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Endophytic fungi associated with the medicinally important aromatic plant *Artemisia nilagirica* (Clarke) Pamp. and antimicrobial activity of selected endophytic fungi against *Rhizoctonia solani*

Sarma Parismita*[1], Kumar Manoj[5], Dkhar Mamtaj S.[2], Kayang Highland[3], Laldinthar Ruth[4], Dubey Nawal K.[6], Raghuwanshi Richa[7]

1,2,3,4 Department of Botany, North Eastern Hill University, Shillong - 793 022, Meghalaya, India.
5,6,7 Department of Botany, Banaras Hindu University, Varanasi- 221005, Uttar Pradesh, India.

E-mail : sarmapari@gmail.com

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Abstract

Endophytic fungi associated with the medicinally important aromatic plant *Artemisia nilagirica* (Clarke) Pamp. were isolated following the method of Suryanaryanan *et al*. [1]. The colonization frequency (% CF) of each endophytic fungal species was calculated following the method of Hata and Futai (1995). The highest colonization frequency in the leaf of *A. nilagirica* was exhibited by *Phoma eupyrena* (17.36%), and *Tichoderma viride* exhibited highest colonization frequency in the stem (14.58%) and root (15.28%) each. The Shannon- Weiner's diversity index ranged from 2.6- 2.8 and Simpson's diversity index ranged from 0.8- 1. The method of Skidmore and Dickinson [2] was followed for antimicrobial activity of selected endophytic fungi against *Rhizoctonia solani.* Altogether, 45 endophytic fungi belonging to 25 genera were isolated from the leaf, stem and root of *A. nilagirica*, of which 25 from leaf, 20 from stem and 24 from root. Among the isolates, seven endophytic fungi *Acremonium cerealis*, *Aspergillus fumigatus*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Phoma eupyrena*, *Pythium irregulare* and *Trichoderma viride* were selected to study the antimicrobial activities against *Rhizoctonia solani*. *T. viride* (62.5 %) was found to have the highest percentage inhibition, followed by *P. irregulare* (58.75 %), *Acremonium cerealis* (57.77 %), *P. chrysogenum* (55.55 %) and *A. fumigatus* (49.3 %). *C. cladosporioides* and *P. eupyrena* did not show any inhibition. From the present investigation, out of the seven fungal isolates, only five isolates showed inhibitory efficacy against the pathogen *R. solani*, the causal organism of root rot and damping off of seedlings in many economically important plants.

Key words : Antimicrobial activity, *Artemisia nilagirica,* Endophytic fungi *Rhizoctonia solani.*

INTRODUCTION

Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host [3]. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites [4, 5].

Endophytic fungi are a polyphyletic group of highly diverse primarily ascomyetous fungi that are found to be symbiotically associated within the tissues of almost all classes of plants [6, 7]. The structure and composition of endophytic fungal communities inhabiting the plant tissues is influenced by various factors such as environmental conditions, climatic patterns, physiology of the host and the type of tissue colonized [8]. In the plant and endophytic fungi symbiosis, the former produces and feeds the latter and in return the latter produces bioactive compounds which are beneficial to the plants [9]. Some species of endophytic fungi have been identified as potential sources of anticancer, antidiabetic, insecticidal and immuno-suppressive compounds. Taxomyces andrenae, an endophytic fungi known to be associated with yew tree species Taxus brevifolia produces a chemical compound called taxol, a well known anticancer agent [10]. Recently, endophytic fungi residing in medicinal plants have gained unequivocal attention, thus requiring their systematic identification and characterization.

Artemisia nilagirica (Clarke) Pamp. is an important aromatic medicinal plant found throughout the hilly regions of India ascending to an altitude of 3600 m in the western Himalayas and between an altitude range of 1500- 2400 m in Sikkim and Khasi hills, it is also found in Mount Abu in Rajasthan and in the western Ghats from Konkan southwards to Kerala (www.envis.frlht.org). The plant belongs to the family Asteraceae, and is rich in many important medicinal properties. In traditional medicine, it is generally used for the treatment of diabetes, epilepsy, depression, insomnia and anxiety stress [11]. Since all the parts are of medicinal value, the entire plant is used as antihelmintic, antiseptic, antispasmodic, carminative, cholagogue, digestive, expectorant, purgative and stimulant also. The essential oils of the plant were reported to exhibit 90% mosquito repellency against the mosquito, Aedes aegypti that transmits yellow fever [12].

Despite several reports on beneficial activities of A. nilagirica, the present work was carried to understand the association of the endophytic fungi of *Artemisia nilagirica* and antimicrobial activity of selected endophytes against the phytopathogen Rhizoctonia solani, the causal organism of root rot and damping off diseases of many commercially important plants.

MATERIALS AND METHODS

Plant materials and Study site:

The collection of the plant Artemisia nilagirica (Clarke) Pamp. for the present study was carried out in the permanent campus (1430 meters from sea level), North Eastern Hill University, Shillong, Meghalaya, India. *A. nilagirica* (Clarke) Pamp. belongs to the family Asteraceae and is a tall aromatic perennial herb, which grows in the hilly districts of India in areas up to 2400 m elevation. In herbal medicine, aerial parts of A. nilagirica are used as an anthelminthic, an antiseptic, an

PLATE 1: *Artemisia nilagirica* in natural habitat

antispasmodic, and a tonic for vital organs and for various disorders including hepatosis. With the different parts of the plant collected aseptically, the following investigations were carried out:

Isolation and identification of endophytic fungi:

Different plant parts such as leaves, stems and roots of the selected medicinally important aromatic plant *Artemisia nilagirica* Clarke were collected aseptically. Surface sterilization of the plant parts were done following the method of Fisher et al. [13] and processed following the methods of Suryanarayanan et al. [14] using potato dextrose agar medium (PDA) for the isolation and identification of endophytic fungi. All the samples were washed in running tap water followed by distilled water before processing. The samples were cut into small pieces (for stem and roots pieces 1.0 x 1.0 cm and for leaves 5 x 5 mm). To eliminate epiphytic microorganisms, all the samples were surface sterilized. The samples were immersed in 70% alcohol for 1-3 minutes and then sterilized with aqueous sodium hypochlorite (4% available chlorine) for 3-5 minutes and then rinsed in 70% alcohol for 2-5 seconds before a final rinse in sterilized distilled water. Each sample was dried under aseptic conditions. Segments (4 to 6 per Petri plate) of each sample were placed on potato dextrose agar (PDA) medium amended with streptomycin (100mg/l) to inhibit the bacterial colony growth. The parafilm sealed Petri plates were incubated in an incubator and observed every day for growth of fungal colonies. Hyphal tips of emerging colonies were identified based on their morphological and reproductive structures using standards manuals [15, 16, 17, 18]. Pure cultures of the isolates using Czapek dox agar medium were maintained.

Data analysis

The colonization frequency (CF %) of each endophytic fungi was calculated following the method of Hata and Futai [19].

$$CF\ (\%) = (Ncol / Nt) \times 100$$

Where, Ncol = the number of segments colonized by each endophytic fungi

Nt = the total number of segments observed

Utilizing the data of percentage colony frequency in leaves, stem and roots Shanon- Wiener diversity index and Simpsons's diversity index was calculated.

Shannon- Wiener diversity index= $-\Sigma[(pi)*\ln(pi)]$

Simpson's diversity index=$1-\Sigma(pi)^2$

Where pi(n/N)= proportion of frequency of colonization of the ith species in a sample.

Antimicrobial Activity

Antagonistic activity of endophytic fungi against Rhizoctonia solani was studied following the method of Skidmore and Dickinson [20]. Seven endophytic fungi such as *Acremonium cerealis, Aspergillus fumigatus, Cladosporium cladosporioides, Penicillium chrysogenum, Phoma eupyrena, Pythium irregulare* and *Trichoderma viride* were tested for antagonism against the selected phytopathogen, *Rhizoctonia solani*. PDA plates were inoculated with a 5 mm diameter disc of the antagonist positioned diametrically opposite to the pathogen. Three replicates were maintained for each pathogen. Monoculture plates of the pathogen served as controls. All the plates were incubated at room temperature. The growth of antagonist and pathogen colonies were measured after 5 days of incubation at regular intervals of 24 hours till the 8th day. The % inhibition of the pathogen was calculated using the following equation based on Keshavachandran et al. [21].

$$\%\ \text{inhibition}\ \frac{Y - Z}{Y} \times 100$$

Where, Y= mycelial growth of the pathogen alone

Z= mycelial growth of the pathogen along with the antagonist

RESULTS

Colonization and Diversity of endophytic fungi

A total of 45 species of endophytic fungi belonging to 25 genera were isolated from the leaf, stem and root of Artemisia nilagirica. Amongst the endophytic fungi isolated, 38 species (20 genera) belonged to Ascomycota, 3 species (2 genera) belonged to Oomycota and 2 species (2 genera) belonged to Zygomycota. The maximum number of species was isolated from the leaf (25 species) followed by the root (24 species). The Shannon- Weiner diversity index was calculated for different plant parts i.e. leaf, stem and root. The root has the highest diversity index (0.72).

Among the endophytic fungi isolated, *Trichoderma viride* was found to possess highest colonization frequency in all the plant samples. *Cladosporium cladosporioides, Phoma eupyrena* and *Trichoderma viride* showed highest colonization frequency

Table 1: List of endophytic fungi isolated from the leaf, stem and root of *A. nilagirica* Clarke.

Sl. No.	Endophytic fungi	Division	No. of species
1.	*Gongronella* sp.	Zygomycota	2 genera, 2 species
2.	*Mucor hiemalis*		
3.	*Acremonium cerealis*	Ascomycota	20 genera, 38 species
4.	*A. fusidoides*		
5.	*A. killiense*		
6.	*A. murorum*		
7.	*A. strictum*		
8.	*Apiospora montagnei*		
9.	*Aspergillus candidus*		
10.	*A. fumigatus*		
11.	*A. niger*		
12.	*Aureobasidium pullulans*		
13.	*Chaetomium globosum*		
14.	*Cladosporium cladosporioides*		
15.	*C. herbarum*		
16.	*C. macrocarpum*		
17.	*C. sphaerospermum*		
18.	*Colletotrichum gloeosporioides*		
19.	*Eupenicillium brefeldianum*		
20.	*Fusarium oxysporum*		
21.	*Humicola fuscoatra*		
22.	*Nigrospora sphaerica*		
23.	*Nectria inventa*		
24.	*N. ventricosa*		
25.	*Paecilomyces carneus*		
26.	*P. farinosus*		
27.	*Penicillium chrysogenum*		
28.	*P. jensenii*		
29.	*P. rubrum*		
30.	*P. verrucosum*		
31.	*Phoma betae*		
32.	*P. chrysanthemicola*		
33.	*P. eupyrena*		
34.	*P. exigua*		
35.	*P. glomerata*		
36.	*Phymatotrichopsis omnivora*		
37.	*Taeniolella exilis*		
38.	*Trichoderma viride*		
39.	*Trichothecium roseum*		
40.	*Verticillium alboatrum*		
41.	*Phytophthora cinnamomi*	Oomycota	2 genera, 3 species
42.	*Pythium aphanidermatum*		
43.	*P. irregulare*		

Table 2: Colonization frequency of endophytic fungi isolated from the leaf, stem and root of *A. nilagirica* Clarke.

Sl. No.	Endophytic fungi	Colonization frequency (%)		
		LEAF	STEM	ROOT
	Zygomycota			
1.	*Gongronella* sp.	2.08	3.47	2.08
2.	*Mucor hiemalis*	2.78	1.39	5.56
	Ascomycota			
3.	*Acremonium cerealis*	5.56	0.69	4.86
4.	*A. fusidoides*	-	-	2.08
5.	*A. killiense*	-	-	2.08
6.	*A. murorum*	-	0.69	-
7.	*A. strictum*	0.69	-	-
8.	*Apiospora montagnei*	-	-	1.39
9.	*Aspergillus candidus*	-	0.69	-
10.	*A. fumigatus*	-	-	0.69
11.	*A. niger*	-	-	1.39
12.	*Aureobasidium pullulans*	4.86	2.78	-
13.	*Chaetomium globosum*	-	0.69	-
14.	*Cladosporium cladosporioides*	14.58	2.78	0.69
15.	*C. herbarum*	1.39	-	-
16.	*C. macrocarpum*	0.69	6.94	-
17.	*C. sphaerospermum*	-	-	0.69
18.	*Colletotrichum gloeosporioides*	-	0.69	-
19.	*Eupenicillium brefeldianum*	-	0.69	-
20.	*Fusarium oxysporum*	-	-	2.08
21.	*Humicola fuscoatra*	-	-	0.69
22.	*Nigrospora sphaerica*	0.69	-	-
23.	*Nectria inventa*	-	1.39	-
24.	*N. ventricosa*	0.69	0.69	4.17
25.	*Paecilomyces carneus*	-	-	0.69
26.	*P. farinosus*	-	0.69	-
27.	*Penicillium chrysogenum*	-	-	6.25
28.	*P. jensenii*	1.39	-	-
29.	*P. rubrum*	1.39	0.69	-
30.	*P. verrucosum*	6.94	-	2.08
31.	*Phoma betae*	0.69	-	-
32.	*P. chrysanthemicola*	0.69	-	-
33.	*P. eupyrena*	17.36	6.94	1.39
34.	*P. exigua*	1.39	-	-
35.	*P. glomerata*	0.69	-	-
36.	*Phymatotrichopsis omnivora*	2.08	-	0.69
37.	*Taeniolella exilis*	-	-	0.69
38.	*Trichoderma viride*	11.11	14.58	15.28
39.	*Trichothecium roseum*	0.69	-	-
40.	*Verticillium alboatrum*	0.69	-	-
	Oomycota			
41.	*Phytophthora cinnamomi*	1.39	-	0.69
42.	*Pythium aphanidermatum*	-	1.39	-
43.	*P. irregulare*	-	-	7.64
	Mycelia sterilia			
44	Black	1.39	1.39	1.39
45.	White	4.86	6.25	5.56

in the leaf. Highest colonization frequency in the stem was shown by *Cladosporium macrocarpum, Phoma eupyrena* and *T. viride. Penicillium chrysogenum, Pythium irregulare* and *Trichoderma viride* in the root.

Antimicrobial activity of selected endophytic fungi

The endophytic fungi viz., *Acremonium cerealis, Aspergillus fumigatus, Cladosporium cladosporioides, Penicillium chrysogenum, Phoma eupyrena, Pythium irregulare* and *Trichoderma viride* were selected to test their antimicrobial activity against the selected phytopathogen, *Rhizoctonia solani.* Highest percentage of inhibition was exhibited by *T. viride* (62.5 %) followed by *P. irregulare* (58.75 %), *A. cerealis* (57.77 %). *P. chrysogenum* (55.55 %) and *A. fumigatus* (49.3 %) respectively. *C. cladosporioides* and *P. eupyrena* did not show any inhibition.

DISCUSSION

It was observed that the highest group of endophytic fungi isolated belonged to Ascomycota, similar findings were also reported [22] from Coscinium fenestratum- a red list endangered medicinal plant. It could be due to their ability to compete with other groups of endophytic fungi in terms of better substrate colonization and ability to tolerate wide fluctuations in environmental conditions. The endophytic fungi isolated are mostly organ- specific. However, *Acremonium cerealis, Cladosporium cladosporioides, Gongronella sp. Mucor hiemalis* and *Nectria ventricosa* are isolated in all the plant samples studied. This is in agreement with the findings of Thrower and Lewis [23, 24] who reported that there is sufficient evidence that endophytic fungi play an important role in host- plant physiology. They also received nutrition, protection and propagation opportunities from their hosts.

In our study, highest colonization was recorded in the leaf, followed by the root and the least in the stem. This may be due to large surface area exposed to the outer environment and the presence of stomata providing passage to the entry of fungal mycelia. This may also be one of the reasons why most endophytic fungi of leaf had greater colonization frequency than that of stem. This could be due to repeated reinfection of leaf tissue over time [25]. The species compositions of endophytes are known to vary with different tissues of host plants [26, 27]. This has prompted Petrini et al. [28] to suggest that plant organs resemble distinct microhabitats with reference to endophyte infections.

Trichoderma viride showed the best antagonism against R. solani by showing highest inhibition percentage of 62.50% as calculated by the formula of Keshavachandran et al. [21]. Growth inhibition of the pathogen occurred soon after the contact with the antagonist due to the efficient coiling process followed by substantial production of hydrolytic enzymes. According to Lin et al. [29], *T. viride* secreted some fungicides namely Tricholin (a ribosome inactivating protein) which inhibited the growth of R. solani. Further the work was supported by the study of Shalini et al. [30] who opined that *T. aureoviride, T. harzianum* and *T. viride* inhibited the growth of R. solani, as a result of formation of coiling structure formed around the hyphae and formed hook like structure known as appresoria. The inhibition of pathogen may also be attributed to the production of secondary metabolites by antagonists such as glioviridin, viridin and gliotoxin [31, 32, 33]. It could be also due to the utilization of the protoplasm as a source of

Table 3: Shannon- Weiner index and Simpson's diversity index for leaf, stem and root of *A. nilagirica* Clarke.

Plant parts	Shannon-Weiner index	Simpson's diversity index
Leaf	2.61	0.89
Stem	2.45	0.87
Root	2.72	0.91

Table 4: Antagonistic activity of selected endophytic fungi on *R. solani.*

Sl. No.	Endophytic fungi (antagonist)	Growth of *R. solani* with antagonist (z) (mm)	Growth of *R. solani* in control (y) (mm)	% Inhibition (y-z)/y x 100
1.	*Acremonium cerealis*	38.00	90.00	57.77
2.	*Aspergillus fumigatus*	45.60	90.00	49.30
3.	*Cladosporium cladosporioides*	-	90.00	-
4.	*Penicillium chrysogenum*	40.00	90.00	55.55
5.	*Phoma eupyrena*	-	90.00	-
6.	*Pythium irregulare*	33.00	80.00	58.75
7.	*Trichoderma viride*	30.00	80.00	62.50

Note: '-'= no inhibition zone

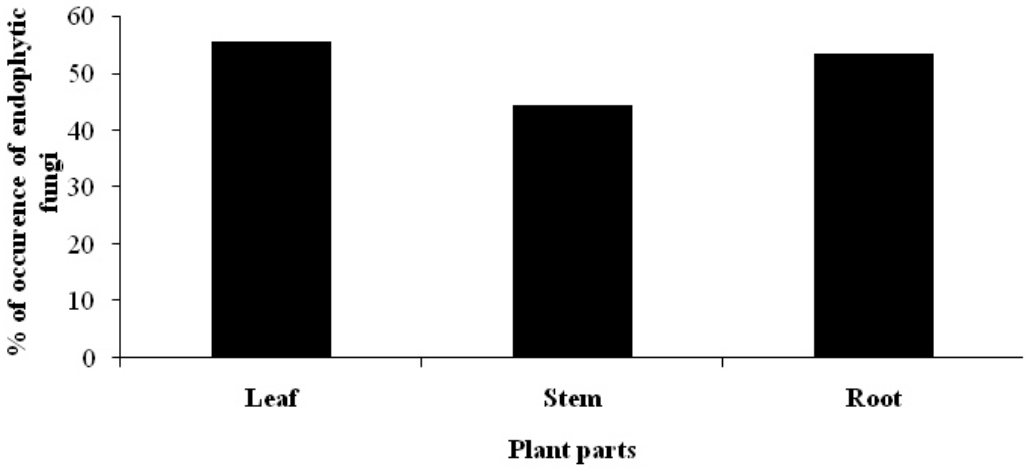

Figure 1: % of occurrence of endophytic fungi of leaf, stem and root of *A. nilagirica.*

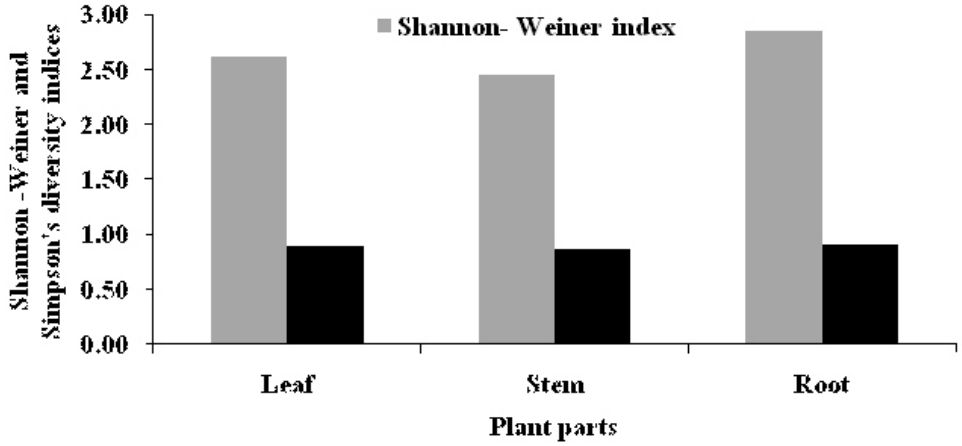

Figure 2: Shannon-Weiner and Simpson's diversity indices of endophytic fungi colonizing the leaf, stem and root of *A. nilagirica.*

food by *T. viride* and multiply its spores. By this method the spores of the pathogen are destroyed. However, the results for *T. viride* may also depend on the ability of producing antimicrobial compounds and degradative enzymes by the tested antagonistic organism. It produces antibiotics and toxins such as trichothecin, sesquiterpine and trichodermin, which have a direct effect on other organisms. *T. viride* hyphae either grow along the host hyphae or coil around it and secrete different lytic enzymes such as chitinase, glucanase and pectinase that are involved in the process of mycoparasitism. On the other hand, the absence of inhibition zone in *C. cladosporioides* and *P. eupyrena* may indicate that their antagonistic metabolites were not inhibitory to each other.

CONCLUSION

It was observed that the highest percentage of inhibition against *Rhizoctonia solani* was shown by *Trichoderma viride* (62.5%) followed by *Pythium irregulare* (58.75%), *Acremonium cerealis* (57.77%) and *Penicillium chrysogenum* (55.55%), whereas, *Aspergillus fumigatus* showed least activity against R. solani. *Cladosporium cladosporioides* and *Phoma eupyrena* did not show any activity against the test pathogen. Therefore it can be concluded that three of the test organisms *T. viride, P. irregulare* and *P. chrysogenum* can be used as good biocontrol agent against *R. solani* causing 'damping off' of seedlings of commercially important plants.

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