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Investigation about seasonal variation and tissue specificity by Endophytic fungi from fifteen Indian medicinal plants

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Abstract
Endophytic fungi infect host plant without causing any visible symptoms, these groups of fungi are pervasive and plant species are host to numbers of diverse endophytes. In all fifteen Indian medicinal plants were selected for isolation of endophytic fungi. About 600 total fungal endophytes were isolated from different parts such as, 173 were from root, 211 from stem, and 216 from leaf tissues. Data obtained was statistically evaluated by analysis of variance, pooled standard deviation, value box plot and null hypothesis. This investigation proves that endophytes are tissues specific and climatic conditions affect endophytic population in selected host plants.

Keywords: Endophytes, standard deviation, tissue specificity

1. Introduction
Plants are considered as a complex and dynamic system composed of interacting networks among microbial populations. Such type of interactions exists on the surface of plants as well as in the internal tissues and type of association ranges from strongly positive mutualism to negative parasitism and also plant diseases[1]. Endophyte includes specific group of microorganisms such as bacteria, fungi and actinomycetes that inhabit, some period of their life in the interior of a host plant without causing any visible harm and even not producing any type of external structures [2].It is now confirm that in nature, each single plant is the host to one or more type of endophyte. Endophytic fungi live internally, moreover intercellularly or intracellularly. They usually occur in above-ground plant tissues, as well as in below-ground plant tissues. Fungal endophytes are distinguished from mycorrhizae by lacking external hyphae. Studies of fungal endophytes in trees, shrubs, and ferns show that individual species and even individual plants typically harbor scores of fungal species. Studies have shown that the leaves of tropical host plants are densely colonized by endophytes[3]. The exact role of endophytic fungi with respect to their hosts has not been evidently proved; to date very few efforts have been made to measure the effects of endophytes on plant physiology. The purpose of this research is to study the impact of different seasons and tissue specificity with respect to naturally occurring endophytic fungi from selected medicinal plants.

2. Materials and Methods
2.1 Selection of plant for isolation of endophytic fungi
Several reasonable hypothesis governs plant selection strategies and these includes assortment of plants that have ethano botanical history, used by indigenous peoples and that are related to the specific uses. Also plants that are endemic and having an unusual longevity, also occupied a certain ancient land mass and growing in temperate region.

2.2 Sampling area and collection of plant materials
Healthy plants were selected growing in different regions of Jalgaon geographically located at 21.01 0 N75.56 0 E and average elevation about 209 meters, during monsoon, winter and summer seasons. Endophytic fungal flora was isolated from roots and rhizomes of *Aloe vera* (L.) Burm.f.(BTE01) and *Curcuma longa* (L.) (BTE02) respectively. Plant tissues such as leaves, stem and roots were broadly screened for presence of endophytic fungi.from *Azadirachta indica* A. Juss. (BTE03), *Coriandrum sativam* L. (BTE04), *Eucalyptus globules* Dehnh (BTE05), *Hibiscus rosa sinensis* L. (BTE06), *Ixora coccinea* L. (BTE07), *Murrayo koenginii* (L.) Sprengel (BTE08), *Musa paradiasica* L. (BTE09), *Ocimum sanctum* (L.) (BTE10), *Pongamia glabra* Vent. (BTE11), *Sphaeranthus indicus* Linn (BTE12),

Vinca rosea (L.) G. Don. (BTE13), *Vitex nigundo* (L.) (BTE14) and *Withania somniphera* (L.) Dunal. (BTE15). Herbarium codes (in round brackets) were given during plant collection. Samples were immediately brought to laboratory and were used within 8 hrs.

2.3 Processing of sample for isolation of endophytic fungi

Sampling different parts of the selected medicinal plants, ten samples were collected from roots, stems and leaves. These samples were wash in running tap water to remove soil particles and adhered debris, and finally washed with distilled water. Sub samples were prepared from each sample for further isolation of endophytic microbes.

2.4 Disinfecting plant sections

Samples were treated with 70% ethanol for 3-4 min and 4% aqueous solution of sodium hypochlorite 2 min, 0.1% mercury chloride for 1.5 min and rinsed with sterile distilled water [4, 5, 6]. Surface sterile samples were selected by aseptic cutting using sterile knife and inner tissues were excised. Later the segments were rinsed three times with sterile distilled water and were blotted on sterile blotting paper. Size of tissue section varies from 1 cm long to 3-4 mm broad. The efficiency of surface sterilization procedure was necessarily checked by imprint method [7].

2.5 Isolation of endophytic fungi

The fungi discussed in this report were isolated by decontaminating the endophytic bacteria. The dissected tissues were placed on the potato dextrose agar, with 50mg/l chloramphenicol. Endophytic fungi usually began to produce hypal filaments after 5-6 days of incubation at 30^0C. The hypal tips appeared were carefully transferred to fresh sterile potato dextrose agar plates[5,6] . Colonization frequency (CF) was calculated as described by Hata [8]. Colonization frequency (%) of an endophyte species was equal to the number of segments colonized by a single endophyte divided by the total number of segments observed X 100

$$\% \text{ Colonization frequency} = \frac{\text{Number of segments colonized endophyte}}{\text{Total number of segments}} \times 100$$

3. Results and discussion

Fungal endophytes are generally ubiquitous in all plant species studied till the date and medicinal plants are also known to harbor endophytic fungi. During course of investigation fungal endophyte were isolated at definite conditions using specific growth media. Presence of endophytic fungi demonstrates prevalence of endophytes in plant tissues under taken for investigation. Both aerial and beneath ground parts exhibit presence of endophytes. Out of 600 total fungal endophytes isolated, 173 were from roots, 211 from stems, and 216 from leaves. It is evidence from results that plant organs are highly colonized by endophytic fungi. Isolated endophytic fungi were distributed in different groups according to their colonization frequency as shown below in the table.

Table 1. Colonization frequency by endophytic fungi in different groups

Groups	Host Plant	% CF	Range
I	*S.indicus*	12	10-20
II	*P.glabra*	22	20-30
III	*A.vera*	31	30-40
	H.sinesis	32	
	W. somniphera	35	
	I. coccinea	37	
	C.longa	39	
IV	*E.globules*	41	40-50
	M. paradiasica	42	
	C.sativum	45	
	O.sanctum	47	
	M.koenginii	48	
V	*A.indica*	53	50-60
	V.nigundo	54	
VI	*V.rosea*	62	60-70

% CF: Per cent colonization frequency

The total colonization frequency during the isolation period of different groups is described on the basis of frequency range (Table 1.). The frequency range is found from 10 to 70 %. Highest colonization frequency is 62% in plant *V.rosea* followed by *V.nigundo* (54) and *A.indica* (53). This frequency is reduced in *P.glabra* (22%), and *S.indicus* (12%), while other plants *A.vera, H.sinesis, W. somniphera, I. coccinea, C.longa, E.globules, M. paradiasica, C.sativum, O.sanctum, M.koenginii* were moderately colonized by endophytes. In these plants it varies from 31% to 48 %.

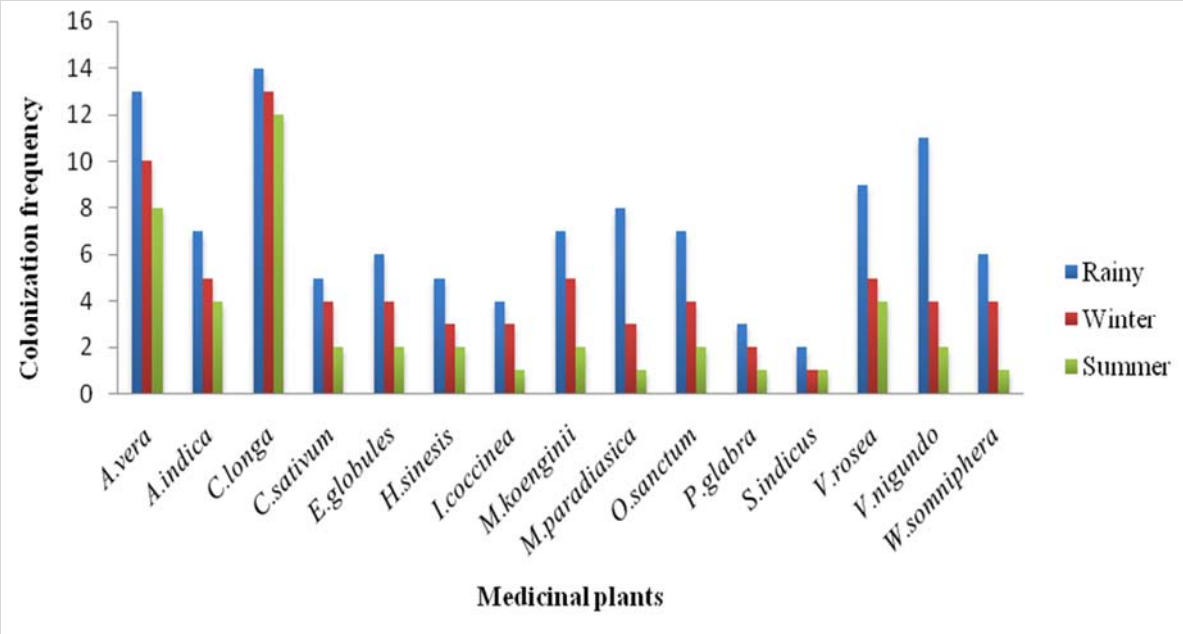

Fig 1: Colonization frequency by endophytic fungi isolated from root/rhizomes

Above Figure 1. depicts graphical representation of root endophytic fungi isolated during rainy, winter and summer seasons. Colonization frequency ranges from 1-14%. In consideration with root fungi, highest colonization frequency attributes in rhizomes of *C.longa* (14%), followed by roots of *A.vera* (13%), *V.nigundo* (11), *V.rosea* (9%), *M.paradiasica* (8%), *O.sanctum* (7), *M.koenginii* (7%) and *A.indica* (7%) during period of rainy season. Root tissue of remaining plants has abstemiously colonized by endophytic fungi.

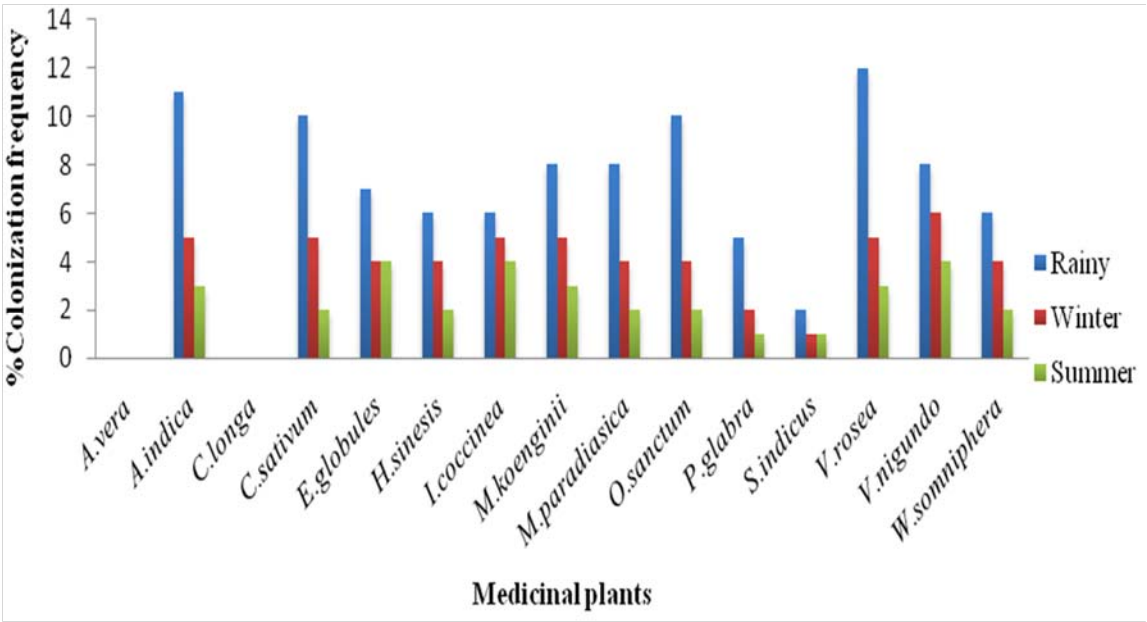

Fig 2: Colonization frequency by endophytic fungi isolated from stem tissues

Similarly Figure 2. Represents colonization frequency of fungal endophytes from stem tissues during various seasons. Colonization frequency ranges from 1-12%, and was found maximum in rainy season in comparison of other seasons. Host plants *V.rosea* occupy first rank (12%), followed by *A.indica* (11%), *C.sativum* (10%), *O.sanctum* (10%), *M.koenginii* (8%), and *M.paradiasica* (8%). Other plants were fairly colonized by endophytic fungi with respect to stem tissues.

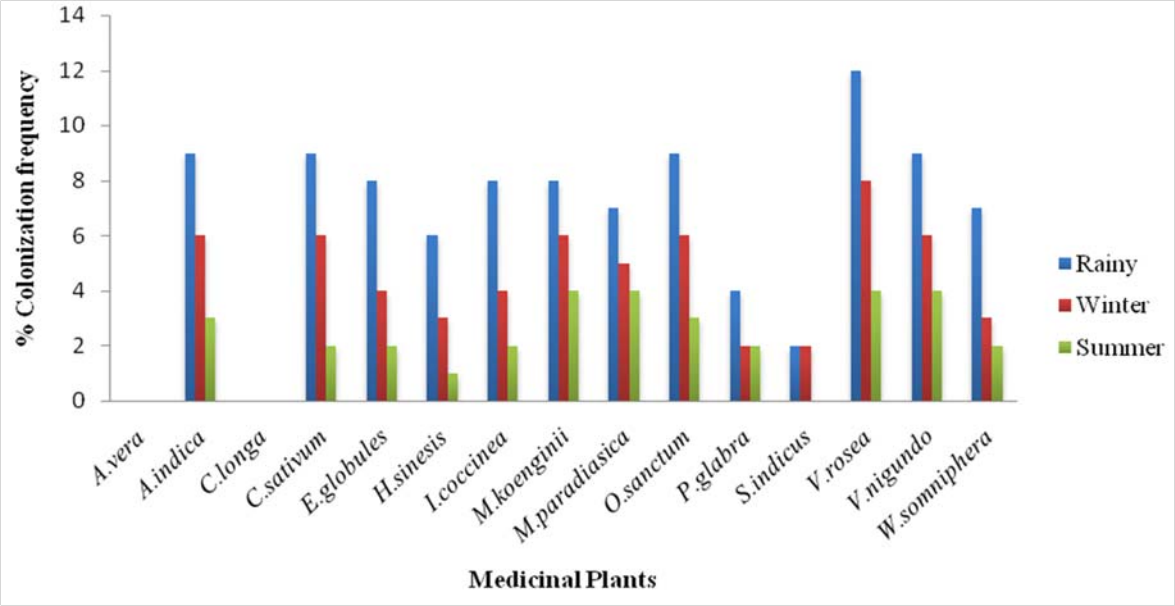

Fig 3: Colonization frequency by endophytic fungi isolated from leaf tissues

Figure 3**.** indicates that leaf tissues were also colonized vastly with endophytic fungi. Per cent colonization frequency is found from 1-12% in summer and rainy seasons respectively. Leaf tissue from host plant *V.rosea* harbor more fungal flora (12%), which is followed by *V.nigundo*(9%), *O.sanctum*(9%), *C.sativum* (9%), *A.indica* (9%), *E.globules* (8%), *I.coccinea* (8%), *M.koenginii* (8%), *M.paradiasica* (7%), *W.somniphera* (7%), *H.sinesis* (6%), *P.glabra* (4%) and *S.indicus* (2%) for the duration of rainy season.

Table 2. Analysis of variance for fungi of root tissues (one way ANOVA)

Source	DF	SS	MS	F	P
Factor	2	129.7	64.9	6.35	0.004
Error	42	429.1	10.2		
Total	44	558.8			

DF:Degree of freedom;**SS**:Sum of squares; **MS**:Mean sum of square; **F**:Ratio; **P**:Value

Table 3. Pooled Standard deviation for fungi of root tissues

Level	N	Mean	St Dev
Rainy	15	7.133	3.441
Winter	15	4.667	3.039
Summer	15	3.000	3.094

Pooled St Dev = 3.196; *N:Number of observation; StDev:Standard deviation

H0=M1=M2=M3 Vs H1= M1$\neq$ M2$\neq$ M3 (1)

Here, Ho: For all three seasons fungi in roots are same

H1: For all three seasons fungi in roots are not same, (M=Mean); (If P value< 0.05, then reject H0), P value=0.004<0.05.

Hence,Ho is rejected,Therefore,fungi in roots for three seasons (rainy,winter,summer) are not same. (Table 2.and 3.)

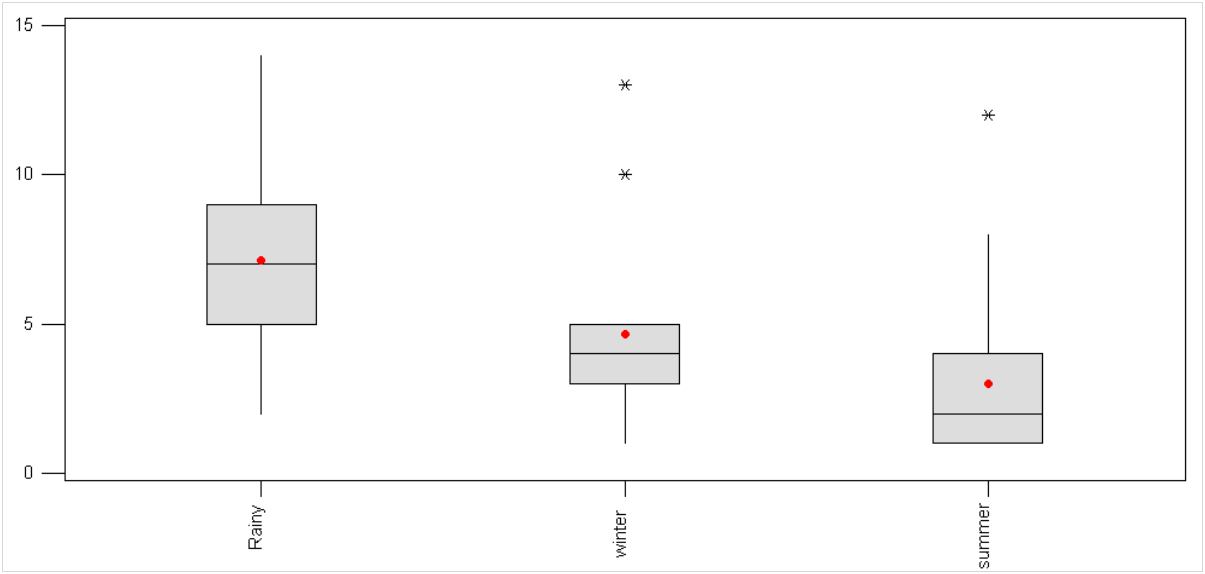

Fig 4: Box plot of root endophytic fungi in different seasons

Value box plot analysis represents alteration between isolated fungi during three different seasons

Table 4. Analysis of variance for fungi of stem tissues (one way ANOVA)

Source	DF	SS	MS	F	P
Factor	2	174.92	87.46	25.39	0.000
Error	36	124.00	3.44		
Total	38	298.92			

DF:Degree of freedom;**SS**:Sum of squares; **MS**:Mean sum of square; **F**:Ratio; **P**:Value

Table 5. Pooled standard deviation for fungi of stem tissues

Level	N	Mean	St Dev
Rainy	13	7.615	2.725
Winter	13	4.154	1.345
Summer	13	2.538	1.050

Pooled StDev = 1.856 *N:Number of observation; StDev:Standard deviation

H0=M1=M2=M3 Vs H1= M1≠ M2≠ M3 (1)

Here, Ho: For all three seasons fungi in stem are same

H1: For all three seasons fungi in stem are not same, (M=Mean);(If P value< 0.05 , then reject H0), P value=0.000<0.05. Hence,Ho is rejected,Therefore,fungi in stem for three seasons (rainy,winter,summer) not same. (Table 4. and 5.)

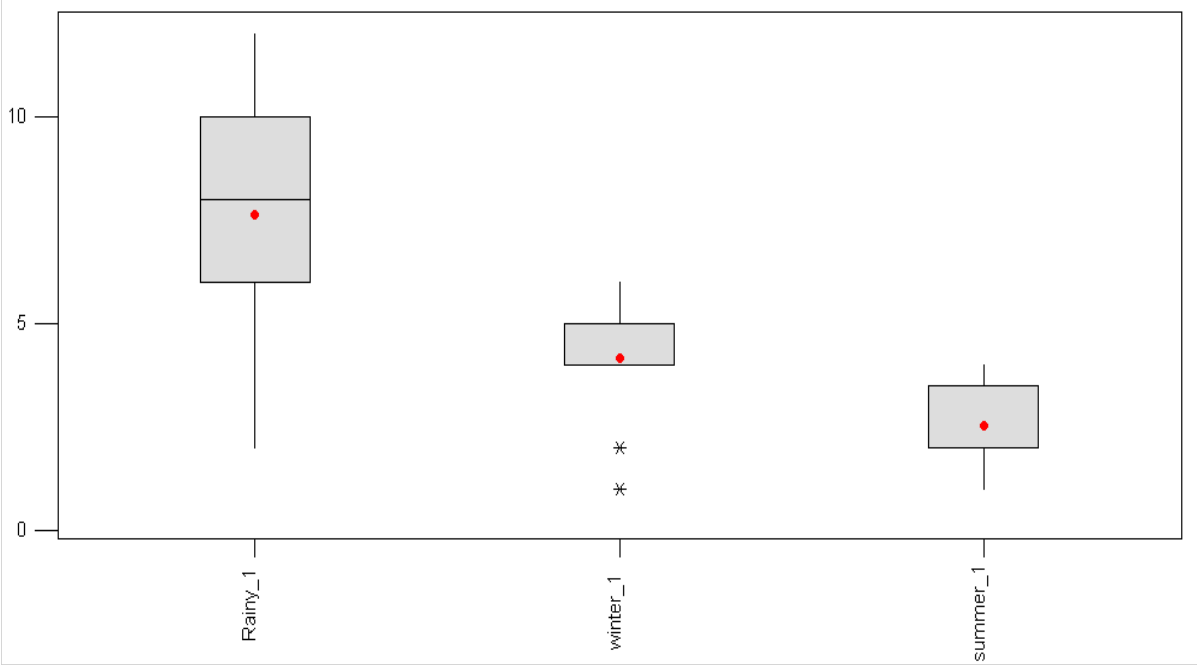

Fig 5: Box plot of stem endophytic fungi in different seasons

Box plot study signifies change in endophytic fungi isolated from stem tissues during different seasons.

Table 6. Analysis of variance of leaf tissues (one way ANOVA)

Source	DF	SS	MS	F	P
Factor	2	163.54	81.77	21.77	0.000
Error	36	135.23	3.76		
Total	38	298.77			

DF:Degree of freedom;**SS**:Sum of squares;**MS**:Mean sum of square;**F**:Ratio;**P**:Value

Table 7. Based on pooled Standard deviation of leaf tissues

Level	N	Mean	StDev
Rainy	13	7.538	2.504
Winter	13	4.692	1.843
Summer	13	2.538	1.266

Pooled StDev = 1.938 *N:Number of observation; StDev:Standard deviation

H0=M1=M2=M3 Vs H1= M1≠ M2≠ M3 (1)

Here, Ho: For all three seasons fungi in leaf are same

H1: For all three seasons fungi in leaf are not same, (M=Mean);(If P value< 0.05 , then reject H0), P value=0.000<0.05.

Hence, Ho is rejected,Therefore, fungi in leaf for three seasons (rainy,winter,summer) not same (Table 6. and 7.)

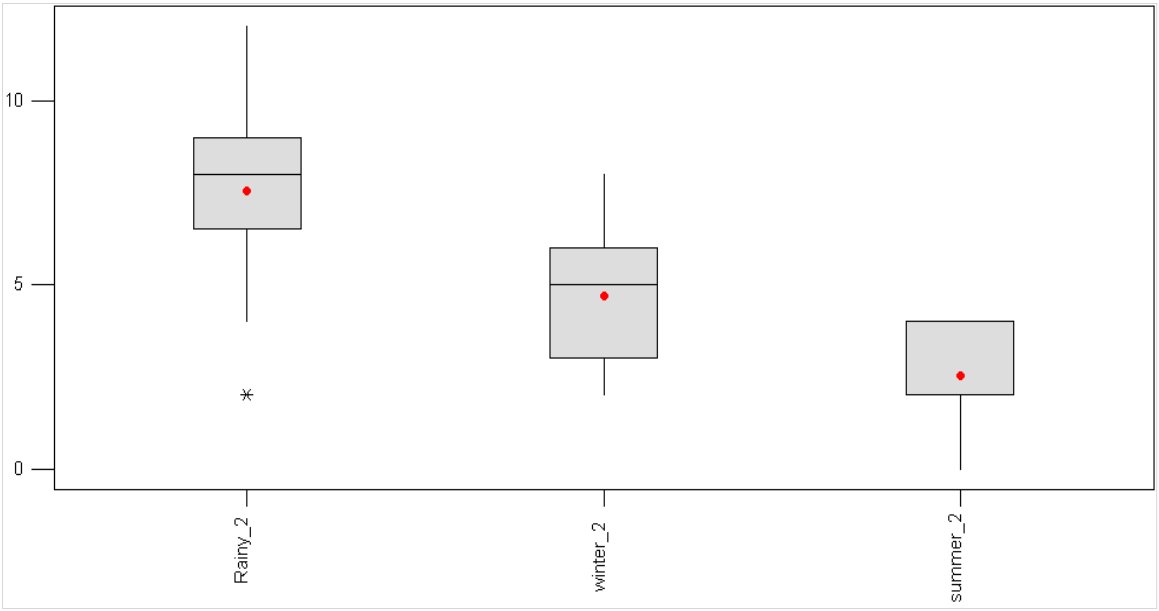

Fig 6: Box plot of leaf endophytic fungi in different seasons
Box plot reading shows significance change of leaf endophytic fungi with respect to change in diverse season.

In the present study fungal endophytes were successfully isolated from fragments of leaf, stem and root of selected fifteen Indian medicinal plants. Statistical data obtained demonstrated how the frequency of colonization of these microorganisms fluctuates during different seasons. The hypotheses established at the beginning of this study clearly recognized, that the colonization frequency with respect to fungal endophytes from diverse host plants varies according to change in seasons. The Per cent colonization frequency with respect to isolated fungal flora was high in monsoon when temperature was low with rise in moisture of the atmosphere. This is followed by dry winter season when temperature was low. In hot, dry summer with elevation in temperature, colonization frequency decreases. Huge numbers of endophytes were isolated from leafs and stems while less number of endophytes isolated from roots. Indicating that endophyte occurrence is not depend on the tissues only but also on seasonal variations.

This neglected group of organism is important component of biodiversity, they play vital physiological and ecological role in their life, by increasing the adaptability of their host [9, 10]. These microorganisms found to affect by change in climate and environmental conditions. In this research we addressed mechanism of outcome about interaction between fungal endophyte and host plant. At time of progressive research, we found that endophytes are tissue specific and affected by seasonal variations. Statistically it is proved that in rainy season endophytes were active and isolated more in number comparative to winter and summer. This finding also collate with work done by earlier researchers [11, 12]. The low colonization during the summer months may attributed to reduce fungal activity and the proportion of microbial biomass formed by microbes appear to be reduced[13]. Distribution pattern of endophytes within plant tissues are studied at length, previously occurrence of endophytes in inner bark and root examined by Bills [14, 15] extended studies of previous authors with correspond to foliar endophytes. After a decade Cannon and Simmons[16] confirmed endophytic flora majorly from leaves. This information was insufficient for us, therefore, in the present study based on endophytic investigation of fifteen medicinal plants massive flora was isolated together from upper ground and under ground parts. Outcome of these work lead to finding that endophytic microbes are generally tissue specific. This confirms finding of Fisher[17, 18] that frequency of colonization varied according to host and type of tissues.

In this study, a larger percent of colonization of endophytic fungi has been observed during the wet season compared to the dry season. A similar configuration was observed in a previous work where the isolation frequency of fungal endophytes increased during the rainy season[19,20]. The alteration in frequency of colonization is probably due to environmental changing aspects that should be affecting the microbial populations. It appears that the environmental factors should have an influence on endophytes recovered from different tissue fragments; it was evident that frequency of colonization differs seasonally.

4. Conclusion

Climatic conditions affect endophytic population and variation in their occurrence is proved statistically. The results of present study indicate that endophytes are not host specific, provides evidence that isolates were capable to survive inside medicinal plants. As wet conditions are generally encouraging for fungal sporulation and this may be reasons for more colonization frequency during wet season.

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