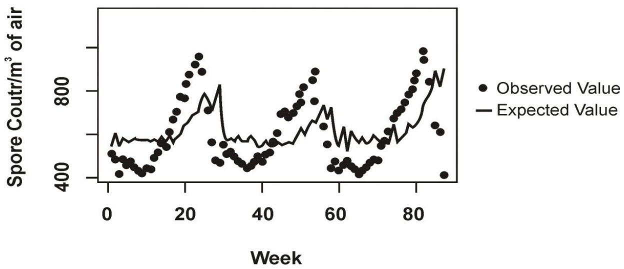

Fig. 5: Fitted values against observed spore/m³ of air

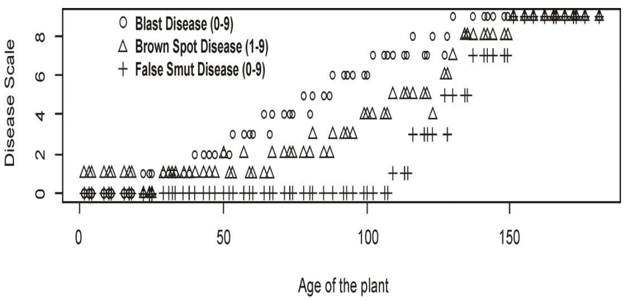

Fig. 6: Relationship between disease scale and age of the crop plant for three major fungal diseases.

number of fungal spores a generalized linear model (GLM) was fitted. Since the response variable was number of fungal spores, Poisson regression was assumed with log link. For each sampler, the following GLM was fitted:

$$log\ (Spore\ Count\ /\ m^3\ of\ air) = Intercept + \alpha_1 *\ age + \alpha_2\ maxT + \alpha_3 *minT\ RH + \alpha_4 *\ RH + \alpha_5 *\ Rain + \alpha_6\ wind\ speed$$

Results in Table 3 show that for culturable and non-culturable fungal types, age of the plant, maximum temperature, relative humidity, rain fall and wind speed are significant with spore count/ m³ of air and for culturable mold types, age of the plant, maximum temperature, relatve humidity are significant with the CFU count/ m³ of air in the atmosphere of Barasat. Rain fall and wind speed showed significant negative correlation with culturable mold types but they had no significant impact on non-culturable fungal types (Table 3). Moreover, it is found that in both the cases, the maximum temperature, relative humidity and wind speed showed negative correlation and age of the plant show positive correlation with the spore count or CFU count. So, as these meteorological factors rain fall and wind speed increase, the mean spore count or CFU count decreases but as age of the plant increases the mean spore count or CFU count increases sigificantly (Table 3). The regression equation for both the samplers can be expressed as:

For sampler 1

log(spore count/ m^3 of air) = Intercept + 0.004*age - 0.0114*maxT - 0.0028* RH - 0.0124*Rain - 0.0122* wind speed with AIC value 1741.524.

From the above equation it can be inferred that if age of the plant increased by one unit the log(spore count/ m^3 of air) increased by 0.004 unit, when all other fctors remain constant.

For sampler 2

log(CFU/ m^3 of air) = Inteecept + 0.0024* age - 0.0159*maxT - 0.0048* RH - 0.0065Rain with AIC value 1557.026.

From this above equation for sampler-2, it can be conclutedthat if maxT increased by one unit the log(CFU/ m^3 of air) decreased by 0.0159 unit, when age and other significant meteorological factors remain constant. So, from these two equations we can predict the spore/ CFU concentrations.

Generalised linear model

By using three-year pooled data, the expected values were fitted along with their observed values for three consecutive *kharif* season (Fig. 5). Here the dots denotes observed values and line indicates the expected or fitted values. The spore count shows three peaks during three consecutive crop seasons. In these peak portion the observed values were much higher than the expected values which may be due to secondary infection during these time periods (Fig. 5).

Major fungal diseases
Pathogenic spore concentrations

The seasonal spore concentration of *Pyricularia. oryzae* in Barasat ranged between 5.14-5.89. Highest *P. oryzae* spore concentration observed in 2015 (502/$m^3$of air) and lowest in 2016 (452/ $m^3$of air). The highest spore contribution (4.33%) of *Helminthosporium oryzae* was recorded in 2015 followed by 2017, 2016. The seasonal spore concentration of *Ustilaginoidea virens* ranged 3.09-4.21 in Barasat and number of spores ranges from 347/m^3 of air -386/m^3 of air.

In *kharif* season, *Pyricularia oryzae* spore type was first appeared in the air over rice field from 3rd week of July, but the disease appeared in the 3rd to 4th week of August. Maximum incidence of disease was recorded during September to October. Susceptible growth phases of rice crop were panicle initiation to flowering (Table 4).

Helminthosporium oryzae spore was first came in view in the air over rice field from 2nd to 3rd week of August, while the symptoms appeared within 1st to 2nd week of September. Maximum disease incidence was recorded from September to November. Susceptible growth phases of rice crop ranged from flowering to early ripening (Table 4).

Ustilaginodea virens spore first came into existence in the air over rice field from 3rd to 4th week of October, while the disease appeared within the 2nd to 3rd week of November with maximum recorded incidence during November to December. Susceptible growth phases of rice crop was from early ripening to pre – harvest (Table 4).

Relationship between the three disease scales along with the growth phases

A relation has been drawn between first incidence and gradual severiety of these three diseases along with the age of the crop plant (disease scale as suggested by IRRI 2002) (Fig. 6). The three diseases of rice crop namely, blast, brown spot and false smut have three different disease scales. Blast and false smut disease have 0-9 scale while brown spot disease have 1-9 disease scale. Here these three disease scales were plotted along with the age of the plant (Fig.6)From these plots, disease development along with the crop period can be understood. The blast appear in the early vegetative stage (at 25 days) followed by brown spot (at 72 days) and smut disease (

at 113 days in fruiting stage). It has been observed that the severiety of these three diseases was high in between 155 to 180 days of crop plant (Fig.6).

The composition of culturable and non-culturable fungal types were more or less homogeneous during three kharif seasons in Barasat except for *Alternaria petroselini* and *Aspergillus candidus.* This observation is in agreement with the findings of Das and Gupta Bhattacharya (2007), where the authors found significant variation in *Aspergillus flavus* and *Aspergillus japonicus. Alternaria and Aspergillus* were found to be the most abundant spores over this Swarna variety of rice appearing from the very beginning, particularly from the maximum vegetative growth stage onwards. Chavan (2012) also reported occurrence of Ascomycetes fungal spores over paddy field at Raigad. Deka (2013) suggested that high occurrence of *Cladosporium* and *Alternaria* was associated with the rice crop which also resemble with the present investigation.

The present study revealed that *Cladosporium* sp. is the most prevalent fungal type. Similar volumetric surveys over rice field were also reported in India (Uddin 2004; Saha and Bhattacharya 2015; Saha *et al.* 2015a) where *Cladosporium* sp. was the most dominant airborne fungus. *Cladosporium* sp. is a saprophytic fungus as it thrives on decaying organic materials. In agricultural field huge amount of decomposing leaves and plant debris are found which in turn provide ideal growth substrata for *Cladosporium*. Actually this fungal types produce dry spore which can be easily spread in air by wind.

It has been observed in 'Aman' variety of paddy that *Helminthosporium oryzae* and *Fusarium* were the most effective disease causing agents in the field (Devi and Singh 2009; Saha 2016; Saha *et al.* 2015b). The above observation is in agreement with the findings of present study.

The three years pooled data analysed for regression analysis and correlation matrix showed that the maximum temperatue, relative humidity, rain fall and wind speed could explain seasonal dynamics of airborne culturable and non-culturable fungal spore types over rice field in the study area. The highest spore/ CFU count are observed in October for both the sampling years probably due to moderate temperature (17°-32°C), low relative humidity (58%-71%), low wind speed (< 5 km/ h) and complete absence of rainfall. Since, the daily temperature during May – June in tropical plains including Barasat, reaches as high as 41°C to prevent fungal growth, so in the present study we noticed low spore count/ CFU count were noted during summer. Earlier workers (Das and Gupta Bhattacharya 2007; Chakraborty *et al.* 2003) found negative association between airborne fungi and temperature, which was in agreement with the observations of the present study.

Three major fungal diseases viz. blast , brown spot and smut disease incidence and severity were observed where blast appeared at the early stage followed by brown spot while smut disease appear at the later stage like fruiting stage. It was observed that fungal types responsible for initiating diseases in the crop occurred in the air over rice field 2-3 weeks in advance (Saha *et al.* 2020; Mishra, 2006). Deka (2013) reported that there was a highly positive correlation between the number of trapped *Pyricularia oryzae* conidia with 4 to 14 days prior to lesions formation. Maximum concentration of *P. oryzae* conidia coincided with booting and panicle initiation stages of crop growth in October, while panicle initiation and flowering stages occurred in November over rice field. Susceptible growth phases of rice crop for brown spot was from flowering to early ripening stage. *Helminthosporium oryzae* spore was first came into view in the air from 2nd to 3rd week of August, while the symptoms appeared within 1st to 2nd week of September. *Ustilaginoidea virens* spore was first trapped in the air from 3rd to 4th week of October. Susceptible growth phases for false smut disease was from early ripening to pre-harvest period (Saha, 2022). Lara *et al.* (2005) studied climatic conditions of *Pyricularia oryzae* spore concentration and leaf symptoms in rice crop. Devi and Singh (2009) found three major diseases of rice viz. false smut. brown leaf spot and blast disease in in Thoubal district, Manipur for two crop seasons. The above findings are in agreement with the findings of present study.

CONCLUSION

The three year monitoring of airborne culturable and nonculturable fungal spora of suburban West Bengal produces a fungal spore calendar for this region. A total of 21 viable and non-viable and 17 viable fungal types were recorded in the atmosphere of Barasat. The relationship between meteorological factors and spore count or CFU count has been established. The maximum temperature, relative humidity and wind speed showed negative correlation and age of the plant shown positive correlation with the spore count or CFU count The regression model would be beneficial to the farmers for prediction of fungal diseases of rice in the study area. The first incidence and severiety of the three major fungal diseases viz. blast, brown spot and smut disease were observed where blast appeared at the early stage followed by brown spot while smut disease appear at the later stage like fruiting stage.

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DECLARATIONS

Conflict of interest: Author declares no conflict of interest.

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