Plant Archives Vol. 20, Supplement 2, 2020 pp. 4374-4381 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

DIVERSITY AND CYTOTOXIC ACTIVITY OF FUNGAL ENDOPHYTES OF AN ENDANGERED PLANT *MAPPIA FOETIDA*

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Abstract

A total of 20 endophytic fungal species were isolated from an endangered medicinal plant *M. foetida*, which belongs to the Western Ghats of Mookambika Wildlife Sanctuary, Shivamogga, Karnataka. *M. foetida* is listed as an endangered plant in Red data book. The endophytic fungal diversity was analyzed using Absolute frequency, Relative frequency, Isolation rate, Colonization rate, Simpson's dominance (D) and diversity index, Shannon-Wiener diversity index (H) and Evenness. The fungal endophytes were further screened for their cytotoxicity using *Allium cepa* root tips and *Vigna radiata*. The mitotic index of treated endophytes was found to be 81.0 % in leaf and 62.0 % in twig and the control 93% respectively. The leaf endophytic fungi, *Alternaria alternata*during 24-hour incubation period showed average shoot length around 50mm(24h), 23.25mm (48h) and 38.75mm (72h) than the twig endophytic fungi, *Fusarium species* that is 3.33mm (24h), 7mm (48h) and 15mm (72h). The standard values showed that the average shoot length of leaf 8mm(24h), 18mm (48h) and 31mm(72h) than twig 2mm(24h), 7mm(48h) and 9mm(72h).

Keywords**:** Endophytic fungi, diversity analysis, Cytotoxicity

Introduction

Endophytic fungi were studied in the plants of temperate regions, but recently these studies were extended to tropical plants as well. All plants are having fungal endophytes and epibionts. These sources between fungi and plants are generally a cryptic phenomenon in nature. Endophytic fungi inhibit tissues of stems, bark, branches, roots, petioles, flowers, seeds, and fruits which includes xylem of all plant organs. Therefore, it is to be believed that the endophytes play an important role in the protection of plant which acts against insects, pathogens of host and herbivores and also it may increase plant resistance to pathogens and abiotic and biotic stresses (Ahlholm *et al.,* 2002; Kogel *et al.,* 2006).

The biological diversity of endophytic fungi which occurs naturally in the temperate regions as well as tropical rainforests around 3,00,000 terrestrial host plant species were distributed. These endophytic fungi belong to the meiosporic and mitosporic ascomycetes (Bacon and White, 2000), "asymptomatically reside in the internal tissues of plants beneath the epidermal cell layer, where they colonize healthy and living tissue via quiescent infections". In this study, the main aim is to examine the endophytic fungal diversity and their cytotoxic effect on mung bean (*V. radiata*) as well as on onion (*A. cepa* root tips)

Materials and Methods

Collection of plant material

Plant material, *M. foetida* was collected from the Western Ghats of Mookambika Wildlife Sanctuary, Shivamogga district, Karnataka, India during the year 2018-2019 (March-June, July-Aug, Sep-Oct, Nov-Feb for summer, monsoon, mid monsoon and winter seasons respectively) (Fig. 1).

Fig 1.: Endangered plant *M. foetida* from Western Ghats of Mookambika Wildlife Sanctuary, Karnataka.

Isolation and morphological identification of an endophytic fungi

Surface sterilization was done. Leaves and twigs were surface sterilized (Nithya and Muthumary, 2010) under aseptic condition in sequential steps by immersing with mercuric chloride (1mg/1ml) for 10min and 70% ethanol for 1min followed by washing finally with sterile distilled water. The endophytic fungi were identified based on

morphological characters such as surface texture, margin character, aerial mycelium, mechanism of spore production, growth pattern using standard manuals (Barnett, 1972).

Diversity analysis of endophytic fungi

In this study, endangered plant *M. foetida*, was analyzed for the presence of endophytic fungi from their natural habitat during summer (Mar-Jun), monsoon (July-Aug), mid-monsoon (Sep-Oct) and winter (Nov-Feb) seasons during the year 2018-19. The study was under taken to investigate the diversity of endophytic fungal isolates and their colonization pattern across four different seasons in the endangered plant, used for the treatment of various diseases especially in the treatment of cancer. Fungal endophytes were isolated from the healthy endangered plant tissues using standard isolation procedures and the data analysis were performed for each season individually.

Data analysis of endophytic fungi:

a. **Absolute frequency:** The absolute frequency (f) was calculated as the total number of endophytes isolated (Larrsn*et al.,* 2002).
b. **Relative frequency :** Relative frequencies (fr) of isolation, used to represent fungal species density was calculated as the number of isolates of each species of the endophytic fungi divided by the total number of isolates and expressed in percentage.
c. **Isolation rate :** Isolation rate (IR) of the endophytic fungi was calculated as the number of isolates obtained from tissue segments divided by total number of tissue segments (Huang *et al.,* 2008).
d. **Colonization rate :** The colonization rate (CR) of endophytic fungi was expressed as percentage of total number of isolates obtained from different tissue segments divided by total number of isolates obtained from overall tissue segments incubated (Mahapatra and Banerjee, 2010)
e. **Simpson's dominance (D) and diversity index :** Simpson's index of diversity was calculated using the formula: 1-D

Where,

$$\Sigma(n/N)^2$$

f. **Shannon-Wiener diversity index (H):** Shannon-Wiener diversity index (H) was calculated using the formula

$$H_S = \sum_{i=1}^{S} (P_i)(\ln P_i)$$

Where,

H_S- symbol for the diversity in a sample

S- Number of species in a sample

P_i- relative abundance of the ith species

Ln- log to base 2

g **Evenness:** Evenness (E) was expressed by

$$E = \frac{H}{\text{Log}(S)}$$

The Simpson's diversity index, Simpson's dominance index (D), species richness (S),Shannon-Wiener index (H) and Evenness (E) were calculated (Jena and Tayung, 2013)

Evaluation of *In vitro* cytotoxicity of endophytic fungal extract of *M. foetida*.

Antimitotic activity using *A. cepa* root tips.

a. **Growing *A. cepa* root tips :** The fresh and healthy bulbs of onion were obtained from the local market of Shivamogga, Karnataka, India. To achieve sprouting, the bulbs were placed in contact with distilled water and extract in a 25ml beaker at room temperature (26 ± 2°C) for 2 days in the dark separately. The distilled water was changed every 24 hours between 9 to 10 hours. Bulbs with root length of 2cm and above (range= 2.2-3cm) were selected for the studies.
b. **Antimitotic studies :** The bulbs that developed uniform roots were used for the experiment. These roots were incubated with endophytic fungal extracts. A control was set with distilled water. Mitotic index was recorded after 24-48 hours of incubation and compared with that of control.
c. **Microscopic investigations :** The root tip cells were fixed with stains and examined using a stereo microscope. Each treated rootwas rinsed in distilled water and cut into segments of about 1-2cm length from the tips and fixed in ethanol: glacial acetic acid (3:1), hydrolyzed for 5 min with 1N HCl at 70°C and stained with 2% acetocarmine for 1 hour. Stained root tips were excised and squashed on a clean glass slide with a drop of 45% acetic acid and examined under microscope. In all the slides, 100-300 cells were counted to determine the number of cells in interphase and dividing phase. Changes in chromosome morphology were photographed under (100 X).

The mitotic index was calculated by using the formula:

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

Evaluation of cytotoxic effect of *M. foetida* endophytic fungal extract on seed germination of *V. radiata.*

Inexpensive cytotoxic assay of endophytic fungal extract on sprouting seeds has been carried out using mung bean (*V. radiata*) seeds (Satyanarayana *et al.,* 2011). Good quality seeds were purchased from a grocery store. For germination assay the seeds were surface sterilized with 0.1% mercuric chloride solution for 2 min and washed thoroughly with tap water and then distilled water for 30 min, seeds were placed in solutions of endophytic fungal leaf and twig extracts, taken in test tubes containing 10 number imbibed seeds and inoculated in petri plates and this can be taken as 0th hour incubation period. Further seeds were dipped in endophytic fungal leaf and twig extracts and kept for 24 hours incubation period. After 24 hours seeds were inoculated in petri plates. At the end of the test period (24 h), and kept for incubation around 24-72h for seed germination. The length of the radicals was measured in mm at the end of 24, 48, 72h and growth inhibitory effect of extracts were identified and photographs were taken.

Results

Diversity analysis of endophytic fungi from medicinal plant during all the four seasons

A total of 20 endophytic fungal isolates are identified. During summer season the isolation rates (IR) were recorded from leaf and twig as 0.833 and 1.2, monsoon season 0.66 and 1.5, mid monsoon season 0.714 and 1.4 and winter season 1.4 and 0.714 (Fig. 2). The fungal colonization rate (CR) differed amongst the plant seasons (Fig 3). The fungal taxa *Saprophytic fungi, Alternaria alternata, Fusarium oxysporum, Cladosporium sp.* had high relative frequency of occurrence with wide distributions but fungal taxa of *Drechslera dematioidea, Fusarium sp., Cladosporium cladosporioides* were found in less frequency (Table 1). Endangered plant of *M. foetida* of all the different seasons were found to be associated with various endophytic fungi with different isolation rates (IR) and colonization rates (CR) as depicted in the (Table 2). The absolute and relative frequencies of occurrence of each endophytic fungal species were calculated and are depicted in (Table 1). The diversity of the endophytic community isolated from the different tissues of *M. foetida* was compared using diversity indices. The Simpson's dominance of endophytic fungi was 0.262 (twigs) and 0.255 (leaves). There was little difference in species evenness among the tissues studied but showed more in leaves (Table 3).

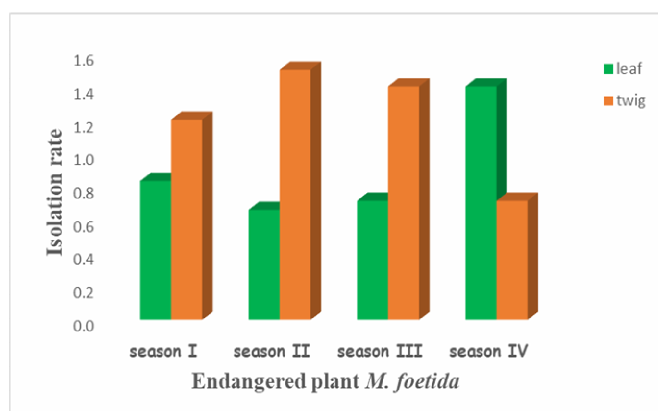


Fig. 2 : Isolation rate of endophytic fungi during all the four seasons

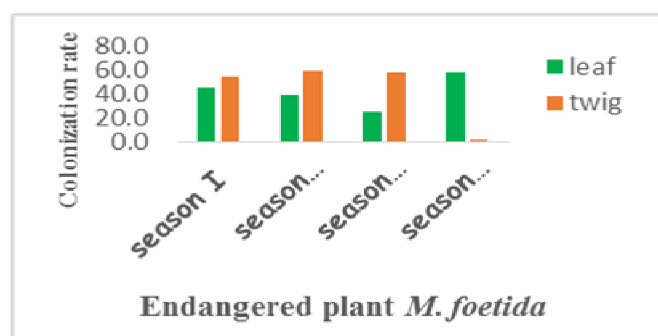


Fig. 3: Colonization rate of fungal endophytes during all the four seasons

Table 1: Number of isolates, Absolute (f) and Relative frequency (fr) of endophytic fungi isolated from selected endangered medicinal plant (Summer, monsoon, mid monsoon and winter season).

Endophytic Diversity	Endophytic fungi	*M. foetida* Leaf	Twig	Absolute frequency (f)	Relative frequency fr (%)
Season I (Summer)	*Saprophytic fungi*	5		5	10
	Cladosporium cladosporioides		2	2	4
	Fusarium sp.		4	4	8
Season II (Monsoon)	*Alternaria alternata*	3		3	6
	Fusarium oxysporum	3		3	6
	Cladosporium sp.		4	4	8
	Fusarium sp.		3	3	6
	Alternaria alternata		2	2	4
Season III (Mid-monsoon)	*Alternaria alternata*	2		2	4
	Fusarium moniliforme	1		1	2
	Colletotrichum sp.	2		2	4
	Fusarium sp.		3	3	6
	Cladosporium sp.		3	3	6
	Drechslera dematioidea		1	1	2
Season IV (Winter)	*Alternaria alternata*	3		3	6
	Fusarium moniliforme	2		2	4
	Bipolaris sp.	2		2	4
	Cladosporium sp.		2	2	4
	Fusarium verticillioides		1	1	2
	Fusarium sp.		2	2	4
	Total	23	27	50	100
	Species richness	23	27		

Table 2: Colonization and isolation rates of endophytic fungi in endangered medicinal plant *M. foetida*

Sl. No.	Diversity of *M. foetida*	Tissue segments	Colonization rate CR (%)	Isolation rate (IR)
1	Season I	Leaf	45	0.833
		Twig	54.54	1.2
2	Season II	Leaf	40	0.66
		Twig	60	1.5
3	Season III	Leaf	25	0.714
		Twig	58.33	1.4
4	Season IV	Leaf	58.33	1.4
		Twig	41.66	0.714

Table 3: Diversity indices of endophytic fungi from endangered medicinal plant isolated from *M. foetida.*

SL. No.	Indices	*M. foetida*	
		Leaf	**Twig**
1	Simpson's dominance	0.255	0.262
2	Simpson's diversity	0.745	0.738
3	Species Richness	23	27
4	Shannon- Wiener	0.595	0.508
5	Evenness	0.988	0.843

Effect on antimitotic activity

The antimitotic assay revealed that the endophytic extracts have good inhibition of meristematic cell in different stages of mitotic phases like Prophase, Anaphase, Metaphase and Telophase due to various processes during observation under microscope. There were chromosomal and cellular changes which will indicate their anti-mitotic activity. The treated cells showed the different cellular and chromosomal abnormalities like nucleolar opening and cellular multiplication at interphase, cell shrinkage, arrest of cellular multiplication and chromosomal condense at metaphase, lagging chromosome and chromosomal bridge at anaphase.

The mitotic index of treated endophytes was found to be 81.0 % in leaf and 62.0 % in twig and the controls 93% respectively (Fig 4-7 and Table 4).

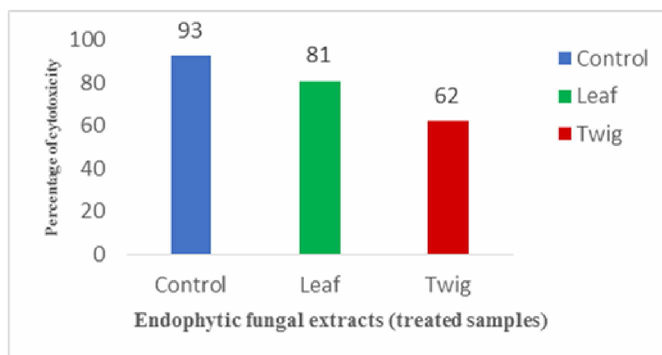
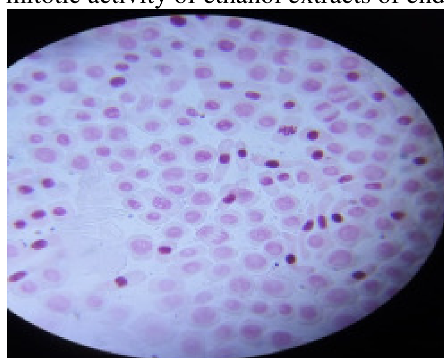


Fig. 4: Anti-mitotic activity of ethanol extracts of endophytic fungi



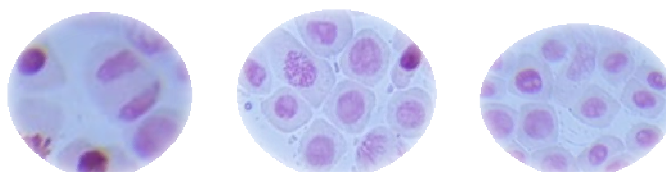


Fig. 5: Anti-mitotic activity of ethanol extracts of endophytic fungi isolated from leaf *Alternaria alternata* extract

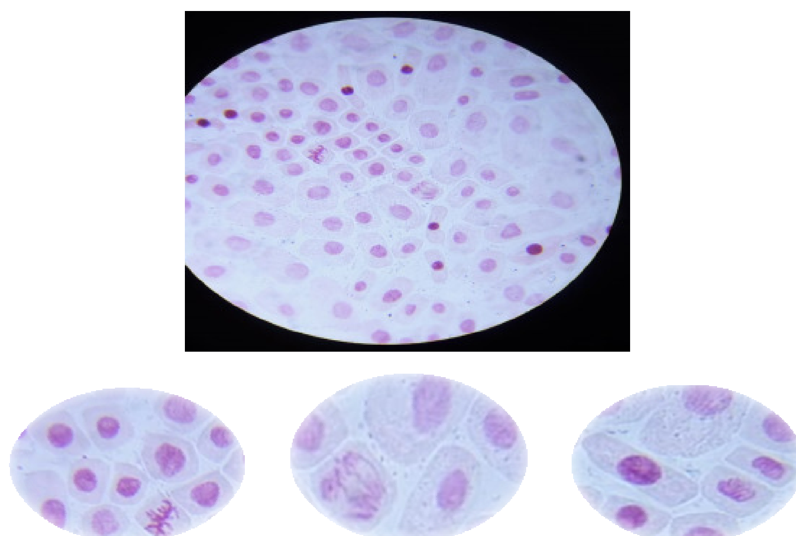


Fig. 6: Anti-mitotic activity of ethanol extracts of endophytic fungi isolated from twig *Fusarium species* extract

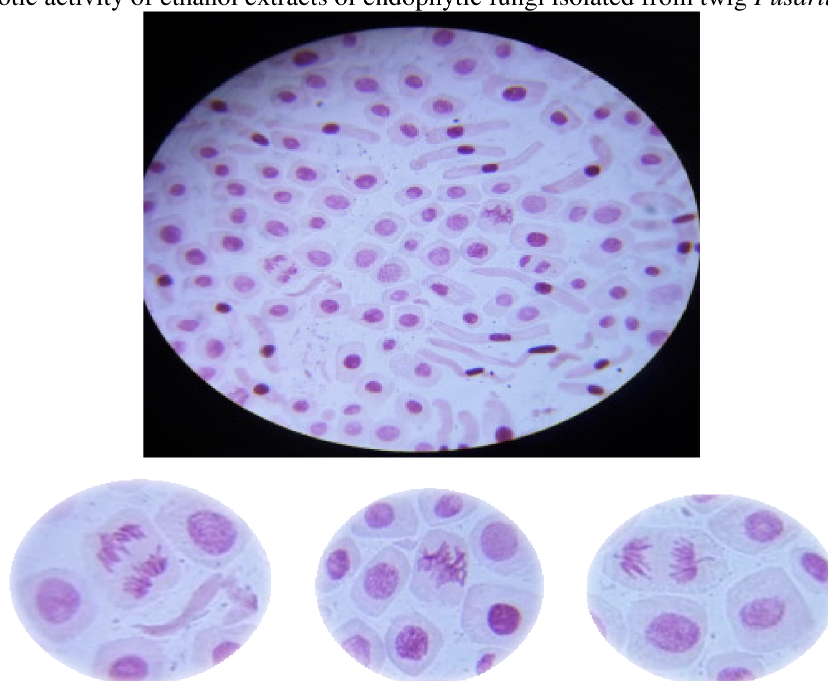


Fig. 7: Anti-mitotic activity of Control (water)

Table 4: Anti-mitotic activity of ethanol extracts of endophytic fungi isolated from leaf and twig species

Samples	Concentration	Total no. of cells	No. of non-dividing cells	P	M	A	T	Dividing cells	Mitotic Index (%)
Leaf (*Alternaria alternata)*	100µg/ml	100	19	64	5	8	4	81	81
Twig (*Fusarium species*)	100µg/ml	100	38	55	2	4	1	62	62
Control (Water)	100µg/ml	100	07	78	3	9	3	93	93

Where, P=Prophase, M= Metaphase, A= Anaphase, T= Telophase

Effect on seed germination of *V. radiata* seeds:

The sequential observation was done using treated (24 hours incubation period) and non-treated (zero-hour incubation period) *V. radiata seeds* of endophytic fungal extracts. The cytotoxicity effect of *V. radiata* after 24, 48 and 72h during 24 hours incubation period has given the significant results when compared with zero-hour incubation period. Germinated shoot length was observed. The leaf endophytic fungi, *Alternaria alternata* showed average shoot length around 50mm(24h), 23.25mm(48h) and 38.75mm(72h) (Fig 8-10 and table 5-6) than the twig endophytic fungi, *Fusarium species*that is 3.33mm (24h), 7mm (48h) and 15mm(72h).The standard values showed that the average shoot length of leaf 8 mm(24h), 18 mm (48h) and 31 mm (72h) than twig 2 mm (24h), 7mm(48h) and 9mm (72h).

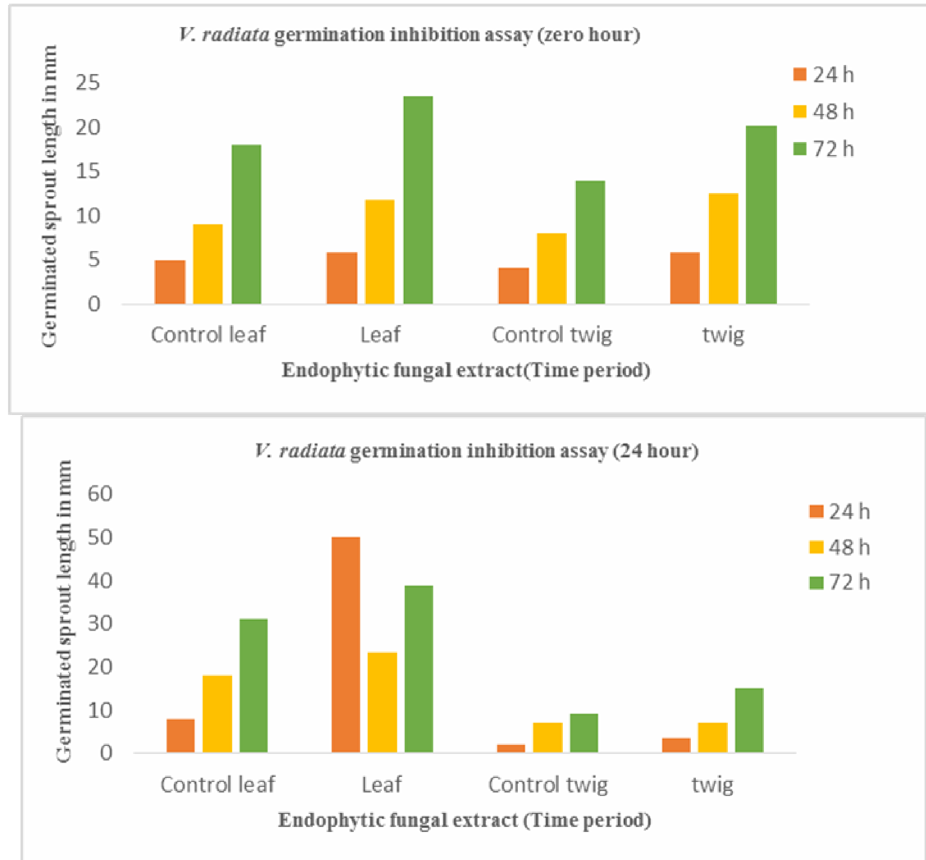


Fig. 8: Comparison of the potential of germination inhibition of *V. radiata* seeds: Compared to water control, ethanol extract of leaf and twig endophytes (zero-hour and 24-hour time period).

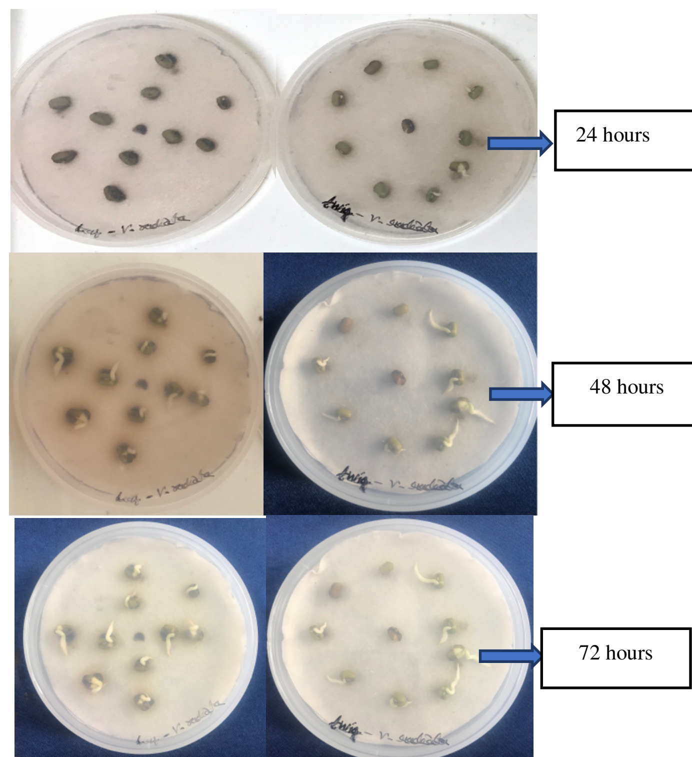


Fig. 9: *V. radiata* treated seeds of endophytic fungal extracts (leaf- *Alternaria alternata* and twig- *Fusarium species*) along with control (zero-hour incubation).

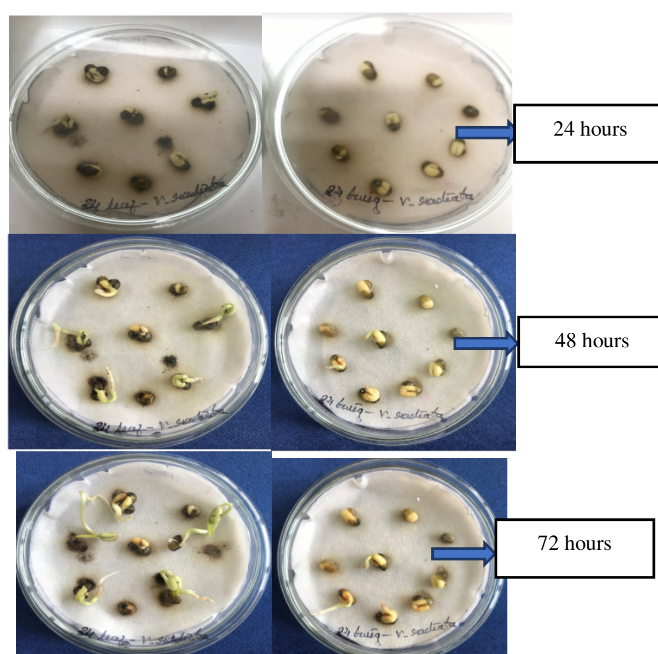


Fig. 10: *V. radiata* treated seeds of endophytic fungal extracts (leaf- *Alternaria alternata* and twig- *Fusarium species*) along with control (24-hour incubation).

Table 5: Observation of treated *V. radiata* seeds along with control seeds (zero-hour incubation)

Samples	Time period in mm		
	24 hours	48 hours	72 hours
Leaf	5.75	11.75	23.5
Twig	5.75	12.5	20.25
Control (leaf)	5	9	18
Control (twig)	4	8	14

Table 6: Observation of treated *V. radiata* seeds along with control seeds (24-hour incubation)

Samples	Time period in mm		
	24 hours	48 hours	72 hours
Leaf	50	23.25	38.75
Twig	3.33	7	15
Control (leaf)	8	18	31
Control (twig)	2	7	9

Discussion

M. foetida plant is a well-known endangered species which are listed in Red data book. Diversity and data analysis have not studied on this plant so far. In previous reports on this plant majority of endophytes are reported in leaf when compared to twigs. The fungal colonization rate (CR) differed amongst the plant seasons. The fungal taxa *Saprophytic fungi, Alternaria alternata, Fusarium oxysporum, Cladosporium sp.* had high relative frequency of occurrence with wide distributions but fungal taxa of *Drechslera dematioidea, Fusarium sp., Cladosporium cladosporioides* were found in less frequency.Simpson's and Shannon- Wiener's diversity indices were higher in leaves compared to twigs. The species richness showed more in twigs than leaves. There was little difference in species evenness among the tissues studied but showed more in leaves. Diversity indices of fungal endophytes varied between plant species as well as within tissue fragments. The values of diversity indices suggest that the endophytic colonization in the tissues of the medicinal plants were even, indicating uniform occurrence of various species. Observation of nuclear abnormalities such as binucleated cells and micronuclei in *A. cepa* root tip cells is a clearindication of genotoxicity (Dash *et al.,* 1988). Binucleated cells may a riseas a result of an incomplete process of cell division, that is, karyokinesis with incomplete cytokinesis. These cells also may arise due to the suppression of cell plate formation between cells in early telophase. The endophytic fungal extracts (leaf- *Alternaria alternata and* twig- *Fusarium species*) showed strong antimitotic activity through various mechanisms in onion actively growing root cells. *V. radiata* were found to be cytotoxic and shoot length were inhibited. The endophytic fungal extracts (leaf- *Alternaria alternata and* twig- *Fusarium species*) also showed inhibition of *V. radiata,* which acts as a toxic.

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