



## Pervasiveness of endophytic fungal diversity in *Anisomeles malabarica* from Aliyar, Western Ghats, South India

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### Abstract

A total of 616 fungal isolates were obtained from 1600 segments of leaves and stems of the medicinal plant *Anisomeles malabarica* during four seasons (2009–2010). Fourteen different morphospecies were isolated: *Botryodiplodia theobromae*, *Colletotrichum dematium*, *Pestalotiopsis microspora*, *Phoma betae*, *Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp., *Phomopsis tersa*, *Phyllosticta murnadensis*, *Septoria* sp., *Penicillium* sp., *Nigrospora* sp. and sterile forms. *Phomopsis* spp. was the most dominant and frequently isolated taxon. Colonization frequency (%) differed significantly between the seasons. The total colonization frequency of endophytes during south–west monsoon comprised young leaves 6.6%, mature leaves 15.7%, young stems 3.6% and mature stems 13.4%; north–east monsoon comprised young leaves 4.3%, mature leaves 16%, young stems 7% and mature stems 15.9%; winter comprised young leaves 14%, mature leaves 21.7%, young stems 12.5% and mature stems 17.1%; summer comprised young leaves 1.2%, mature leaves 4.4%, young stems 0.9% and mature stems 2.6%. The colonization rate was higher during the winter than in the monsoon and summer seasons. Moreover, the higher colonization frequency was found in mature leaves than in young leaves. The maximum relative percentage occurrence was coelomycetes (97.91%) followed by agnomycetes (1.33%) and hyphomycetes (0.76%).

**Key words** – *Anisomeles malabarica* – diversity – endophytic fungi – total colonization frequency percentage – relative percentage occurrence

### Introduction

Strobel (2003) noted that approximately 300,000 plant species growing in an unexplored area on the earth are host to one or more endophytes and the presence of biodiverse endophytes in huge number plays an important role in ecosystems with the greatest biodiversity in tropical and temperate rainforests (Strobel & Daisy 2003). A single endophytic fungal species may form relationships with two related plant species, but demonstrate a preference for one particular host, and this phenomenon is categorized as host selectivity (Cohen 2004, 2006). Evidence of plant–associated microorganisms found in the fossilized tissues of stems and leaves has revealed that the symbiosis of endophyte–host most probably dates back to the emergence of vascular plants (Strobel

2003). Zhang et al. (2006) found that the endophytes are detected in plants growing in tropical, subtropical, temperate, and boreal forests with the hosts ranging from herbaceous plants in various habitats, like arctic, alpine and xeric to mesic temperate and tropical forests.

Suryanarayanan et al. (2002) studied tropical forests in the Nilgiri Biosphere Reserve of the Western Ghats for endophyte assemblages based on host recurrence and spatial heterogeneity of their endophytes and concluded that the dry tropical forests had much less endophyte diversity compared to wet tropical forests. However, the study of endophytic fungal biodiversity is very meager. To date, only a few plants have been broadly investigated for their endophytic biodiversity and their probable to produce bioactive secondary metabolites. One of the important roles of endophytic fungi for nutrient recycling is to initiate the biological degradation of dead host-plant (Strobel 2002a). Medicinal plants are reported to harbour endophytes (Strobel 2002b), which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. Hence it is important to explore the endophyte diversity of medicinal plants. Many reports confirmed that endophytic fungi such as *Pestalotiopsis* spp., *Pestalotia* spp., *Phomopsis* spp., *Phyllosticta* spp., *Melanconium* spp., *Colletotrichum* spp., *Phoma* spp., *Chaetomium* spp., *Botryodiplodia* spp., *Bartalinia* spp. and *Monochaetia* spp. can produce medicinally important bioactive secondary metabolites (Firakova et al. 2007). Interestingly, more endophytic coelomycetous fungi have been reported than hyphyomycetes (Petrini 1986, Wang et al. 2000, Zhou et al. 2001, Yuan et al. 2006, Muthumary & Sashirekha 2007). Jayanthi et al. (2011) isolated an endophytic *Phomopsis* sp. from *Mesua ferrea* which recorded antimicrobial activity against various human pathogens. The present study focused on endophytes from the medicinal plant *Anisomeles malabarica* (L.) R.Br., family Lamiaceae. It is used to treat for dyspepsia, fever, rheumatism, snakebite, epilepsy, hysteria, dementia, anorexia, dyspepsia, colic, flatulence, intestinal worms, intermittent fevers, arthritis and diarrhea.

## **Materials & Methods**

### **Collection of plant samples**

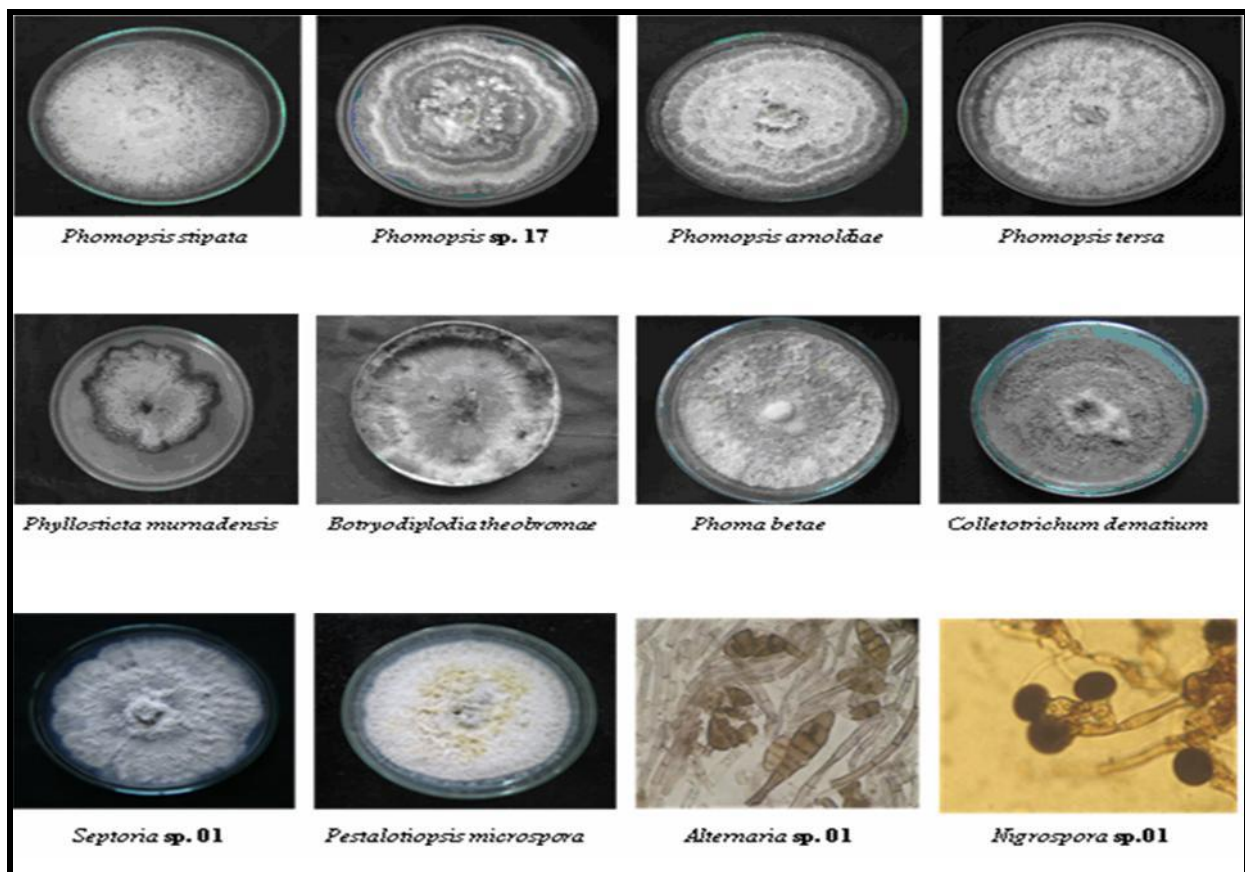
Material of *Anisomeles malabarica* was collected from Azhiyar (10.4739°N, 76.9728°E, located in Anamalai foothills of the Western Ghats), Pollachi Taluk, Coimbatore District, Tamil Nadu, India between May 2009 and April 2010. The samples were collected during different seasons viz., Summer (March–May), South–West Monsoon (June–August), North–East Monsoon (September–November) and Winter (December–February). Young stems (YS), mature stems (MS), young leaves (YL) and mature leaves (ML) were collected. The plant samples were gathered from their natural habitat, placed into sterile polythene bags and taken to the laboratory. The samples were processed within 24 h of collection (Fisher & Petrini 1987).

### **Isolation of endophytic fungi**

The samples were washed meticulously by running tap water. To eliminate the superfluous phylloplane fungal propagules adhering to the cuticle of the leaves, the samples were surface sterilized following the protocol described by Dobranic et al. (1995) with slight alterations. Four hundred segments (approx. 0.5 cm<sup>2</sup>) per different tissues (young stems and mature stems, young leaves and mature leaves) were taken for the study of fungal endophytes (Fisher et al. 1993, Dobranic et al. 1995). The inoculated Petri plates were left in a light chamber for 3–4 weeks (Bills & Polishook 1992) at 23 ± 2°C. Fungal endophytes were maintained on PDA slants in a refrigerator.

### **Preparation of semi-permanent slides**

Specimens with fruit bodies were sectioned with the help of a sterile scalpel blade. Based on the nature of the specimen, the selected sections (10–15 µm) were placed in lactophenol, lactophenol cotton blue or modified Leifson's flagella stain and observed under a light microscope. For the preparation of semi-permanent slide, the slides were sealed with DPX mount. The vertical



**Fig. 1** – Culture plates of endophytic fungi obtained from *Anisomeles malabarica*

sections of the conidiomata were taken to confirm the nature and position of the fructification and conidia in the distilled water was observed under microscope and then photographed.

### Identification and Data analysis

The fungi were identified on the basis of the structure of the conidiomata, conidiogenous cells and conidia (Sutton 1973a, 1973b, 1980). The presence of appendages on the conidia is recognized as an important taxonomic criterion (Guba 1961, Ellis 1971, Onions et al. 1981, Nag Raj 1993) and the conidia were measured. Identification of coelomycetes was done by using standard manuals (Guba 1961, Sutton 1980, Nag Raj 1993).

### Colonization Frequency (CF %)

The colonization frequency for endophytic fungi in the leaves and stems was calculated by using the following formula (Hata & Futai 1995).

$$\text{CF \%} = \frac{\text{No. of segments colonized by an endophytic sp.}}{\text{Total number of segments}} \times 100$$

### Relative Percentage Occurrence (RPO %) of each group of fungi

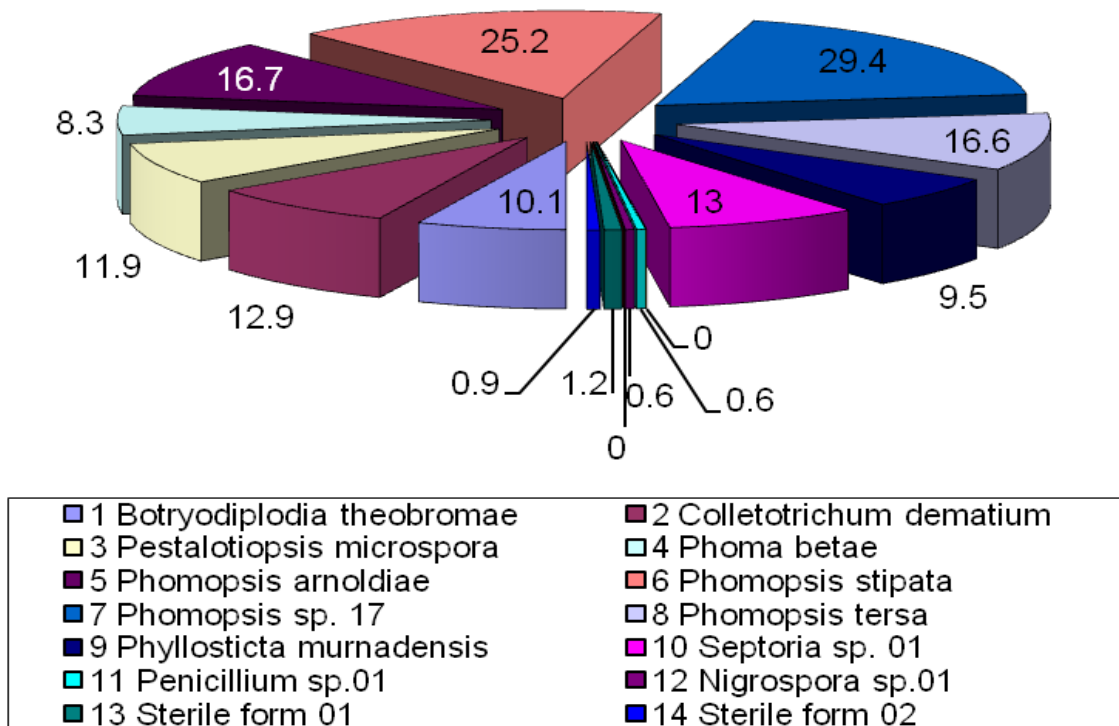
Relative percentage occurrence of each group of fungi (*viz.*, coelomycetes, hyphomycetes and agnomycetes) was calculated using the following formula,

$$\text{RPO \%} = \frac{\text{Density of colonization of one group}}{\text{Total density of colonization of all groups}} \times 100$$

**Table 1** Colonization frequency percentage of endophytic fungi isolated from *Anisomeles malabarica* during different seasons (2009–2010)

S. No	Endophytes	Colonization frequency of endophytes															
		South West monsoon				North East monsoon				Winter season				Summer season			
		(June–Sep)				(Oct–Nov)				(Dec–Feb)				(Mar–May)			
		YL	ML	YS	MS	YL	ML	YS	MS	YL	ML	YS	MS	YL	ML	YS	MS
<b>COELOMYCETES</b>																	
1	<i>Botryodiplodia theobromae</i>	–	–	–	–	2.0	3.0	1.0	2.2	–	0.8	–	0.5	0.3	0.3	–	–
2	<i>Colletotrichum dematium</i>	3.0	3.8	1.3	2.2	–	1.3	–	0.5	–	–	–	–	–	0.5	–	0.3
3	<i>Pestalotiopsis microspora</i>	0.8	1.3	–	0.5	–	0.8	–	2.0	0.8	2.0	1.3	1.8	0.3	0.3	–	–
4	<i>Phoma betae</i>	1.5	2.2	1.0	2.0	–	–	–	–	–	–	–	–	–	1.3	–	0.3
5	<i>Phomopsis arnoldiae</i>	–	1.8	–	2.2	0.5	1.5	1.0	1.3	2.0	2.2	1.3	1.8	–	0.5	0.3	0.3
6	<i>Phomopsis stipata</i>	0.3	1.3	–	–	–	3.8	1.5	3.0	3.0	3.8	3.5	3.8	0.3	0.3	0.3	0.3
7	<i>Phomopsis</i> sp. 17	–	1.0	–	1.5	1.8	3.5	2.2	3.8	4.5	5.0	2.5	3.0	–	0.3	–	0.3
8	<i>Phomopsis tersa</i>	–	0.8	0.5	1.3	–	–	1.3	2.8	2.2	2.8	1.8	2.5	0.3	0.3	–	–
9	<i>Phyllosticta murnadensis</i>	0.5	1.5	0.5	1.3	–	0.5	–	–	0.5	1.8	0.8	1.3	–	–	0.3	0.5
10	<i>Septoria</i> sp. 01	0.5	2.0	–	1.8	–	1.0	–	–	1.0	3.0	1.3	1.8	–	0.3	–	0.3
<b>HYPHOMYCETES</b>																	
1	<i>Penicillium</i> sp.01	–	–	–	0.3	–	0.3	–	–	–	–	–	–	–	–	–	–
2	<i>Nigrospora</i> sp.01	–	–	–	–	–	–	–	–	–	0.3	–	0.3	–	–	–	–
<b>AGNOMYCETES</b>																	
1	Sterile form 01	–	–	0.3	0.3	–	–	–	–	–	–	–	–	–	0.3	–	0.3
2	Sterile form 02	–	–	–	–	–	0.3	–	0.3	–	–	–	0.3	–	–	–	–
No. of species recorded		6	9	5	10	3	10	5	8	7	9	7	10	4	10	3	8
Total no. of isolates observed		26	62	14	53	17	63	28	63	56	86	49	67	4	16	3	9
Total CF% per season		6.6	15.7	3.6	13.4	4.3	16	6.7	15.9	14	21.7	12.5	17.1	1.2	4.4	0.9	2.6
Total		39.3				42.9				65.3				9.1			

YL–young leaf; ML–mature leaf; YS–young stem; MS–mature stem; CF%–Colony frequency percentage



**Fig.2** – Total colonization frequency percentage per year in *Anisomeles malabarica*

## Results

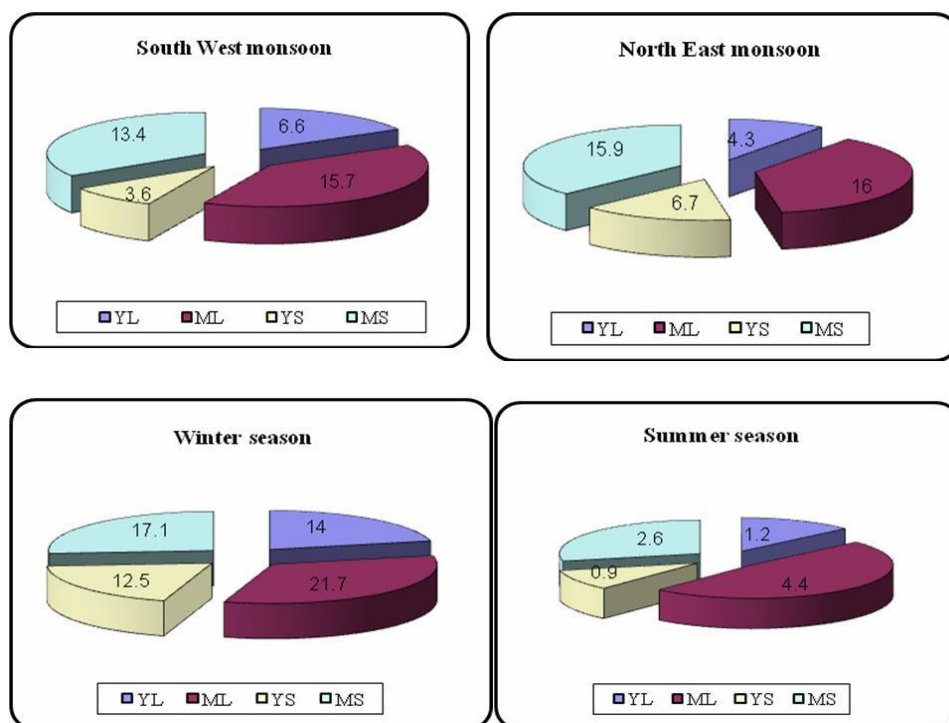
A total of 155, 171, 258 and 32 fungal isolates (i.e., 616 isolates) from 1600 segments (approx. 0.5 cm<sup>2</sup>) of young leaves, mature leaves, young stems and mature stems were isolated during 2009–2010 and their colonization frequencies and relative percentage occurrence were shown in Table. 1. The endophytic fungi isolated are represented in figure 1. Coelomycetes were found to be the dominant group (Fig. 2).

### South west monsoon

A total of 155 isolates, i.e. young leaves (26), mature leaves (62), young stem (14) and mature stem (53) were isolated from 400 segments. Eleven different morphospecies were isolated and identified from leaves and stems. The total colonization frequency of endophytes of young leaves 6.6%, mature leaves 15.7%, young stem 3.6% and mature stem 13.4% were recorded. The most frequently isolated genera were *Phomopsis* (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*). Single species of *Colletotrichum dematium*, *Pestalotiopsis microspora*, *Phoma betae*, *Phyllosticta murnadensis* and *Septoria* sp. 01 of coelomycetes were also recorded, followed by the sterile form 01 (agnomycetes) and *Penicillium* sp.01 (hyphomycetes).

### North east monsoon

A total of 171 isolates, i.e. young leaves (17), mature leaves (63), young stem (28) and mature stem (63) were isolated from 400 segments. Eleven different morphospecies were isolated and identified from leaves and stems. The total colonization frequency of endophytes of young leaves 4.3%, mature leaves 16%, young stem 7% and mature stem 15.9% were recorded. The most frequently isolated genera were *Phomopsis* (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*) and single species of *Botryodiplodia theobromae*, *Colletotrichum dematium*, *Pestalotiopsis microspora*, *Phyllosticta murnadensis* and *Septoria* sp.01 of coelomycetes were also recorded, followed by the sterile form 02 (agnomycetes) and *Penicillium* sp. 01 (hyphomycetes).



**Fig.3** – Distribution of endophytic mycobiota in leaves and stems of *Anisomeles malabarica*

### Winter season

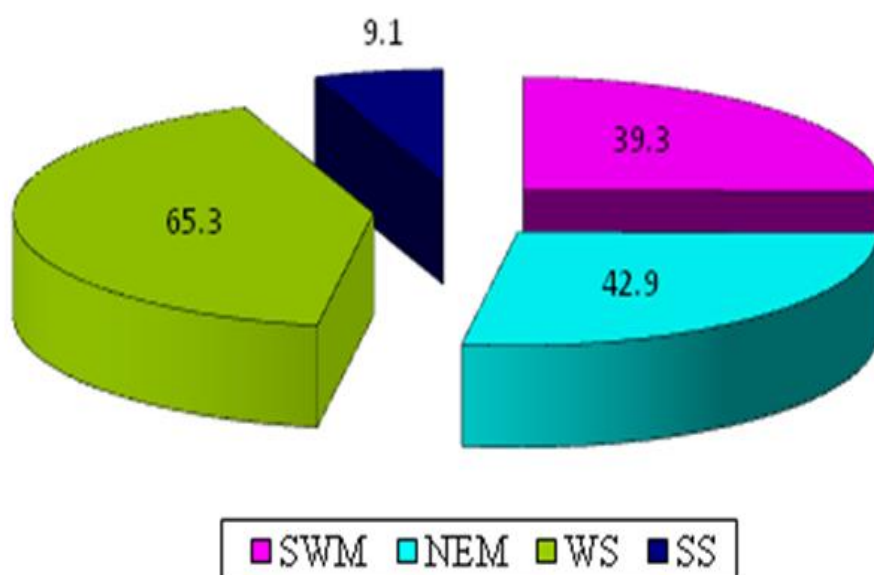
A total of 258 isolate, i.e. young leaves (56), mature leaves (86), young stem (49) and mature stem (67) were isolated from 400 segments. Ten different morphospecies were isolated and identified from leaves and stems. The total colonization frequency of endophytes of young leaves 14%, mature leaves 21.7%, young stem 12.5% and mature stem 17.1% were recorded. The most frequently isolated genera were *Phomopsis* (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*) and single species of *Botryodiplodia theobromae*, *Pestalotiopsis microspora*, *Phyllosticta murnadensis* and *Septoria* sp.01 of coelomycetes were also recorded, followed by *Nigrospora* sp. 01 (hyphomycetes) and sterile form 02 (agnomycetes).

### Summer season

A total of 32 isolates i.e. young leaves (4), mature leaves (16), young stem (3) and mature stem (9) were isolated from 400 segments. Eleven different morphospecies were isolated and identified from leaves and stems. The total colonization frequency of endophytes of young leaves 1.2%, mature leaves 4.4%, young stem 0.9% and mature stem 2.6% were recorded. The most frequently isolated genera were *Phomopsis* (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*) and single species of *Botryodiplodia theobromae*, *Colletotrichum dematium*, *Pestalotiopsis microspora*, *Phoma betae*, *Phyllosticta murnadensis* and *Septoria* sp. 01 of coelomycetes were also recorded, followed by the sterile form 01 (agnomycetes).

### Discussion

The study of endophytes of tropical plants has received much attention because endophytes are believed to be a potential source of biologically active novel compounds (Dreyfuss & Petrini 1984, Hyde 2001). Medicinal plants of Western Ghats of India (a hot spot of global biodiversity) are reported to have a diverse community of endophytic fungi (Raviraja 2005, Krishnamurthy et al. 2008). Few studies on the endophytic fungi of the plants was carried out to probe the diversity of endophytic fungi and their seasonal colonization pattern in medicinal shrub species commonly used in the Malnad region, Western Ghats of Karnataka, Southern India (Krishnamurthy et al. 2008). Hence, in the present study, an unexplored area was chosen for probing endophytic fungi from unexplored medicinal plant *Anisomeles malabarica*.



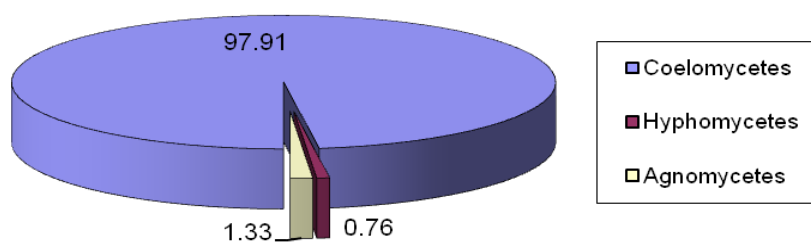
**Fig.4** – TCF percentage of endophytic mycobiota on different seasons in *A. malabarica*

SWM–South West Monsoon; NEM–North East Monsoon; WS–Winter Season; SS–Summer Season.

The increased incidence of endophytic taxa in the older leaves may be due to the increased time of exposure and hence increased accumulation of endophytic fungi from the environment. Older leaves also would have more time to accumulate transmitted colonizers that enter from the petioles (Baun et al. 2003, Osés et al. 2008). The leaves of different maturity showed different rates of colonization; young leaves had 3.33%, mature leaves had 21.02%. The stem, however, showed low percentage frequency of colonization of the endophytic fungi (Goveas et al. 2011). Correspondingly, in the present study, the leaves and stems of different maturity showed different rates of colonization. The result shows that the colonization of endophytic coelomycetes in *Anisomeles malabarica* was significantly higher in leaves than in the stem and also seasonal incidence of endophytes increases with the increasing age of the leaves and stem. Moreover, the highest colonization frequency was found in mature leaves than in the young leaves (Fig. 3) due to the difference in the time of exposure of endophytes. *Alternaria* sp., *Nigrospora* sp. and the sterile forms were found maximum in leaves rather than the stems. The present result also coincides with the findings of Huang et al. (2008), in which some endophytic fungi more frequently colonized the leaves, and other the stems. For example, more endophytic isolates of mycelia sterilia and *Alternaria* sp. were obtained from the leaves than from the stems.

Khan et al. (2007) found eight different species with 8.86% colonization frequency were isolated from *Calotropis procera*. In the present study, 14 different species were observed during South West monsoon, North East monsoon, summer season and winter season and the total colonization frequency was 39.3%, 42.9%, 65.3% and 9.1%, which is slightly more than the above cited study. Thalavaipandian et al. (2011) stated that highest colonization frequency and greater diversity of endophytes was found in monsoon and winter than in summer season. The colonization frequency of endophytic fungi varied and differed significantly between the seasons. Similarly, in the present study, the colonization frequency of leaves and stem was significantly increased during monsoon and winter and decreased during summer season (Fig. 4).

The frequently isolated fungi such as *Chaetomium globosum*, *Colletotrichum gloeosporioides*, *Phyllosticta* spp., and *Cladosporium* spp. are generalist species which grow rapidly on culture medium (Frohlich & Hyde 1999, Suryanarayanan et al. 2003, Krishnamurthy et al. 2008). The coelomycetes such as *Phoma*, *Phomopsis* and *Phyllosticta* are common endophytic fungi (Muller & Hallaksela 1998, Wipornpan et al. 2001, Aveskamp et al. 2008), but are difficult to distinguish from one another due to their similarity in morphological characteristics (Shenoy et



**Fig.5** – Relative percentage occurrence of different group of fungi in *A. malabarica*

al. 2007). Species of *Phomopsis* are widespread plant pathogens and endophytes (Boddy & Griffith 1989). Bussaban et al. (2001) stated that *Colletotrichum "gloeosporioides"*, *Glomerella* spp., Xylariaceous fungi, and *Phomopsis* spp. were found to be dominant endophytes in *Amomum siamense*. Qi et al. (2012) reported that *Alternaria* sp., *Neurospora* sp., *Phomopsis* sp., and *Phoma* sp. were the dominant endophytes in *Acer ginnala*.

In the present study, the most frequently isolated genera were *Phomopsis* (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*). Single species of *Botryodiplodia theobroma*, *Colletotrichum dematium*, *Pestalotiopsis microspora*, *Phoma betae*, *Phyllosticta murnadensis* and *Septoria* sp. 01 (Coelomycetes) were also recorded, followed by the sterile form 01 and 02 (Agnomycetes), *Penicillium* sp. 01 and *Nigrospora* sp. 01 (Hyphomycetes). Mycelia sterilia have been frequently isolated as endophytes from a wide range of tropical and temperate host plants (Suryanarayanan et al. 2004). Since the many sterile fungi do not sporulate in culture and due to the existence of non-culturable endophytes, the real number of endophytic species can be underestimated (Hyde & Soyong 2007). In the current study two sterile forms were isolated. Chareprasert et al. (2006) found that *Phomopsis* was the most frequent endophytic fungus isolated from leaves, both young and adult, of *Tectona grandis* and *Samanea saman* Merr. Similarly, in the present study, *Phomopsis* spp. (*Phomopsis arnoldiae*, *Phomopsis stipata*, *Phomopsis* sp. 17 and *Phomopsis tersa*) were the most dominant and frequently isolated taxa as endophyte from four seasons during the year 2009–2010.

During the four seasons, fourteen different morphospecies were isolated and identified from leaves and stem. *Botryodiplodia theobromae* was not found in South–west monsoon season. *Phoma betae* was absent in North east monsoon and winter season. *Colletotrichum dematium* was absent in winter season. Hyphomycetes were absent in summer season. According to Kumar & Hyde (2004), the vast genera of fungal endophytes were coelomycetes (35%) followed by mycelia sterilia (23.6%), hyphomycetes (15%) and Zygomycetes (0.02%). In the present study, the maximum RPO percentage was found in coelomycetes (97.91%) followed by agnomycetes (1.33%) and hyphomycetes (0.76%) (Fig. 5).

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