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Screening of Endophytic Fungi and Their Diversity in a Lianas of Different Localities from west Medinipur During Summer

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Abstract

An woody lianas- *Bauhinia vahlii*, collected from three localities of West Medinipur district of W.B. during summer season, wasstudied for screening of endophyticfungal diversity. Aerial tissues (leaf, petiole and stem) were selected for endophytic isolation. A total of 154 tissue segments out of 225 were infested with fungi and 158endophytic fungi were isolated. Average colonization frequency (CF%) was 68.44% and leaves of the climber were colonized by a great number of endophytic fungi i.e. 92%. CF% wasmaximum in the plant collected from Godapiasal, it was 86.60% and minimum in the plant of Chilkigarh, it was58.64%. A total of 13 fungal genera with few unknown genera and few sterile fungi were isolated. Highest Shannon-Wiener index (2.267) was shown in the plant of Chilkigarh and with highest Simpson's diversity (0.8751) in the same place. It indicates great species specificity. Fungi of Sordariomycetes weremaximum (32.08%).

Keywords- Endophytes, fungi, diversity, lianas.

1. Introduction

Endophytic fungi are microorganisms that live within the inner tissue of plants without causing apparent symptoms [1]. Although endophytic fungi are primarily mutualistic and commensalisticsymbionts, they may not continue as endophytes throughout their life cycles [2]. Endophytes are ubiquitous in idistribution. Endophytic fungi that infest plants were found in all environments studied [3, 4]. Microorganisms that colonize internal plant tissues without causing any diseases symptoms or apparent injury are called endophytes [5]. Many fungal, bacterial, actinomycetean members are endophytes but most frequently isolated endophytes are fungi [6]. Carrol and Carrol (1988) reported that endophytes live without any symptoms and sometimes systematically within the plant tissues. They have been found infested withevery plant species investigated so far. It is believed that plants from unique environmental settings and which are endemic are likely to accommodate distinct endophytic microorganisms as well as microorganisms making novel bioactive products [6]. Many endophytic fungi present in the root tissues forming mycorrhizalsymbionts play a vital role on phosphate uptake in plant nutition. Others are present in the intercellular- spaces of leaves, petioles and inner tissues of stems [7, 8]. Lianas plants are woody climbers which grow supporting another straight and strong long trees and cover the topmost canopy of it. Sacred grooves are those holy plants which are used to worship in situ condition by races. Different lianas plants harbour some distinct fungal endophytes that are believed to be associated with the production of antimicrobial substances [9] and pharmaceutical products [10]. Huge research work is going on and it has expanded in recent years on isolation of endophytes, its antimicrobial activity and production of novel pharmaceutical products at every corner of the world.

Some endophytes can reportedly reduce plant diseases and enhance plant growth and products [11, 12, 13]. For example, fungal endophytes in Theobroma cacao and Solanummelongena reduced foliar and root diseases respectively, and treatment of Glycine max with culture filtrate of endophyte-Cladosporiumsphaerospermum increased plant height [12, 14, 15, 16].

Inspite of the omnipresence of endophytic fungi in plants, the extent of their contribution to fungal biodiversity and their interaction among themselves and with host still remain unclear. Few studies have been conducted with regards to the diversity and colonization of the endophytes.

Despite the largest diversity of endophytic species in tropical and subtropical rainforests, their biodiversity in tropical country is still poorly studied. It has been speculated that the bioactivity of various plants may be due to its associated

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endophytes. Some researchers isolated very diverse groups of endophytic fungi from plant tissues [17].

Endophytes are the normal microflora of the plant tissues [18]. They protect the plants against pests. Other fungal pathogens help in protection of host plants against grazing animals . They also enhance the defense mechanisms of host plant against unfavourable environments.

There is a very limited knowledge on fungal endophytic communities in the plant community. In the western part of PaschimMedinipur district there are some forest areas with some unique environmental settings and huge plant resources with many lianas in patches. In this area, the study of endophytes in the plant has not been covered till now in a broad way. I have selected a giganticwoody plant which is as long as 100-130 meter in length with much branching and creeping over many other tall trees. The optimul method to identify fungal endophytes is largely based on the research questions to be answered. It is known that endophytic fungi existing in the plant are potential sources of antimicrobial substance [9, 19, 20, 21]. Endophytic fungi show considerable antibacterial and antifungal activity [22]. It has been found that a single plant species may harbour hundreds of endophytes and they may assemble in all available plant tissues like leaf, petiole, stem, twig, bark, xylem, root, fruit, flower and seed [27, 35, 39].Various antifungal agents have been explored, but the control of many of the fungal diseases has not been achieved. Earlier a few lianas plants have been studied for screening of endophytic fungi from various localities of West Medinipur district of W.B. during various seasons i.e. winter, rainy season and summer and a diverse group of various classes of endophytes have been isolated and identified [40, 41, 42].

Plant endosphere is a complex micro-ecosystem where different niches can be occupied by a different variety of microorganisms. It represents a rich and reliable source for novel and bioactive actinobacterial species (Trujillo et al. 2010;Qin et al. 2011) with so many different fungal members. Despite the ever-increasing information on endophytes, the value of functional biodiversity measures in endophyte assemblages has been rarely explored, with few reports focussed mainly on fungi (Yuan et al. 2011). Parrent et al. (2010) suggested that functional biodiversity measures could be more powerful than taxonomic measures for understanding the mechanistic basis of diversity effects on the plant-endophyte relationships. Endophytes are important and particularly have roles for plant growth and development, although not essential. The emergence of efficient physiological systems to enable endophyte residence in the endosphere were suggested by Hardoim et al. (2008) to be the key fitness-enhancing traits that have conditioned the evolutionary success of their host plant species.

Endophytic fungi constitute a major portion of fungal symbionts associated with plants which live inside plant tissues without causing any disease symptoms (Schulz and Boyle, 2005; Rodriguez et al. 2009; Hamilton and Bauerle, 2012). Development of endophytic symbiosis can regulate important functions like host mineral nutrient composition, chemical composition of root exudates, plant hormonal balance, physical modification in soil and host protection against biotic and abiotic stresses (Waller et al. 2005; Khan et al. 2011; Redman et al. 2011). Similarly, fungal symbiosis with host plants facilitate in higher nutrient uptake like potassium, phosphorus, magnesium etc. to bestow greater plant biomass and hence tolerance against stress (Rodriguez and Redman, 2008; Evelin et al. 2009).

Endophytic fungi are asymptomatic or unapparent fungi living within healthy leaves or shoots (Caroll,1991). Fungal species living as symbionts within the tissues of plants (N. S. Raviraja, 2006). They make no apparent infections. This definition excludes the mycorrizal fungi but does not imply that endophytic fungi are not cultivable on artificial media (R. Maheshwari, 2006).

Endophytes are the normal microflora of the plant tissues (Ganley R.J., Brunsfeld S.J. and Newcombe G., 2004). They protect the plants against pests. Other fungal pathogens help in protection of host plants against grazing animals . They also enhance the defense mechanisms of host plant against unfavourable environments and increase host's tolerance power.

The goal of the study was to identify the fungal endophytic communities in leaves, petioles and stems of *Bauhinia vahlii*. The objectives were to: (i) isolate the endophytic fungi (ii) determine the identity and diversity of endophytic fungi (iii) compare the endophytic diversity pattern in different regions.

2. Materials and methods

2.1Study sites and collection of samples

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The study was conducted in PaschimMedinipur district of West Bengal, India during summer, 2013. The district is situated in between the latitude of 22°25′ to 22o57′North and longitude of 87°11′East. The altitude is 23M above from the sea level. The climate is tropical, warm and humid with a mean temperature of 33°C and an average rainfall of 120cm. Two lianas plant *Bauhinia vahlii* (Family-Caesalpiniaceae) was selected from threedifferent localities of forest for present study.

2.2 Sampling procedure

Plant samples (leaves, stems, petioles) were collected randomly from mature, healthy, disease-free plants from each location during summer (May) The samples immediately after collection were kept in zipper-lock plastic bags, brought to the laboratory and stored at 4°C within 2-3 hours of collection until isolation procedure was accomplished.

2.3 Surface disinfection

Samples collected from different localities were thoroughly washed under running tap water before processing and following sequences were followed: leaf, petiole and stem samples were surface sterilized by sequentially dipping into 80% ethanol for 1 min, 1% sodium hypochlorite (NaOcl)(4% available chlorine) for 4 min, 90% ethanol for 20sec. Finally, samples were rinsed with sterile distilled water for 3 times, then allowed to surface dry under sterile condition.

2.4 Placing the samples in media

Sterile leaves were cut into pieces of about 1 square cm size by sterile scizor and placed in plate of water agar (WA), 5 samples in each, equidistant from each other. Similarly 5 sterile petioles of 0.5-1cm long were placed in another WA plate. Stem tissues were cut into short pieces of 4-5 cm long and after sequential sterilization, the outré layer was removed and inner tissues were peeled with sterile scalpel. Thin peels from various depth were placed on another WA plate. Thus, 5 replica plates for each sample from the plant of one locality were made.

2.5 Isolation of endophytic fungi

After placing the samples fungal growth was observed each and every day. Within 2-3 days fungal hyphae were in appearance. Some samples show more than one hyphal growth. From each sample fungal hypha was isolated and transferred to PDA media by cutting a square block of water agar. The plates were incubated in light chamber at 24°C. After 10-15 days huge mycelial and sometimes reproductive growth was occurred in most cases. Culture slants were made and preserved for identification at 4°C and also for further work in future.

2.6 Identification of endophytes

The endophytic fungal organisms were studied under optical compound microscope. The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals [26, 29, 30, 33].

2.7 Data analysis

The relative colonization frequency (CF%) was calculated as the number of sample segments colonized by at least a fungus divided by total number of segments plated x100 using the formula outlined by Hata and Futai: CF = (N_{col}/N_t x100, where N_{col} = number of segments colonized by at least a fungus, N_t = total number of segments plated. Dominant endophytes were calculated as percentage of colony frequency divided by sum of percentage of colony frequency of all endophytes x100 [21]. Dominant endophyte percentage (D) = N_i/N_s x100, where N_i = percentage of colony frequency of individual endophytes, N_s = percentage of colony frequency of all endophytes. Using PAlaeontologicalSTatistics software package (PAST) [31], following diversity indices were calculated:-

(a) Simpson's Diversity Index (1-Dominance) was calculated using the formula 1-D, where D = ∑n(n-1)/N(N-1). Here, n = the total number of organisms of a particular species, N = the total number of organisms of all species.

(b) Shannon-Wiener diversity index was calculated using the following formula: Shannon-Wiener index (H′) = -∑s(Pi)(In Pi), where H′ = Symbol for the diversity in a sample of species or kinds, s = the number of species in the sample, Pi = relative abundance of ith species or kinds and measured by = n/N, N = total number of individuals of all kinds, n_i= number of individuals of ith species, In = log to the base 2.

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(c) Evenness was calculated using the following formula: Evenness (E) = H′/H′max, where H′max is the maximum value of diversity for the number of species.

3. Result and discussion

The lianas plant had a huge number of endophytic fungal association. A total of 154 tissue segments out of 225 were infested with fungi and 158endophytic fungi were isolated. Average colonization frequency (CF%) was 68.44% and leaves of the climber were colonized by a great number of endophytic fungi i.e. 92%. CF% wasmaximum in the plant collected from Godapiasal, it was 86.60% and minimum in the plant of Chilkigarh, it was58.64%. A total of 13 fungal genera with few unknown genera and few sterile fungi were isolated. Fungi of Sordariomycetes weremaximum (32.08%).

Banerjee et al. and Raviraja et al. reported earlier that endophytic colonization is higher in leaf segments than in stem segments of some tropical medicinal plants. In the present study also highest association ofendophytic fungi was obtained from the leaves segments of the plant of all three places and it is 84%, 100% and 92% from the plant of Belpahari, Chilkigarh and Godapiasal respectively (Table 2). Fungi isolated from petioles were 40%, 12% 92% and from stem it was 56%, 64% and 76% and from the three places respectively. It is obvious that for petiole fungal association is lowest. But average colonization frequency is highest in case of plant of Belpahari, 92% and lowest in plant of Chilkigarh, 48% (Table 3). A total of 158endophytic fungi were isolated from 3 different tissue segments of *Bauhinia vahlii*(Family-Caesalpiniaceae). The following dominant genera of endophytic fungi isolated from the plant with a number of unidentified genera and sterile mycelia-Beltrania sp., *Lasiodiplodia* sp., *Arthrinium* sp., *Fusarium*sp*Pestalotiopsis* sp., *Verticillium* sp. and *Arthrobotrys* sp. (table-1). The maximum were under the class Sordariomycetes (32.08%) and Deuteromycetes (30.12%). Average colonization frequency in leaves, petioles and stems were 92%, 48% and 65.33% respectively.Fungi of the class Sordariomycetes are maximum in all three places (Table 4).

Diversity and species richness of endophytic fungi were studied in different tissues of the two plant species. Endophytes of each species were used to calculate diversity indices using PAST (PAleontologicalSTatistics) software packages, ver.-1.89 [31]. Highest Shannon-Wiener index (2.267) was shown in the plant of Chilkigarh and with highest Simpson's diversity (0.8751) in the same place (Table 2). It indicates great species specificity. The result showed that the plant species is rich in endophytic fungi. Various diversity indices in respect of regions (Table-2).Figures shows few light compound microscopic and sterio microscopic pictures of isolated fungi.

4. Conclusions

There is a diverse groups of fungal endophytes in the lianas plant found from my study. Majority has been identified with some unknown genera and some mycelia sterilata. We may draw conclusion that there has an environmental effect to colonize by endophytesin a particular plant and also they have organ and tissue specificity.Moistured, humid and shady place accelerate the mutualistic association of fungi with plant species with a diverse types of fungi.Plants of arid, dry, hot place with higher temperature accommodate relatively lower number of fungal endophytes. Most probably the endophytes give aparmanent structure to the plant community and help to survive in adverse condition, of environment. Pie diagram shows various classes of isolated fungi.

Acknowledgements

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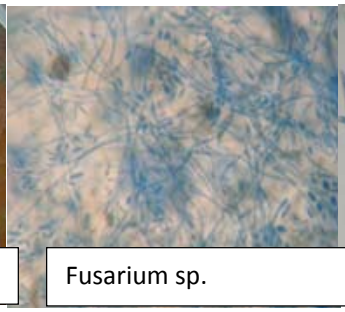
Plate pic.of Fusarium sp.



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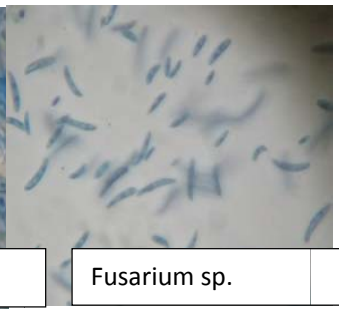

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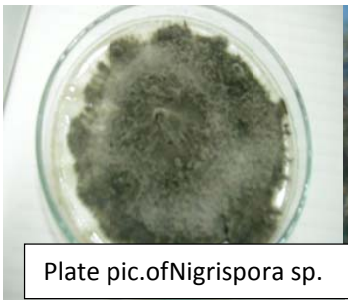
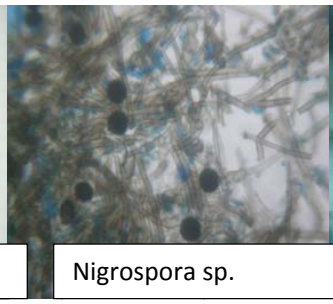


Plate pic.ofNigrispora sp.



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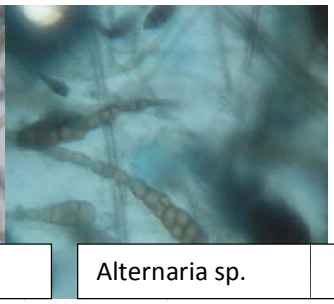
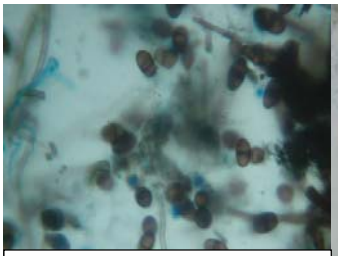
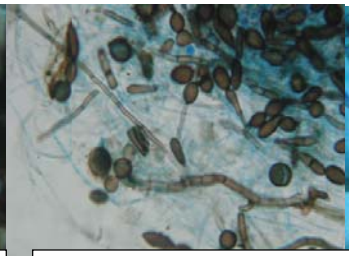


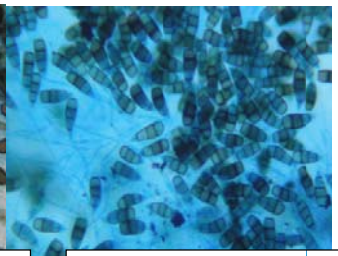
Alternaria sp.



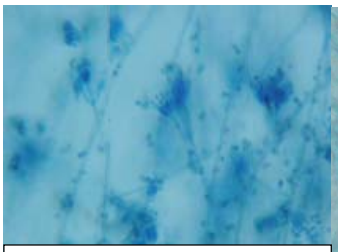
Lasiodiplodia sp.



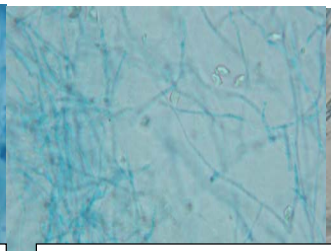
Arthrinium sp.



Pestalotiopsis sp.

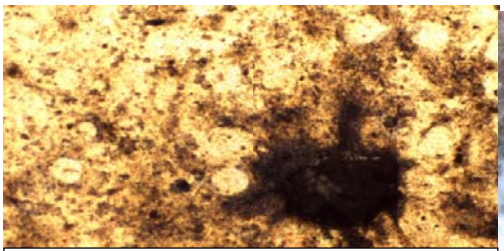


Penicillium sp.



Beltrania sp.

Torula sp.



Sterio picture of Pestalotiopsis sp.

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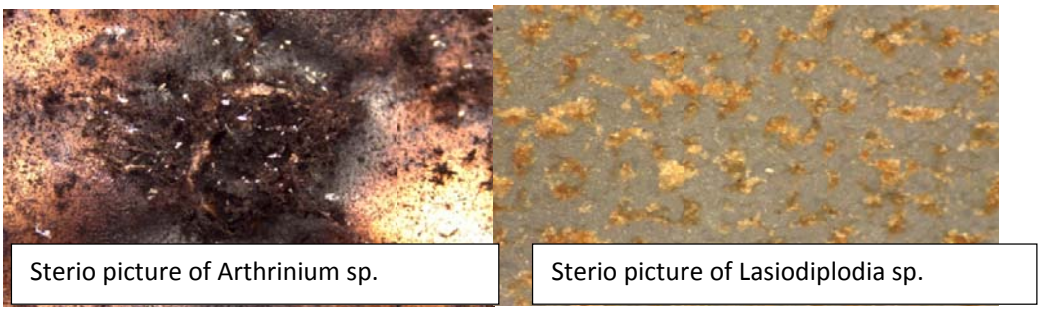

Sterio picture of Arthrinium sp.

Sterio picture of Lasiodiplodia sp.

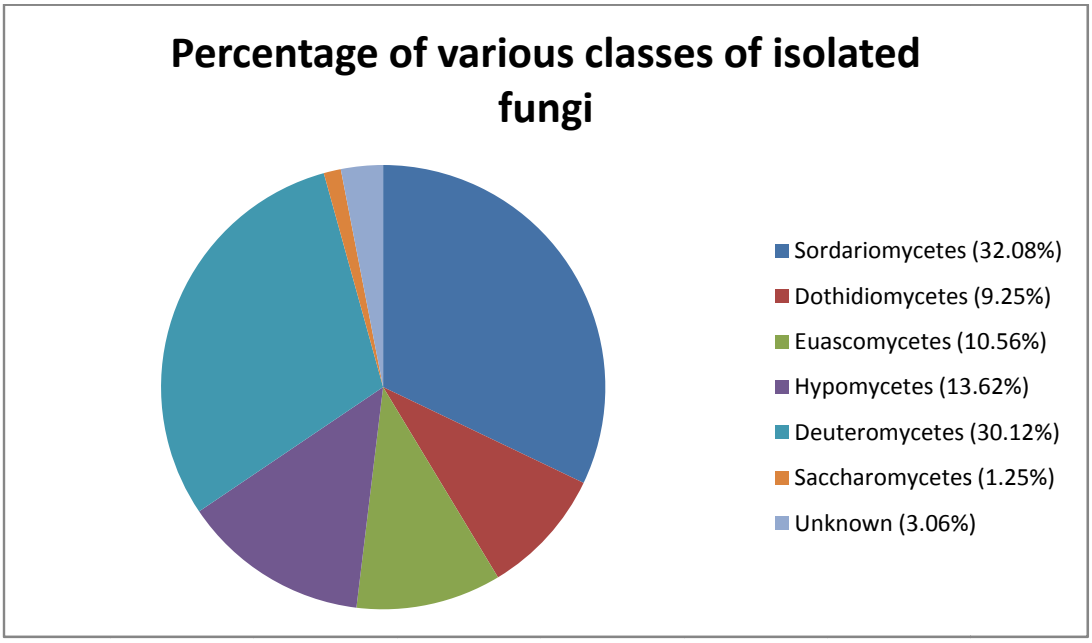

Diagram 1: Pie chart showing percentage of various classes of fungi isolated

Table 1: Endophytic fungi isolated from Bauhinia vahlii of 3 localities in summer

Endophytic fungi	Total Isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	s
Acremonium sp.	2	0	0	0	2	0	0	0	0	0
Alternaria sp.	6	0	1	1	4	0	0	0	0	0
Arthrinium sp.	14	1	1	3	2	0	0	0	5	2
Arthrobotrys sp.	22	0	0	0	0	0	0	20	2	0
Betrania sp.	29	16	1	0	9	0	2	0	1	0
Fusarium sp.	17	0	2	2	1	1	4	1	6	0
Lasiodiplodia sp.	15	2	1	3	2	0	1	1	5	0
Nigrospora sp.	8	0	0	2	0	1	1	0	2	2
Papularia sp.	1	0	0	0	1	0	0	0	0	0
Penicillium sp.	3	0	3	0	0	0	0	0	0	0
Pestalotiopsis sp.	12	0	1	4	4	0	1	0	1	1
Torula sp.	2	0	0	0	0	0	0	0	0	2

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Verticillium sp.	15	0	0	0	0	0	4	0	0	11
Mycelia	7	3	0	0	1	0	3	0	0	0
Unknown genera	5	1	0	0	0	1	0	1	1	1
Total	158	23	10	15	26	3	16	23	23	19

Table 2: Various diversity indices of isolated fungal genera

parameters	Belpahari	Chilkigarh	Godapiasal
Taxa_S	10	12	10
Individuals	48	45	65
Dominance_D	0.1814	0.1249	0.183
Simpson_1-D	0.8186	0.8751	0.817
Shannon_H	1.998	2.267	1.959
Evenness_e^H/S	0.7373	0.8046	0.7094
Brillouin	1.726	1.93	1.744
Menhinick	1.443	1.789	1.24
Margalef	2.325	2.89	2.156
Equitability_J	0.8677	0.9125	0.8509
Fisher_alpha	3.843	5.354	3.3
Berger-Parker	0.3542	0.2444	0.3385
Chao-1	10	12.25	10

Table 3: Colonization frequency (CF) of Bauhinia vahlii collected from three localities in respect of leaves, petioles, stems and totality

Places of Isolation	CF of Leaves	CF of Petioles	CF of Stems	Total CF
Plant of Belpahari	84%	40%	56%	60%
Plant of Chilkigarh	100%	12%	64%	58.65%
Plant of Godapiasal	92%	92%	76%	86.62%
Total CF	92%	48%	65.33%	68.45%

Table 4: Percentage of various classes of fungi in the plant of the respect of three localities

Classes of Fungi	Plant of Belpahari		Plant of Chilkigarh		Plant of Godapiasal		Total	
	Number	%age	Number	%age	Number	%age	Number	%age
Sordariomycetes	21	14.07	20	14.30	8	3.96	49	32.33
Dothidiomycetes	6	1.15	3	0.88	6	0.85	15	2.88
Euascomycetes	8	1.76	2	0.59	7	1.14	17	3.49
Deuteromycetes	12	7.53	19	12.71	17	7.87	48	28.11
Hyphomycetes	0	0	0	0	22	4.61	22	4.61
Saccharomycetes	0	0	0	0	2	0.04	2	0.04
Unknown	1	0.06	1	0.06	3	0.14	5	0.26
Total	48	24.57	45	28.54	65	18.61	158	71.72

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