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Article

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Diversity of endophytic fungi associated with the medicinally important aromatic plant *Gaultheria fragrantissima*

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Abstract

A total of thirty three (33) endophytic fungi including sterile mycelia were isolated from the leaf, stem and roots of medicinally important aromatic plant *Gaultheria fragrantissima*. Majority of the fungi belonged to the phylum Ascomycota. The total percentage colonization frequency (% CF) of each endophytic fungal species was calculated of which *Juxtiphoma eupyrena* showed highest colonization frequency (20.14%) in the leaf whereas *Globisporangium irregulare* was found to have highest colonization frequency in the stem (14.58%) and in the roots, *Trichoderma viride* was observed to have the highest colonization frequency of 21.43%. Diversity of endophytic fungi in the roots was found to be higher as compared to the other plant tissues.

Key words – Colonization frequency – endophytic fungi – Jaccards's index – relative abundance

Introduction

Endophytic fungi colonise their host plants without causing and specific disease symptoms at any moment of their life cycle (Schulz & Boyle 2006). These microorganisms are virtually found in almost all the plants (Promputtha et al 2005, Arnold 2007) and have been isolated from almost all the plant tissues of gymnosperms and angiosperms (Stone et al. 2000, Jeewon et al 2013, Doilom et al 2017, Vinit et al 2018). Endophytic microorganisms reside in protected environment in the plant tissues hence they have competitive advantage over other phyllosphere and rhizosphere microorganisms and are beneficial for the flow of nutrients flow, maintaining pH and humidity for their hosts (Backman & Sikora 2008). Interestingly, the frequently encountered endophytic microorganisms mainly belong to the Kingdom Fungi (Staniek et al. 2008). Majority of the endophytic fungi establish mutualistic relationship with their host by protecting the latter from phytopathogens, herbivory and also increase plant growth (Clay 1987, Cheplick & Clay 1988, Christensen & Latch 1991). Endophytic fungi are also known to help their hosts in coping with different biotic and abiotic stresses (Gond et al. 2010, Kharwar et al. 2010). It was reported that endophytic fungi isolated from wheat protected the host from infection caused by *Gaeumannomyces graminis* var. *tritici*, the causal organism of 'take all' disease of wheat (Dewan & Sivasithaparam 1989). Many of the endophytes also produce secondary metabolites which are

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known to have novel bioactive properties (Jeewon et al 2008, Porras–Alfaro & Bayman 2011) and the multimillion dollar anticancer agent 'taxol' is one of them. The discovery of taxol from fungal endophyte served as a breakthrough in the field of the study of endophytic microbes. Taxol is produced by a fungus known as *Taxomyces andrenae* which is an endophyte of the plant *Taxus brevifolia* (Stierle et al. 1993). Podophyllotoxin, a potential anticancerous, antiviral, antibacterial agent is obtained from *Alternaria* sp., an endophytic fungus isolated from *Sinopodophyllum* and *Fusarium oxysporum* obtained from *Sabina recurva* (Gao et al. 2007, Kour et al. 2008). An anti–neoplastic agent called Campothecin is obtained from *Enterophosphora infrequens* (Puri et al. 2005). More than 8600 bioactive metabolites attributing to their fungal origin have been described till date (Berdy 2005).

Endophytes of medicinal plants are an important source of discovery of novel products and perhaps might help in replacing synthetic drugs in the near future. Studies on endophytic fungi of several medicinal plants for their antimicrobial activities have been reported till now by many workers (Gond et al. 2010, Tejesvi et al. 2011, Basha et al. 2012, Tayung et al. 2012, Bezerra et al. 2013, Desale & Bodhankar 2013, Myrchiang et al. 2014, Rampadarath et al 2018). However their classification is still obscure (Jeewon et al 2017).

Endophytic fungi colonizing the roots of *Gaultheria fragrantissima* was studied by Das & Kayang (2012) which reported the association of dark ericoid mycrrhizal fungi (DEMF), arbuscular mycorrhizal fungi (AMF) and Dark septate endophytic fungi (DSEF). The present work was conducted to study the diversity of endophytic fungi inhabiting the tissues of *Gaultheria fragrantissima* Wall. *G. fragrantissima* belonging to the family Ericaceae is an aromatic medicinal plant commonly known as wintergreen for its production of the wintergreen oil (Apte et al. 2006). Important constituent of wintergreen oil is gaultherin which has many medicinal properties and is used in the treatment of diseases such as rheumatic arthritis and joint pains. It is also used in many pain relief ointments as well as toothpastes (Apte et al. 2006, Vijayakumar & Paulsamy 2010).

Materials & Methods

Plant Material

Fresh plant parts (leaves, stems and roots) of *Gaultheria fragrantissima* were collected from the forests of Upper Shillong, East Khasi Hills District, Meghalaya situated at an altitude of 1785.82 msl and lies at 25^{o} 32' 16.0" E longitude and 91^{o} 50' 54.3" N latitude. The sampling was carried out during Jan 2015- Dec 2015.

Culture, identification & isolation

Leaf, stem and roots were collected aseptically in sterile plastic bags from the plant *G. fragrantissima* and were processed within 24 hours of collection following the methods of Suryanarayanan et al. (2003).

Potato Dextrose Agar medium (PDA) was used for the isolation and identification of endophytic fungi. The samples were thoroughly washed in tap water and were cut into small pieces (5mm for leaf, 1cm for stem and roots). To eliminate the epiphytic microorganisms all the samples were surface sterilized by immersing in 70% alcohol for 1–3 minutes, followed by Sodium hypochlorite (4% available chlorine) for 3 to 5 minutes and again washed in alcohol for 2–5 seconds before a final rinse in distilled water. The samples were dried in laminar airflow before placing them in the petriplates containing PDA medium amended with streptomycin sulphate (100 mg/L). The petriplates were incubated for 7 to 10 days in incubator at 25±2˚ C. Hyphae from the colonies emerging on the tissues only were identified and again subcultured in Czapek Dox Agar media for 5 to 7 days to obtain pure culture. Identification of the isolated endophytic fungi was carried out based on their morphological and reproductive structures using standard manuals (Ellis 1976, Domsch et al. 1980, Barnett & Hunter 1998).

Data analysis

The colonization frequency (CF %) of each endophytic fungi was calculated following the method of Hata & Futai 1995.

$$CF(\%) = (N_{col} / N_t) \times 100$$

Where, N_{col} = the number of segments colonized by each endophytic fungi

N_t = the total number of segments observed

Relative Abundance (RA) was calculated by the following formula:

$$RA(\%) = N'/N_t \times 100$$

Where, N'= Number of endophytic isolates from each class, order, genera

N_t = Total number of endophytic fungal isolates

Shanon–Wiener diversity index and Simpsons's index was calculated.

$$\text{Shanon–Wiener diversity index} = -\Sigma[(pi) \times \ln(pi)]$$

$$\text{Simpson's dominance index} = \Sigma(pi)^2$$

Where pi= (n/N), pi= proportion of colonization of the *i*th species in a sample.

Shannon's Diversity and Simpsons dominance indices were calculated using the software PAST

$$\text{Jaccard's index (JI)} = c/(a+b+c)$$

a= No. of species in community 1

b= No. of species in community 2

c= No. of species common between the two assemblages.

The results were expressed in percentages.

Results

Diversity & identification of endophytic fungi

A total of 287 fungal isolates were obtained from 432 tissue segments. Highest number of species were isolated from the roots (19 species) followed by the stem (15 species) and leaf (14 species). Identification of endophytic fungi was carried out using standard taxonomic keys which included colony colour, texture, morphology of hyphae and conidia (Hyde et al. 2000) Isolated fungi belonged to the classes Dothideomycetes, Eurotiomycetes, Mucoromycetes, Oomycetes, Saccharomycetes and Sordariomycetes (Table 1). The class Saccharomyceteswas observed only in the roots with relative abundance of 5%. Eurotiomycetes was observed to be the main community member with its relative abundance comparatively higher than other classes of fungi although it showed different relative abundance in different tissues followed by Sordariomycetes and Dothideomycetes. Eurotiomycetes accounted for 29% in the leaves, 20% in the stem and 37% in the roots (Figs 1–3).

Overall frequency of fungal endophytes in *Gaultheria fragrantissima* tissues was 66.44%. *Globisporangium irregulare* was the most frequent species (13.24%) followed by *Juxtiphoma eupyrena* (12.20%). The third most abundant species was *Trichoderma viride* with 7.31%. (Table 2) Fourteen species showed abundance ranging from 0.5 to 5%. Highest colonization frequency in the leaf was shown by *Phoma eupyrena* (20.14%), in the stem was shown by *Globisporangium irregulare* (14.58%) and in the roots was shown by *Trichoderma viride* (14.58%) (Table 3). The total colonization frequency was found to be highest in the autumn season (70.37%) and lowest in the spring season (59.26%) (Fig. 4).

The diversity indices of endophytic fungi of *G. fragrantissima* are presented in Table 4. The higher the values of Shannons diversity index (1.5–4.5) and closer the Simpsons diversity index to 1 the more diversified is the microbial assemblage. Margalef's index indicates the species richness.

Diversity of endophytic fungi was found to be higher in the roots with H' index of 2.65 as compared to the leaf and stem. The lowest being recorded in the stem with H' index of 1.98. Species Evenness index was also found to be highest in the roots (0.64) followed by the leaf (0.43) and Stem (0.43) (Table 4). Shannons index was found to be higher in the winter season (2.35) and lowest in the autumn season (H'= 2.15) (Table 5). Jaccard's similarity index showed that species composition of endophytic assemblage of leaf tissue overlapped stem tissue by 36%, species composition of stem tissue overlapped the root tissue by 30% and composition of root tissue and leaf tissue overlapped by 39.28%.

Discussion

Majority of the endophytic fungi isolated belonged to Ascomycota (83.33%) which is similar to the findings of Goveas et al. (2011) from an endangered medicinal plant, *Coscinium fenestratum*. Only 0.08% belonged to Zygomycota and 0.11% belonged to Oomycota. Sordariomycetes and Dothideomycetes were the predominant classes of Ascomycota in the endophytic assemblage as shown from the findings of Li et al. (2016). The predominant genus was *Penicillium.* Wu et al. (2013) reported that *Aspergillus*, *Nectria* and *Penicillium* were the predominant genera in the roots of *Panax ginseng.* The genera *Aspergillus* and *Penicillium* are generally cosmopolitan and epiphytic inhabitants but are also reported to be endophytic in nature by Schulthess & Faeth (1998). It was found that only one or a few species dominated the endophytic community of the host while majority of them were rare (Petrini et al. 1992). Bezerra et al. (2013), Kusari et al. (2013) also have reported the isolation of species belonging to these genera viz. *Aspergillus, Nectria* and *Penicillium* as endophytes from many plants including plants with medicinal properties. Similar results have also been reported by (Geris dos Santos et al. 2003, Gond et al. 2012, Wu et al. 2013, Kaul et al. 2013, Jariwala & Desai 2018). It was observed that the roots harboured maximum number of endophytic fungi than the other plant parts which may be due to the exposure of the roots to the soil microflora which may facilitate the entry of certain fungi into the plant (Jin et al. 2013). Das & Kayang (2012) also reported the presence of the beneficial root endophytic fungi *Pirisformospora indica* in the roots of *G. fragrantissima.* Bayman et al. (1997), Yuan et al. (2010) also reported rich assemblage of endophytic fungi in the roots of the orchid *Lepanthes* and Wild rice (*Oryza granulata*). The present findings also show highest Shannon- Wiener diversity index in the roots which indicates that the roots have more diverse endophytic fungal community as compared to the leaves and stem. A seasonal variation of Shannon's diversity index was also observed which showed that the diversity was higher in the winter season and lowest in the monsoon season which is in accordance to the findings of Kim et al. (2013). Endophytic fungi such as *Acremonium cerealis, Cladosporium cladosporioides, Penicillium chrysogenum, Juxtiphoma eupyrena* and *Pythium aphanidermatum* were found to be common in all the plant parts however rest of them were not evenly distributed (e.g. Liu et al 2010). Numerous factors such as biotic, abiotic factors, chemical composition and structure of host tissues are involved in the variation of colonization of endophytic fungi in different tissues (Sanchez–Azofeifa 2012, Liu et al 2012). The presence of *Cladosporium cladosporioides* and *Juxtiphoma eupyrena* in all the plant parts is similar to the findings of Myrchiang et al. (2014). Among the genera of endophytic fungi isolated from *G. fragrantissima* the genera *Phytophthora* and *Pythium* are commonly associated with disease symptoms of many commercially important plants and are common soil borne pathogen causing wilting and necrosis in plants. It was observed that certain species of fungi which are pathogenic to certain plants may act as endophytes in other plants without causing any disease symptoms (Promputtha et al 2007, D'Amico et al. 2008). Endophytic fungi are known to establish symbiotic relationship with their hosts, they receive all the necessary nutrition from their hosts while protecting the hosts from phytopathogens, stress and also herbivory (Thrower & Lewis 1973, Clay & Schardl 2002, Yichen et al. 2018) by producing certain useful metabolites in the hosts. The present study throws a light on the various species of endophytic fungi of *G. fragrantissima* and thus offering a scope for the further utilization of the untapped potential of these fungi.

Table 1 Classification of endophytic fungi isolated from *Gaultheria fragrantissima*

Phylum	Class	Order	Genera	Species
Oomycota	Oomycetes	Peronosporales	3	4
Zygomycota	Mucoromycetes	Mucorales	2	4
Ascomycota	Sordariomycetes	Hypocreales	4	5
		Sordariales	1	1
		Incertae sedis	1	1
	Dothideomycetes	Pleosporales	2	2
		Capnodiales	1	2
	Eurotiomycetes	Eurotiales	1	9
	Saccharomycetes	Saccharomycetales	1	1

Table 2 Total percentage colonization frequency and relative abundance of endophytic fungi of *G. fragrantissima*

Sl. No	Endophytic fungi	Total no. of isolates	%CF	RA(%)
1	*Absidia cylindrospora*	1	0.23	0.35
2	*Acremonium cerealis*	12	2.78	4.18
3	*A. kiliense*	1	0.23	0.35
4	*Acrostalagmus luteoalbum*	2	0.46	0.70
5	*Alternaria alternata*	5	1.16	1.74
6	*Apiospora montagnei*	2	0.46	0.70
7	*Cladosporium cladosporioides*	4	0.93	1.39
8	*C. sphaerospermum*	1	0.23	0.35
9	*Eupenicillium javanicum*	2	0.46	0.70
10	*Globisporangium intermedium*	1	0.23	0.35
11	*G. irregulare*	38	8.80	13.24
12	*Humicola fuscoatra*	7	1.62	2.44
13	*Isaria farinosa*	1	0.23	0.35
14	*Juxtiphoma eupyrena*	35	8.10	12.20
15	*Mucor hiemalis*	6	1.39	2.09
16	*M. mucedo*	1	0.23	0.35
17	*M. piriformes*	1	0.23	0.35
18	*Penicillium canescens*	1	0.23	0.35
19	*P. chrysogenum*	4	0.93	1.39
20	*P. daleae*	1	0.23	0.35
21	*P. glabrum*	2	0.46	0.70
22	*P. lanosum*	2	0.46	0.70
23	*P. restrictum*	3	0.69	1.05
24	*P. rubrum*	2	0.46	0.70
25	*P. simplicissimum*	4	0.93	1.39
26	*P. verrucosum*	2	0.46	0.70
27	*Phytophthora cinnamomii*	8	1.85	2.79

Table 2 Continued.

Sl. No	Endophytic fungi	Total no. of isolates	%CF	RA(%)
28	*Pythium aphanidermatum*	10	2.31	3.48
29	*Rectifusarium ventricosum*	13	3.01	4.53
30	*Trichoderma viride*	21	4.86	7.32
31	*Mycelia sterilia* (Black)	10	2.31	3.48
32	*Mycelia sterilia* (White)	82	18.98	28.57
33	*Mycelia sterilia* (Yellow)	2	0.46	0.70
	Total isolates	**287**		

%CF= Percentage colonization frequency, RA= Relative abundance

Table 3 Percentage colonization frequency of endophytic fungi of *G. fragrantissima*

Sl. No.	Endophytic fungi	% Colonisation frequency		
		Leaf	Stem	Root
1	*Absidia cylindrospora*	0.69	–	–
2	*Acremonium cerealis*	2.08	4.86	2.04
3	*A. kiliense*	–	–	1.02
4	*Acrostalagmus luteoalbum*	–	1.39	–
5	*Alternaria alternata*	1.39	2.08	–
6	*Apiospora montagnei*	1.39	–	–
7	*Cladosporium cladosporioides*	1.39	–	2.04
8	*C. sphaerospermum*	–	0.69	–
9	*Eupenicillium javanicum*	–	–	2.04
10	*Globiosporangium intermedium*	0.69	–	–
11	*G. irregulare*	6.25	14.53	8.16
12	*Humicola fuscoatra*	–	0.69	6.12
13	*Isaria farinosa*	0.69	–	–
14	*Juxtiphoma eupyrena*	20.14	2.08	3.06
15	*Mucor hiemalis*	–	3.47	1.02
16	*M. mucedo*	–	–	1.02
17	*M. piriformes*	–	0.69	–
18	*Penicillium canescens*	–	0.69	–
19	*P. chrysogenum*	0.69	1.39	1.20
20	*P. daleae*	–	–	1.20
21	*P. glabrum*	–	–	2.04
22	*P. lanosum*	–	–	2.04
23	*P. restrictum*	0.69	–	2.04
24	*P. rubrum*	–	1.39	–
25	*P. simplicissimum*	1.39	–	2.04
26	*P. verrucosum*	–	–	2.04
27	*Phytophthora cinnamomii*	–	2.08	5.10
28	*Pythium aphanidermatum*	3.47	0.69	4.08
29	*Rectifusarium ventricosum*	1.39	0.69	10.20

Table 3 Continued.

Sl. No.	Endophytic fungi	% Colonisation frequency		
		Leaf	Stem	Root
30	*Trichoderma viride*	–	–	21.43
31	*Mycelia sterilia* (Black)	0.69	1.39	7.14
32	*Mycelia sterilia* (White)	20.83	27.08	13.26
33	*Mycelia sterilia* (Yellow)	1.39	–	–

'-'= absent

Table 4 Diversity indices of the endophytic fungi of *G. fragrantissima*

Diversity Indices	Leaf	Stem	Root
Shannon index (H)	2.00	1.98	2.65
Simpsons Dominance (D)	0.21	0.23	0.09
Simpsons Diversity (1- D)	0.79	0.77	0.09
Margalef's index (D')	3.52	3.51	4.58
Evenness	0.43	0.43	0.64

Table 5 Seasonal variation of diversity indices of *G. fragrantissima*

Seasons	Shannon's index	Simpson's index
Winter	2.35	0.12
Spring	2.29	0.15
Summer	2.23	0.15
Autumn	1.99	0.21

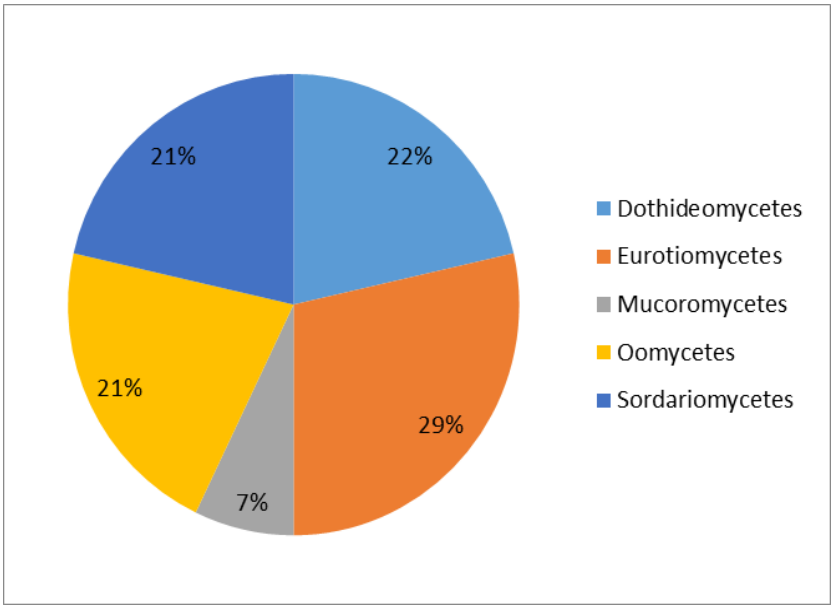

Fig. 1 – Relative abundance of different classes of fungi in the leaf of *G. fragrantissima*

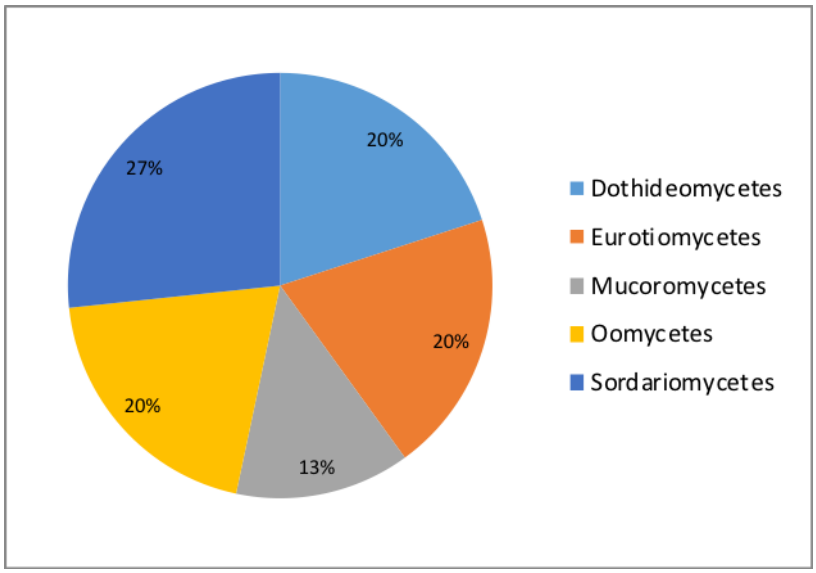

Fig. 2 – Relative abundance of different classes of fungi in the stem of *G. fragrantissima*

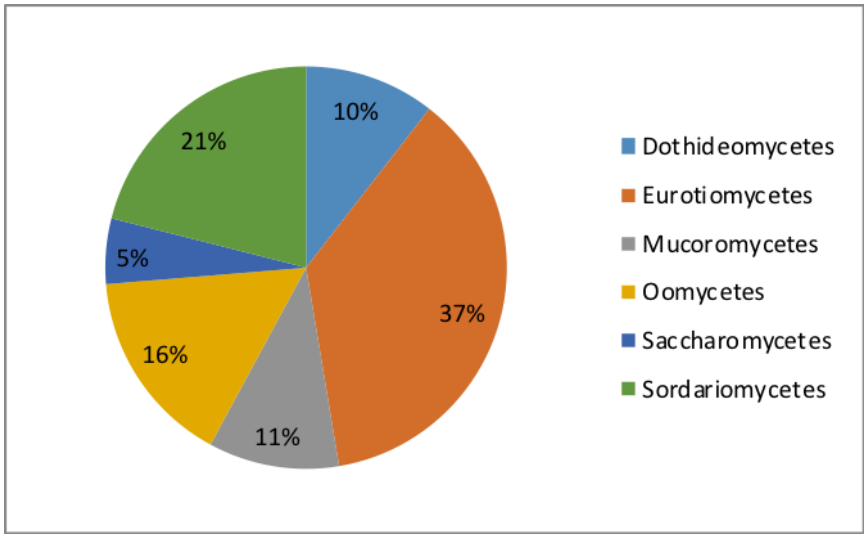

Fig. 3 – Relative abundance of different classes of fungi in the root of *G. fragrantissima*

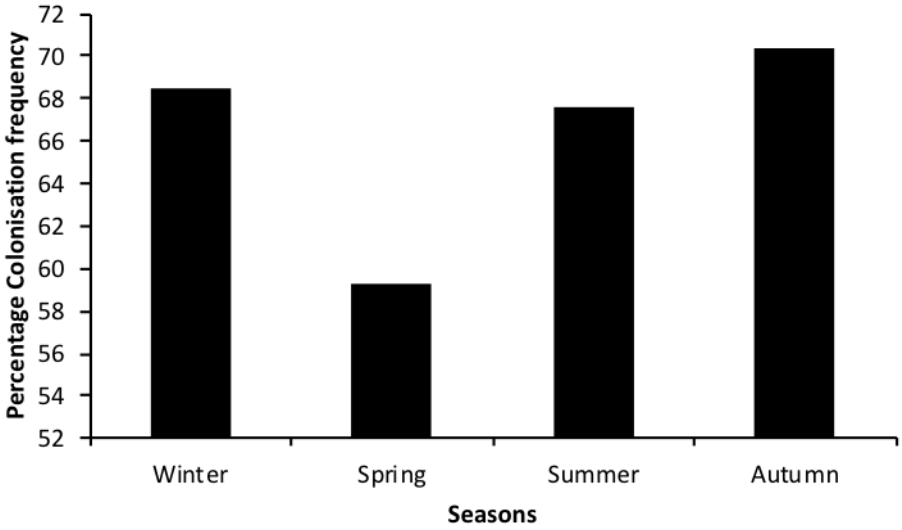

Fig. 4 – Total colonization frequency of endophytic fungi of *G. fragrantissima* during different seasons

Conclusion

The study gives an insight to the diverse populations of endophytic fungi residing in the tissues of *Gaultheria fragrantissima*. It can be concluded that majority of the endophytic belonged to the phylum Ascomycota. Diversity was found to be higher in the roots. The endophytic assemblage consisted of diverse populations of fungi belonging to the Eurotiomycetes. With respect to the colonization of endophytic fungi during different seasons, autumn season showed highest colonisation frequency however diversity was higher during the winter season.

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