Diversity and distribution of endophytic fungi associated with *Nothapodytes nimmoniana* (J. Graham) Mabb.: An endangered medicinal plant of Western Ghats, Maharashtra

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Diversity and distribution of endophytic fungi associated with *Nothapodytes nimmoniana* (J. Graham) Mabb.: An endangered medicinal plant of Western Ghats, Maharashtra

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Endophytic fungal diversity was studied from the *Nothapodytes nimmoniana* (J. Graham) Mabb. (Icacinaceae), an endangered medicinal tree species from five different plant organs and four different localities of Western Ghats of Maharashtra of India. A total of 659 endophytic isolates were obtained from 1200 plant samples/segments of *N. nimmoniana* collected from four different localities. Mitosporic fungi were found to be dominant (55%), followed by agonomycetes (24%) and ascomycetes (21%). The isolates of *Colletotrichum gloeosporioides*, *Phoma* sp. 1 and *Phomopsis* sp. were widely distributed in all the localities. Whereas, some endophytic species, viz. *Alternaria alternata*, *Cladosporium cladosporioides*, *C. oxysporum*, *Colletotrichum* sp., *Cylindrogloeum* sp., *Mycosphaerella* sp., *Myrothecium* sp., *Pestalotiopsis* sp., *Thielaviaica cinacearum* and NS Gr. VI were restricted to only one location. The per cent colonization density (rD%) was higher in a non-sporulating form NS Gr. I (7.1%). The overall colonization and isolation rates of endophytic fungi from leaf lamina were significantly higher (x^2 test, g= 4, P<0.001) than other tissues. The per cent colonization and isolation rates of endophytes recovered in monsoon season were significantly (x^2 test, g = 2, P<0.001) higher (72.75%, 0.71) when compared to summer (41.5%, 0.48) and winter (35.75%, 0.44).

***Key words*:** Colonization frequency, fungal Diversty, *Nothapodytes nimmonina*, Western Ghats,

INTRODUCTION

Research to date shows that novel compounds from fungal endophytes have huge potential as therapeutic agents. It's wide scope in the area of medicine has made it inevitable to conduct comprehensive study of host plants and its medicinal usage.

Nothapodytes nimmoniana (Graham) Mabb. (Icacinaceae) is a small tree, growing in moist deciduous to evergreen and shola forests, commonly referred as Amruta or Narakya in Maharashtra. The distribution of *N. nimmoniana* is throughout the Western Ghats in Southern India (Singh *et al.,* 2015). It has acquired wide attention in the field of medicine, as bark of *N. nimmoniana* has been used as a source of monoterpene, anticancerous alkaloid Camptothecin (CPT), (Ramesha, 2008). In medicine, it is a noteworthy source of several semi-synthetic derivatives of CPT having clinical use against ovarian, small lung and refractory ovarian cancers. (Ulukan and Swaan, 2002; Cragg and Newman, 2004).

The species has been used widely for extraction of the drug (Camptothecin) causing their very existence under threat in the forests of the Western Ghats. (Hombegowda *et al.* 2002; Ramesha, 2008; Padmanabha *et al*. 2006; Singh *et al*. 2015; Khwajah *et al*. 2021). Therefore, efforts have been made to discover the alternate plants and other endophytic fungal sources that can be used in the production of Campthothecin. (Amna *et al,* 2006; Rehman *et al.*2008, 2009; Nagraja 2011; Ramesha *et al*. 2013; Singh *et al.* 2013; Samaga and Rai 2016; Khwajah *et al.* 2021).

Though multiple studies have been conducted over the years on endophytic diversity from *N. nimmonina* on number of aspects, the localities Tilari Ghat, Tamhini Ghat, Mahabaleshwar and Lonavala of Western Ghat, Maharashtra are underexplored for their comprehensive studies for endophytic diversity. So, intense study was carried

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out in these locations as part of this research. Present study provides insights into diversity and distribution of endophytic fungi from leaf lamina, leaf midrib, petiole, inner bark and stem from these four localities.

MATERIALS AND METHODS

Study sites and Sample Collection

Three symptomless and apparently healthy individual plants were randomly chosen from collection sites viz. Lonavala, Mahabaleshwar, Tamhini Ghat and Tilari Ghat during monsoon, winter and summer seasons for two consecutive years (2006- 2008) (Table 1). The plant samples were collected from different aerial parts, viz. leaf lamina, leaf mid-rib, petiole, stem and inner bark. The samples were collected in pre-sterilized polythene bags except inner bark that was gathered in sterile screw capped vials. The collected samples were brought to the laboratory and processed within 24 h of collection.

Isolation of fungal endophytes

The collected plant samples were washed thoroughly under tap water until the surface adherents were removed. A total of 1200 plant bits [(5 × 5 mm (approx.)]; 300 bits per site and 60 bits per tissue/organ were separately excised and subjected to surface sterilization.

In order to eliminate epiphytic microbes, plant bits were sterilized with ethanol, Sodium hypochlorite (NaOCl) solution and distilled water. Plant bits were first rinsed with 70% ethanol for 1 minute, then with sodium hypochlorite for 30 seconds. After that plant bits are again immersed in 70% ethanol for 30 seconds. After every treatment of sodium hypochlorite and 70% ethanol plant bits were rinsed with sterilized distilled water nearly for 3 minutes (4 times) (Surynarayanan and Vijaykrishna, 2001).

The disinfected plant bits were transferred onto a PDA medium incorporated with streptomycin sulphate (500 mg L^{-1}). The plates were incubated at 28±1°C for 10 days and regularly observed for fungal growth. Individual hyphal tips that emerged from the edges of each treated plant bits were transferred separately onto fresh PDA plates without antibiotics. Eventually, the pure endophytic fungal cultures were transferred onto PDA slant and used as stock culture for further experimental studies.

The non-sporulating forms of endophytes obtained in present study were induced *in vitro* sporulation by the selective grass leaf technique proposed by Srinivasan *et al.* (1971).

Diversity assessment using statistical indices

The data gathered was subjected to statistical analysis. The diversity and richness of fungal endophytes isolated from different tissues of *N. nimmoniana* were quantified using various indices such as Colonisation rate, Isolation rate, Colonization frequency, Simpson's diversity index (1-λ'), Shannon-Wiener index (H') and Pielou's evenness (J').

Colonization rate was calculated as the total number of plant-tissue segments infected by one or more fungi, divided by the total number of inoculated segments ×100 (Petrini *et al.* 1982). Isolation rate was determined as the number of isolates obtained from plant-tissue segments divided by the total number of segments inoculated (Wang and Guo, 2007).The density of colonization (rD%) or colonization frequency (CF%) of a single endophyte species was calculated by the method of Fisher and Petrini (1987).

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

Where

N_{col} = Number of segments colonized by each fungus

N_t = Total number of segments inoculated

Simpson's diversity index (1-ë'), Shannon-Wiener index (H') and Pielou's evenness (J') were calculated using Primer software to determine the endophytic species diversity of *N. nimmoniana* growing in four different localities.

RESULTS AND DISCUSSION

A total of 659 endophytic isolates were obtained from 1200 plant samples/segments of *Nothapodytes nimmoniana* collected from four different localities. All the isolates were ascribed to 22 species in 16 genera of fungi (Table 2). Out of these, mitosporic fungi were found to be dominant (55%), followed by agonomycetes (24%) and ascomycetes (21%) (Fig. 1).

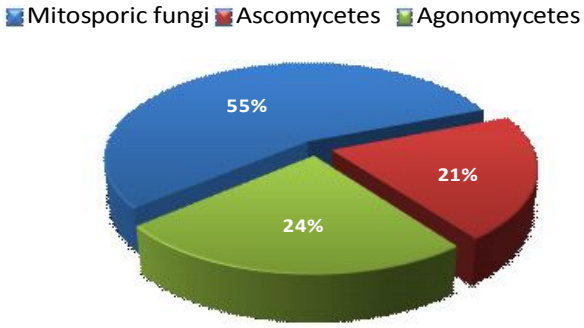

Fig. 1: Group wise distribution of fungal endophytes of *N. nimmoniana*

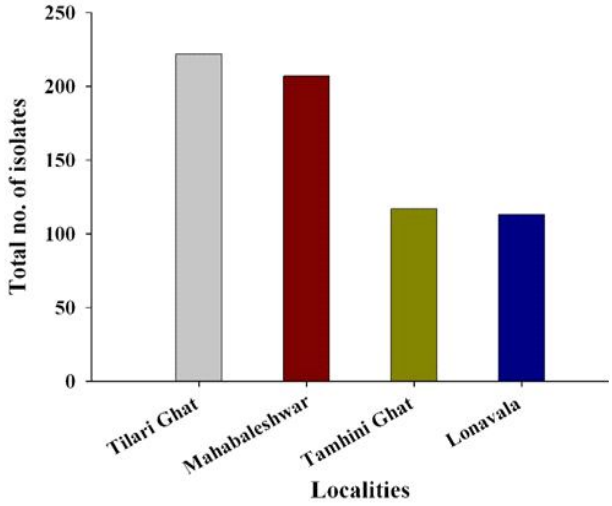

Fig. 2. Endophytic fungal isolates obtained from *N. nimmoniana* collected from different localities.

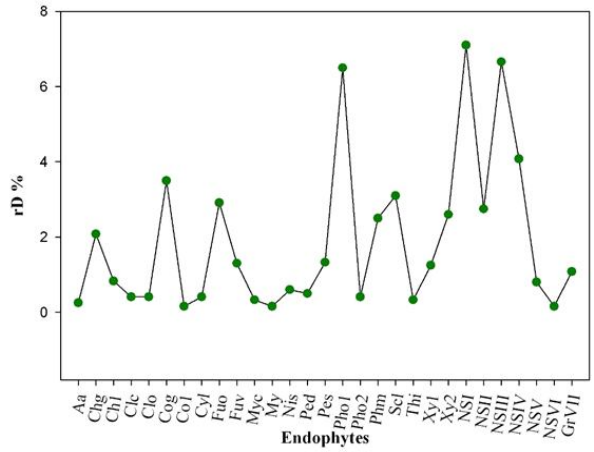

Fig. 3. Colonization density (rD%) of fungal endophytes of *Nothapodytes nimmoniana*

Aa- *Alternaria alternata,* Chg- *Chaetomium globosum,* Ch1- *Chaetomium* sp.1, Clc- *Cladosporium cladosporioides,* Clo- *C. oxysporum,* Cog- *Colletotrichum gloeosporioides,* Co1- *Colletotrichum* sp. 1, Cy1- *Cylindrogloeum* sp., Fuo- *F. oxysporum,* Fuv- *Fusarium verticillioides,* Myc- *Mycosphaerella* sp., My-*Myrothecium* sp., Nis- *Nigrospora sphaerica,* Ped- *Periconia digitata* , Pes- *Pestalotiopsis* sp., Pho1-*Phoma* sp. 1, Pho2-*Phoma* sp. 2, Phm- *Phomopsis* sp. ,Scl- *Scytalidium lignicola,*Thi- *Thielaviaica cinacearum,* Xy1- *Xylaria* sp. 1, NSI- NS Gr. I, NSII- NS Gr. II, NSIII- NS Gr. III, NSIV- NS Gr. IV, NSV- NS Gr. V, NSVI- NS Gr. VI, NSVII- NS Gr. VII.

Plant samples collected from Tilari Ghat have shown maximum recovery of endophytes (222 isolates) as compared to Mahabaleshwar (207), Tamhini Ghat (117) and Lonavala (113) (Fig. 2).

The isolates of *Colletotrichum gloeosporioides*, *Phoma* sp. 1 and *Phomopsis* sp. were widely distributed in all the localities. Whereas, some endophytic species, viz. *Alternaria alternata*, *Cladosporium cladosporioides*, *C. oxysporum*, *Colletotrichum* sp., *Cylindrogloeum* sp., *Mycosphaerella* sp., *Myrothecium* sp., *Pestalotiopsis* sp., *Thielaviaica cinacearum* and NS Gr. VI were interestingly limited to only one location (Table 2).

The per cent colonization density (rD%) was higher in a non-sporulating form NS Gr. I (7.1%), followed by NS Gr III (6.66%), *Phoma* sp. 1 (6.5%), NS Gr IV (4.08%) and *C. gloeosporioides* (3.5%). However, it was recorded between 0.1–2% for most of the other endophytes (Fig. 3).

Species density of colonization (rD%) of endophytes isolated from five different organs ranged between 1 to 17.5. The leaf lamina was highly colonized by the isolates such as NS Gr. I (17.5) and *Phoma* sp. 1 (15) followed by NS Gr. III (10) and *C. gloesporioides* (8.75). The highest species colonization density in inner bark was exhibited by *Scytalidium lignicola* (15.83) followed by NS Gr. IV (13.33), NS Gr. I (10) and *Xylaria* sp. 2 (9.5). Similarly, NS Gr. III (8.3) from leaf mid-rib, *Phoma* sp. 1 (9.58), NS Gr. III (9.5) and NS Gr. IV (7.08) from stem and NS Gr. III (7.08) from petiole showed highest density of colonization. *Colletotrichum gloeosporioides* and NS Gr. I colonized all the tissue segments. Whereas *A. alternata*, *C. oxysporum, Colletotrichum* sp., *Mycosphaerella* sp., *Myrothecium* sp., *Pestalotiopsis* sp., *S. lignicola*, *Xylaria* sp. 1, NS Gr. V and VI were isolated only from tissues of single plant organ (Table 3**).**

The overall colonization and isolation rates of endophytic fungi from leaf lamina was significantly higher (X^2 test, g= 4, P<0.001) than other tissues. The colonization rate of endophytes was very high in leaf lamina (77.91%) followed by inner bark (62.5%) and stem (52.91%). The per cent colonization rate was very less in leaf mid-rib (35.41%) and petiole (21.25%). Similarly, isolation rate of endophytic fungi was significantly higher in leaf lamina (0.82) followed by inner bark (0.7), stem

Table 1. Physiography of sampling sites/localities of *Nothapodytes nimmoniana*

Locality	Latitude/ Longitude	Max. Temp. (Summer)	Min. Temp. (Winter)	Max. Annual rainfall (mm)	Altitude (MSL)	Habitat/ Forest type
Lonavala	N 18º45'03.3" E 73º24'08.7"	38ºC	12ºC	4,500	625	Semi- evergreen
Mahabaleshwar	N 17º55'17.2" E 73º39'18.3"	30ºC	16ºC	6,000 – 7,000	1438	
Tamhini Ghat	N 18º26'01.2" E 73º25'47.5"	38ºC	8ºC	6,511	800 –1,050	
Tilari Ghat	N 15º48'18.2" E 74º10'34.3"	32ºC	8ºC	6,500 – 7,000	400 – 800	

Table 2. Occurrence of fungal endophytes of *N. nimmoniana* collected from different localities and in different seasons

Endophytic Fungi	NFCCI no.	Tilari Ghat	Maha-baleshwar	Tamhini Ghat	Lona-vala	M*	W*	S*
Alternaria alternata	1383	-	+	-	-	+	+	+
Chaetomium globosum	701	+	-	+	+	+	+	-
Chaetomium sp.1	1386	-	+	-	+	-	+	-
Cladosporium cladosporioides	1409	+	-	-	-	+	-	-
C. oxysporum	652	+	-	-	-	+	-	-
Colletotrichum gloeosporioides	690	+	+	+	+	+	-	+
Colletotrichum sp.	1481	-	+	-	-	+	-	-
Cylindrogloeum sp.	660	-	-	-	+	+	+	-
Fusarium oxysporum	651	+	-	+	-	-	-	+
F. verticillioides	653	+	+	+	-	+	+	+
Mycosphaerella sp.	VPG7	-	+	-	-	-	+	+
Myrothecium sp.	655	-	-	-	+	+	-	-
Nigrospora sphaerica	650	+	-	+	-	-	+	-
Periconia digitata	1384	-	+	-	+	-	+	+
Pestalotiopsis sp.	661	-	+	-	-	+	+	+
Phoma sp. 1	702	+	+	+	+	-	-	+
Phoma sp. 2	654	-	+	-	+	-	+	+
Phomopsis sp.	1398	+	+	+	+	-	+	+
Scytalidium lignicola	658	+	+	-	-	-	-	+
Thielaviaica cinacearum	657	+	-	-	-	-	+	+
Xylaria sp. 1	659	-	+	+	-	+	+	+
Xylaria sp. 2	703	+	+	-	-	+	-	-
NS Gr. I	VPG8	-	+	+	+	+	+	+
NS Gr. II	VPG9	+	+	+	-	+	+	-
NS Gr. III	VPG10	+	+	-	+	+	+	+
NS Gr. IV	VPG11	+	+	-	+	+	+	+
NS Gr. V	VPG12	-	+	+	-	+	-	-
NS Gr. VI	VPG13	-	+	-	-	+	-	-
NS Gr. VII	VPG14	+	-	+	+	+	+	-

*M = Monsoon; W =Winter; S = Summer

(0.55) and leaf mid-rib (0.38) whereas, it was minimum in petiole (0.24) (Table 4).

The per cent colonization and isolation rates of endophytes recovered in monsoon season were significantly (X^2 test, g = 2, P<0.001) higher (72.75%, 0.71) when compared to summer (41.5%, 0.48) and winter (35.75%, 0.44) (Table 6). The endophytes *Alternaria alternata, Fusarium verticillioides, Pestalotiopsis* sp., *Xylaria* sp. 1 and NS Gr. I, III and IV were more commonly isolated in all the seasons. Whereas, *Chaetomium* sp. 1, *Cladospirium cladosporioides*, *C. oxysporum, Colletotrichum* sp., *F. oxysporum, Myrothecium* sp., *Phoma*sp. 1, *Nigrospora sphaerica, Scytalidium lignicola, Xylaria* sp. 2,NS Gr. V and VI were recovered only in a particular season (Table 5).

Shannon-Wiener index value for endophytes recorded in Mahabaleshwar (2.607) and Tilari Ghat (2.534) were almost same but comparatively higher than Lonavala (2.255) and Tamhini Ghat (2.089). The evenness of endophytes in Tilari Ghat was recorded higher (0.914) than the three other localities (Mahabaleshwar-0.87, Tamhini Ghat-0.841 and Lonavala-0.879). (Table 6).

Table 3. Organ wise colonization density of fungal endophytes of *Nothapodytes nimmoniana*

Endophytic fungi	Leaf lamina	Leaf mid-rib	Stem	Petiole	Inner bark
Alternaria alternata	1.25	0	0	0	0
Chaetomium globosum	0	2.08	3.33	0	5
Chaetomium sp.1	0	0.8	0.8	2.5	0
Cladosporium cladosporioides	1.25	0.8	0	0	0
Cladosporium oxysporum	2.08	0	0	0	0
Colletotrichum gloeosporioides	8.75	6.66	1.25	1.66	1.25
Colletotrichum sp.	0	0.8	0	0	0
Cylindrogloeum sp.	2.08	0	0	0	0
Fusarium oxysporum	5.4	4.1	2.5	0	2.5
Fusarium verticillioides	2.5	0	0	0	4.1
Mycosphaerella sp.	1.66	0	0	0	0
Myrothecium sp.	0	0.8	0	0	0
Nigrospora sphaerica	1.66	1.66	0	0	0
Periconia digitata	0	0	0	0.41	2.08
Pestalotiopsis sp.	0	0	0	0	6.66
Phoma sp.1	15	0	9.58	6.66	0
Phoma sp.2	0.41	0	0	1.66	0
Phomopsis sp.	6.25	3.33	2.9	0	0
Scytalidium lignicola	0	0	0	0	15.83
Thielaviaica cinacearum	1.66	0	0	0	0
Xylaria sp. 1	0	0	1.66	0	0
Xylaria sp. 2	0	0	3.75	0	9.5
NS Gr. I	17.5	2.9	4.5	2.9	10
NS Gr. II	0	2.9	6.25	1.25	3.75
NS Gr. III	10	8.3	9.5	7.08	0
NS Gr. IV	0	0	7.08	0	13.33
NS Gr. V	4.1	0	0	0	0
NS Gr. VI	0.8	0	0	0	0
NS Gr. VII	0	2.9	2.5	0	0

Table 4. Colonization and isolation rates of fugal endophytes of *Nothapodytes nimmoniana*

	Leaf lamina	Leaf mid-rib	Stem	Petiole	Inner bark
Total no. of plant samples used	240	240	240	240	240
No. of samples yielding isolates	187	85	127	51	150
Total no. of isolates obtained	198	92	134	58	177
Colonization rate (%)	77.91	35.41	52.91	21.25	62.5
Isolation rate	0.82	0.38	0.55	0.24	0.7

Table 5. Seasonal variation in colonization and isolation rates of fungal endophytes of *Nothapodytes nimmoniana*

	Monsoon	Winter	Summer
Total no. of plant samples used	400	400	400
No. of samples yielding isolates	291	143	166
Total no. of endophytic isolates obtained	286	178	195
Colonization rate (%)	72.75	35.75	41.5
Isolation rate	0.71	0.44	0.48

Table 6. Diversity indices and evenness of fungal endophytes of *Nothapodytes nimmoniana* collected from different localities

Study site	Simpson diversity index (1- λ')	Shannon-Wiener index (H')	Evenness index (Pielou's evenness index) J'
Tilari Ghat	0.911	2.534	0.914
Mahabaleshwar	0.909	2.607	0.87
Tamhini Ghat	0.827	2.089	0.841
Lonavala	0.871	2.255	0.879

In the present investigation,mitosporic fungi have been found dominant as compared to ascomycetes and agonomycetes, collected from respective localities and seasons which is in accordance with the other studies (Tejesvi *et al.,* 2005; Gond *et al.* 2007; Sunayana and Prakash, 2012). In comparison to other three localities, maximum isolates were recovered from Tilari Ghat followed by Mahabaleshwar. Maximum annual rainfall is recorded at these locations might be the possible reason for favoring endophytic fungal diversity.

In the present study, number of endophytic fungi recovered from *Nothapodytes nimmoniana* varied seasonally. Higher number of isolates were obtained during monsoon. However, the number of endophytes recovered in winter were low. Similar results were obtained by Nagraj in 2011 while studying seasonal distribution of endophytes of *N. nimmoniana.* He also reported maximum recovery of endophytes during monsoon season followed by winter. The tissues sampled during monsoon have been reported to harbor more endophytes than those screened during dry season (Suryanarayanan *et al.* 2002; Tejesvi *et al.* 2005). The low temperature might be the possible responsible factor for minimum recovery of endophytes. Results obtained in the study suggest that various environmental factors such as climate, water availability, seasons and geographic locations control endophytic fungal communities, which was also reflected in diversity measures. Briefly, endophyte population depends on the physiological condition of the host plant which, in turn, is partly related to the seasonal weather variation. This is in accordance with other studies (Mishra *et al.* 2012; Giauque and Hawkes, 2016; Rampadarath *et al.*, 2018; Costa *et al*. 2018; Slamet *et al.* 2021).

It is also noteworthy that an endophyte, *Cylindrogloeum* sp.was obtained once, from a particular location and exhibited seasonal patterns of distribution. Endophytic distribution in four different patterns based on seasonal appearance has been categorized previously. Fungi showing low colonization frequency can be assigned to the first category (i.e. fungi appearing once in a year and for a short period).

The diversity index is a quantitative measure that provides visibility about the number of different species and the degree of distribution of individuals among those species. Shannon-Wiener index describes the species diversity in a community, whereas Simpsons diversity index indicates species dominance. Shannon-Wiener index, Simpson's diversity index and evenness index varied among all the four localities. Diversity of species increased as the richness and evenness increase. Highest species diversity was recorded at Tilari Ghats and species dominance was higher at Mahabaleswar as compared to other locations. In the present investigation, the highest colonization frequency was recorded in the leaf lamina followed by inner bark, leaf mid rib, petiole and stem. Likewise, the variations in colonization densities of endophytes of selected plant organs of all the host plants were also recorded in the present study. The differences in colonization frequencies may be attributed to the distinct substrate utilization patterns developed by the endophytic fungi. The variation in colonization and isolation rates with respect to their organs has been observed.

The density of the endophytic infection was largely determined by rainfall and humidity that favour the germination and penetration of adherent spores on host tissues. This, in turn leads to increase in infection and colonization rates by endophytes. It is also reported that the precipitation supports the colonization rates of endophytes (Suryanarayanan *et al.,* 2002; Wilson 2000; Wang and Guo, 2007).

In 2005 and 2006, Camptothecin was found in an unidentified endophyte from inner bark of plant *Nothapodytes foetida* (*N. nimmoniana*) in the western coast of India (Puri *et al.* 2005) and endophytes *Entrophospora infrequens* from Jammu and Mahabaleshwar regions of India (Amna *et al.* 2006) respectively. According to Gurudatt *et al.* (2010) report as many as 26 endophytic fungi from the inner bark tissue of 15 individuals of *N. nimmoniana* that generate Camptothetin in culture. These results indicate the ability of the wide diversity of endophyets to produce CPT, a plant-specific secondary metabolite. It is also reported that bark of *N. nimmoniana* has been used as a source of a monoterpene anticancer alkaloid, Camptothecin (Ramesha, 2008). Based on these studies, it is assumed that endophytes *Chaetomium globosum, Pestalotiopsis* sp.*, Periconia digitata, Xylaria* sp.*, Scytalidium lignicola, Fusarium verticillioides, F. oxysporum, Myrothecium* sp. and *C. gloeosporioides* isolated in the present work from inner bark of *N. nimmoniana* could be utilized as potential source of not only Camptothecin but also other alkaloids.

In the present study, it was observed that different endophytic fungi dominated different plant organs of the host plant suggesting that endophytes exhibit some organ specificity. D'souza and Hiremath (2013) isolated endophytes from stem, leaves, leaf petiole, flowers and pedicel of *N. nimmoniana* from the forests in Amboli and reported that the composition and abundance of the endophytes differed according to the host tissues and was found to be dependent on the tissue type. Singh *et al.* (2015) studied the distribution gradient of endophytic fungal diversity from samples of leaves and bark from the stem of *N. nimmoniana* trees from Kerala, Karnataka, Ajivali, and Amboli in Western Ghats of Maharashtara and noticed differences in the endophytic fungal assemblage between leaf and stem bark tissues. Samaga and Rai (2016) isolated around 33 taxa from the leaves and 11 taxa from the stems of *N. nimmoniana* that were collected from Alur, Hassan district, Karnataka, India. They discovered that some of the taxa in *N. nimmoniana* were exclusively inhabited in particular tissues. Several similar studies that were conducted in the past also confirm that many endophytic fungi show a certain degree of tissue specificity.

To sum up, the results presented in this work show appreciable fungal diversity recovered from *N. nimmoniana* from four different localities of Westren Ghat of Maharashtra that are relatively unexplored and need to be explored for their potential. Diverse endophytes isolated from *N. nimmoniana* from the present investigation could be a potential future source of not only Camptothecin but also other alkaloids.

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