International Journal of Experimental Research and Review (IJERR)

ISSN: 2455-4855 (Online) ***Original Article***
Received: 10th January, 2021; **Accepted:** 19th February, 2021; **Published:** 30th April, 2021

DOI: https://doi.org/10.52756/ijerr.2021.v24.001

Diversity and antimicrobial activity of endophytic fungi from *Combretum* sp. collected in monsoon from three regions

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Abstract

Aerial tissues of the woody lianas- *Combretum* sp. was selected and assessed for study of endophytic fungal diversity from three forest areas of West Medinipur and Jhargram districts of West Bengal. In monsoon study it was observed that out of 225 various tissue segments ,165 segments had been colonized by endophytic fungi *Combretum roxburghii* and 209 endophytic isolates were isolated from them. Average colonization frequency (CF)was 73.32 percent. Highest CF was in plant of Belpahari (76%) and in petiole (82.66%). Out of isolated fungal genera *Pestalotiopsis* sp. was highest in number. *Diplodia* sp., *Beltrania* sp., *Chaetomium* sp., *Fusarium* sp., *Arthrinium* sp. were also greater in number. Dominance index was highest in Belpahari (0.1547). Simpson's diversity was maximum in Godapiasal (0.8693) and Shannon-Wiener index was also highest (2.152) in plant of Godapiasal. Highest diversity of endophytic fungi was in plants of Chilkigarh. Isolated endophytic fungi were *Lasiodiplodia* sp., *Diplodia* sp., *Fusarium* sp., *Chaetomium* sp., *Arthrinium* sp., *Aspergillus* sp., *Pestalotiopsis* sp. etc. Simpson's diversity and Shannon-Wiener index were maximum in plants of Godapiasal. The study of antimicrobial activity of isolated fungal endophytes indicated that few plugs showed antimicrobial activity against few pathogenic bacteria. Among all isolates tested only seven showed antimicrobial activity. *Aspergillus* sp. and one unidentified fungi showed the antimicrobial activity against three bacteria i. e., *Bacillus cereus*, *Escherechia coli* and *Vibrio cholera* and maximum inhibition zone was observed against *E. coli,* diameter of inhibition zone is 1.4 cm. Other fungi showed less antimicrobial activity. Diameter of zone of inhibition varied (0.7 – 1.4) and this variation might be due to the difference of bioactive compound produced or the varied concentrations or amount degree of same compound.

Keywords: Antimicrobial activity, endophytes, diversity, diversity index, lianas, symbionts.

Introduction

Endophytes are found in all groups of plant community. The term endophyte is most commonly used for those micro organisms which infect and colonize internally and here the tainted tissues in host plant will not show any instant symptoms, and will be evenly applied for prokaryotic bacteria as well as eukaryotic fungi (Banerjee, 2011). Fungal

endophytes in aerial tissues of host are culturable on synthetic media. *Muscodor vitigenus, M. equiseti* and *M. heveae* were isolated from *Hevea brasiliensis* in Thailand (Siri-Udom et al., 2016).

Endophytic organisms have been exhibited as the key components in symbiotic relationships of plant hosts, influencing tolerance power of host to stressful condition. Endophytic fungi are very important in the biodiversity since they have an effect on structure and defence mechanism of plants and ultimately in the ecosystem). Arnold et al., (2000) isolated extremely abundant and very diverse group of endophytic fungi from plant tissues. Endophytic fungi are ubiquitous in distribution found within the tissues of plants. Ganley et al., (2004) reported that endophytes are the normal inhabitants of the plant tissues. They protect plants against pests (Azevedo et al., 2000) fungal pathogens (Arnold et al., 2003)

The endophytes can play important ecological roles in plant communities. Endophytic fungi can also increase drought resistance and enhance drought resistance (Malinowski et al., 2000). Many symbiotic endophytic fungi have been isolated from three plants of Lamiaceae family (Banerjee et al., 2009*).* Despite the largest diversity of endophytic species in tropical and subtropical rainforests, their biodiversity in tropical country is still poorly studied. Some researchers isolated very diverse groups of endophytic fungi from plant tissues (Arnold et al., 2001). Some fungi show higher degree of antimicrobial activity against few virulent bacteria. Various novel bioactive materials released from fungi are responsible for this inhibition of bacteria. The objectives were to: (i) isolate and identify the endophytic isoaltes determine the diversity of endophytic fungi and to study the antimicrobial activity of isolates.

Materials and Methods

Study sites

The study was conducted in Paschim Medinipur and Jhargram districts of West Bengal, India on the basis of canopy variation. Present study was done in monsoon (June to September, 2018). The districts are situated in between the latitude of 22°25′ to 22°57′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. One woody lianas- *Combretum roxburghii* (Combretaceae) was selected for study of endophytic fungal diversity from three forest areas (Belpahari, Chilkigarh and Godapiasal) of West Medinipur and Jhargram districts, West Bengal.

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

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 minute, 1% sodium hypochlorite (NaOcl)(4% available chlorine) for 4 minutes, 90% ethanol for 20 seconds. Finally, samples were rinsed with sterile distilled water for 3 times, then allowed to surface dry under sterile condition.

Sterile leaves were cut into pieces of about 1 square cm size by sterile scissor 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 outer layer was removed and inner tissues were peeled with sterile scalpel. Thin peels from various depth were placed on another WA plate. Thus, at least 5 replica plates for each sample from the plant of one locality were made.

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 23^{o}C. After 10-15 days huge mycelial and in some cases reproductive growth was observed. Culture slants were made and preserved for identification at 4^{o}C and also for further work in future.

Identification of endophytes

The endophytic fungal organisms were studied under optical compound microscope. The fungal isolates were identified based on their cultural and reproductive characters using the standard identification manuals (Martin et al., 1997; Barnett et al., 1998; Ellis and Ellis, 1997; Gilman, 2001; Magurran, 2004; Nagamani, 2006).

Statistical 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 allendophytes x100. 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 Palaeontological statistics software package (PAST), 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 its species or kinds and measured by = n/N, N = total number of individuals of all kinds, n_i= number of individuals of its species, In = log to the base 2.

(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.

Agar plug diffusion assay

The isolated endophytic fungi from *Combretum* sp. were subjected to preliminary screening and then some fungi were assessed for antibacterial activity through agar plug diffusion method.

Test bacteria collected from Department of Botany, Vidyasagar University, were inoculated earlier in Petri dishes. Then, agar plugs with diameter of approximately eight mm were cut from the PDA plate of actively growing endophytic fungi and were transferred to the test bacteria-*Escherichia coli* (Gram positive), *Bacillus cereus* (Gram positive) and *Vibrio cholerae* (Gram positive). These plates were sealed with parafilm. The plates were then incubated at a temperature of 23^{o}C for 2 days to enable growth of test microorganisms and diffusion of metabolites in media from fungi. After incubation, the diameter of the zones of inhibition was measured using a ruler excluding the size of plug.

Results and Discussion

In the study of *Combretum roxburghii*, out of 225 various tissue segments 165 segments had been colonized by endophytic fungi and 209 endophytic fungi were isolated from them. The endophytic fungal colonization may vary with different seasons and localities (Gond et al., 2007). Average colonization frequency was 73.32%. Leaf, petiole and stem tissues showed colonization frequency of 62.66, 82.66 and 74.66 percent respectively. Highest CF was in plant of Belpahari (76%) and in petiole (82.66%) of all localities. Out of isolated genera *Pestalotiopsis* sp. was highest in number. *Diplodia* sp., *Beltrania* sp., *Chaetomium* sp., *Fusarium* sp., *Arthrinium* sp., *Mucor* sp., *Verticillium* sp. were intermediate in number and are common endophytes reported many times by various workers (Unterseher et al., 2007; Li et al., 2008; Ren et al., 2008). Maximum number of endophytes were isolated from plant samples of Belpahari but plant samples collected from Godapiasal possessed lowest number of endophytes. This difference might be due to variation of physical and chemical properties of soil and microclimate in local environment in different sites. Total genera in Belpahari, Chilkigarh and Godapiasal were 10, 12 and 10 respectively. Dominance index was represented highest in Belpahari (0.1547). Simpson's diversity was maximum in Godapiasal (0.8693) and Shannon-Wiener index was also highest (2.152) in plant of Godapiasal. The highest diversity of endophytic fungi was in plants of Chilkigarh. Average number of isolated endophytic fungi was highest in plants of Belpahari. The isolated endophytic fungi showed their preference for specific tissues. Generally fungal endophytes maximally colonize in leaf tissues, Raviraja and Banerjee et al., but in fewer some cases there is exception to it.

Various diversity indices were calculated to find out the relationships between the endophytes and the host plant. Shannon-Wiener index (2.152) and Simpson's diversity index (0.8693) were maximum in Godapiasal. The highest species richness of isolates was in plant samples of Chilkigarh. Relative abundance of species was highest in plant samples of Godapiasal (Evenness 0.859). At present, endophytes are supposed to be the principal source of novel bioactive compounds functional for human civilization (Raviraja et al., 2005). *Fusarium* sp., *Taxomyces* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Muscodor* sp., *Colletotrichum* sp. are some fungal endophytes reported to produce active bio compounds (Strobel et al., 2003; Gond et al., 2007).

Accordingly, the study of antimicrobial activity of isolated fungal endophytes was carried out. It was observed that few plugs showed antimicrobial activity against few pathogenic bacteria.

Table 1. Colonization frequency of endophytes in various organs of *Combretum roxburghii.*

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF in leaf (%)	CF in petiole (%)	CF in stem (%)
Belpahari	75	57	89	76.00	88	64	76
Chilkigarh	75	55	64	73.33	52	88	80
Godapiasal	75	53	56	70.33	48	96	68
Total	225	165	209	73.32	62.66	82.66	74.66

Table 2. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Combretum roxburghii*.

Isolated Endophytic fungi	Total isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Arthrinium sp.	15	0	0	0	2	1	3	2	7	0
Aspergillus sp.	01	0	0	0	1	0	0	0	0	0
Beltrania sp.	24	12	0	2	2	3	1	1	0	3
Chaetomium sp.	24	1	1	1	4	4	2	2	8	1
Diplodia sp.	25	7	4	0	2	3	1	3	2	3
Fusarium sp.	19	0	5	9	0	1	1	2	0	1
Lasiodiplodia sp.	03	0	2	1	0	0	0	0	0	0
Mucor sp.	13	0	0	3	0	0	7	1	0	2
Mycelia	15	0	1	2	1	0	2	3	5	1
Paecilomyces sp.	01	0	0	0	0	0	1	0	0	0
Penicillium sp.	05	0	0	0	1	2	0	0	0	2
Pestalotiopsis sp.	46	19	0	5	3	8	7	1	2	1
Sordaria sp.	06	5	1	0	0	0	0	0	0	0
Unidentified	04	0	0	0	1	0	0	0	0	3
Verticillium sp.	08	3	4	1	0	0	0	0	0	0
Total	209	47	18	24	17	22	25	15	24	17
L=Leaf, P=Petiole and S=Stem										

Table 3. Diversity indices and species richness of endophytic fungi from *Combretum roxburghii.*

Parameters	Monsoon (June-September)		
	Belpahari	Chilkigarh	Godapiasal
Species richness	10	12	10
Individuals	89	64	56
Simpson diversity	0.8453	0.8521	0.8693
Shannon-Wiener index	2.049	2.145	2.152
Evenness	0.7761	0.7117	0.859
Fisher-alpha diversity	2.891	4.36	3.544

Table 4. Determination of antimicrobial activity of different endophytic fungi isolated from *Combretum roxburghii.*

Isolated endophytic fungi	Diameter of inhibition zone (cm) against 3 bacteria		
	Bacillus cereus	***Escherichia coli***	***Vibrio cholerae***
Aspergillus sp.	1.1	1.4	1.0
Beltrania sp.	0.5	0.3	0
Diplodia sp.	0	0.2	0
Lasiodiplodia sp.	0	0	0.3
Pestalotiopsis sp.	0.4	0	0
Unidentified 1	1.3	0.7	1.1
Unidentified 2	1	0	0

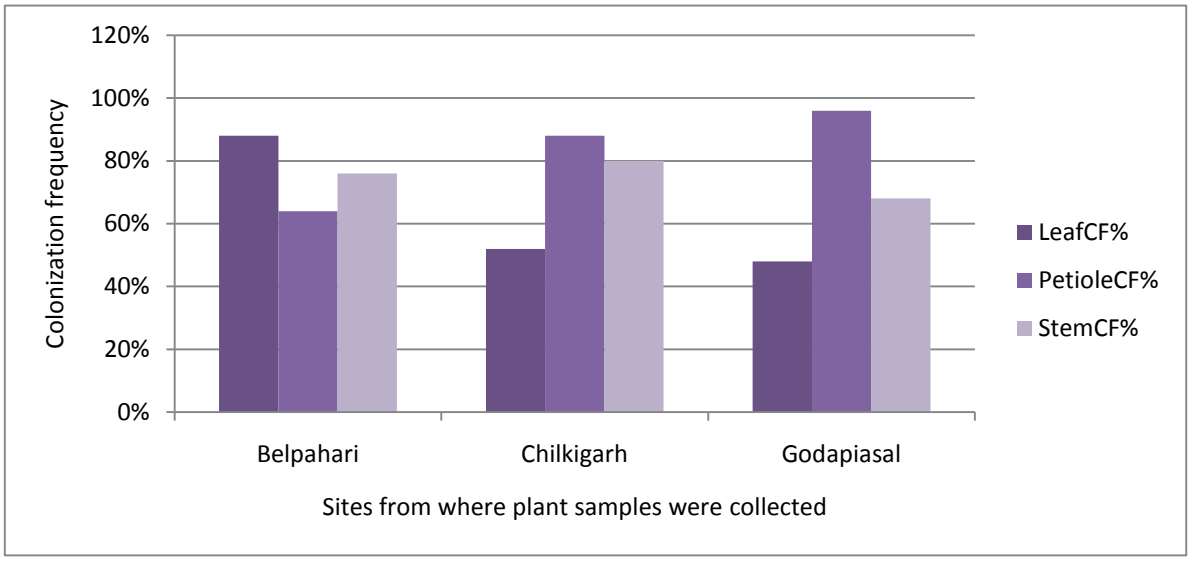

Figure 1. Graph showing comparison of colonization frequency (CF) in various organs.

The growth of fungi produced some crude bioactive compounds that diffused away centrifugally and inhibited the growth of pathogens. Among all isolates tested only seven showed antimicrobial activity (Table 4). Li et al., (2005) reported the antifungal activity of endophytes-*Colletotrichum* sp., *Alternaria* sp. and *Phoma* sp. *Aspergillus* and one unidentified fungi showed the antimicrobial activity against all three bacteria i.e., *Bacillus cereus*, *Escherechia coli* and *Vibrio cholera* and maximum inhibition zone was observed against *Escherichia coli,* diameter of inhibition zone is 1.4 cm. *Beltrania* sp. inhibited the growth of *Bacillus cereus* and *Eecherichia coli*. *Diplodia* sp. and *Lasiodiplodia* sp. retarded the growth of *Escherichia coli* and *Vibrio cholerae* respectively. *Pestalotiopsis* sp. and unidentified 2, both suppressed the growth of *Bacillus cereus*. Mahapatra and Banerjee (2010) also reported antimicrobial activity of some endophytic fungal isolates. The degree of antimicrobial activity from these two endophytes were different. Diameter of zone of inhibition varied 0.7 – 1.4 cm and this variation might be due to the difference of bioactive compound produced or the varied concentrations or amount or degree of same compound. More and more newly discovered

bioactive compounds have immense value in respect of drug resistant pathogens.

Conclusion

Diverse groups of endophytes were isolated with some unknown genera and some mycelia sterilia. *Pestalitiopsis* sp., *Diplodia* sp., *Beltrania* sp., *Chaetomium* sp., *Fusarium* sp., *Arthrinium* sp. are dominant among genera. The plant of *Combretum roxburghii* possesses maximum number of endophytes in Belpahari area and petiole shows highest colonization frequency. Among all isolates tested only seven showed antimicrobial activity *Aspergillus* sp. and one unidentified fungi showed the antimicrobial activity against all three bacteria and maximum inhibition zone was observed against *Escherichia coli,* diameter of inhibition zone is 1.4 cm. Other fungi showed less antimicrobial activity. Diameter of zone of inhibition varied 0.7 – 1.4cm and this variation might be due to the difference of bioactive compound produced or the varied concentrations or amount or degree of same bioactive compound.

Conflict of interest

Author declare that there is no conflict of interest.

Acknowledgement

UGC, New Delhi, is thankfully acknowledged for financial assistance.

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