



RESEARCH ARTICLE

ECOLOGICAL AND SEASONAL VARIATION IN THE OCCURRENCE OF ENDOPHYTIC FUNGI ASSOCIATED WITH *CALOTROPIS GIGANTEA*

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Received 24th December, 2017; Accepted 07th January, 2018; Published Online 28th February, 2018

ABSTRACT

Variation in the distribution and diversity of endophytic fungi associated with *Calotropis gigantea* with respect to ecological and seasonal conditions was assessed. It has been noticed that diversity of endophytic fungi and colonization frequency varied with the seasons with least in summer and highest in winter. A preference of colonization of different fungal species was also observed. It is also evident that diversity of endophytic fungi varied with the geographical distribution of the plant. Similarly, dominance of fungal species varied both with season and plant part. In conclusion it has been pointed out that more attention should be paid to investigating the endophytic fungal diversity in host plant growing under varied ecological and seasonal conditions which is more likely to reveal the behavior of endophytic fungi and the metabolites produced by them.

Key words: *Calotropis gigantea*, Ecological variation, Endophytic fungi, Colonization frequency, Dominant frequency.

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Citation: Aruna, A., Abhinash M., Parvathi, D. and Krishna Reddy, V., 2018. "Ecological and seasonal variation in the occurrence of endophytic fungi associated with *calotropis gigantea*" *International Journal of Current Research in Life Sciences*, 7, (02), 1152-1157

INTRODUCTION

Calotropis gigantea commonly known as gaint milkweed or sodom apple or crown flower belongs to the family Apocyanaceae. It is widely distributed in tropics and sub tropics including India. Though grows as a wild plant, it is recognized as a useful medicinal plant mainly because of its milky latex which contains various active compounds such as is motin and lupeol and other plant parts contain cardiac glycosides, flavonoids, phenolic compounds and terpenoids (Muller *et al.*, 2001) which are responsible for a range of its pharmacological properties. Different parts of this plant are reported to harbor a variety of fungal endophytes (Rodriguez *et al.*, 2008, Arechavaleta *et al.*, 1989) which confer various benefits to host plant. (Zhang *et al.*, 2009).The biological diversity of fugal endophytes is enormous especially in tropical countries. Fungal endophytes are a rich source of novel organic compounds with wide biological activities (Giridharan *et al.*, 2012). Strobel and Daisy (2003) reported that the plants growing in unique environmental settings, having ethno botanical uses, having extreme age or interesting endemic locations generally harbor novel endophytic microorganisms, of which the secondary metabolites are usually unique and with may ultimately be shown to have applicability in medicine. Endophytes appear to have direct and indirect effects on plant responses to biotic agents.

The interaction with biotic agents remains less clear. Environment plays a crucial role both on host and endophytes and also their interactions. Environmentally, the endophyte may be metabolically aggressive by affecting host defense chemicals (Schulz *et al.*, 1999) Endophytes residing in the host tissue in a symptom less state or one that may be beneficial to its host may turn into a pathogen in response to some environment cue (Hendry *et al.*, 2002). In view of impact of environment on endophyte distribution and activity, in the present study an attempt has been made to investigate the effect of ecological and seasonal variations on endophytec distribution and relative dominance of endophytes of *Calotropis gigantean*

MATERIALS AND METHODS

Locality of collection

A total of 333 samples of *Calotropis gigantea* plant parts were collected from different ecological locations of Telangana state, India. Warangal (215) Karimnagar (54) and Khammam (64) in different seasons (summer, rainy, and winter). Healthy and old mature plant parts were carefully chosen for sampling.

Isolation of fungal endophytes

Different parts of the plant, *Calotropis gigantea* such as root, stem, leaves and flower were collected from the plants growing in different edaphic and environmental conditions. The samples were brought to the laboratory in sterilized polythene bags and were washed thoroughly in tap water followed by

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sterilized water for few minutes to remove dirt and debris (Selvanthan *et al.*, 2011). A total 333 of samples (Warangal-215, Karimnagar-54 and Khammam-64) were selected for sampling. In root maturation zone of elongation were cut in to vertically in 4 to 6 cm segments. The mature internodal parts of stem were collected and cut in to 0.4 -0.8 cm. Healthy and mature leaves lateral parts and midrib were cut into approximately 1cm segments (Dobranic *et al.*, 1995). The segments of flower were trimmed to 0.2-0.4 cm. The samples from leaves were dipped in 70 % ethanol for 5 seconds, and then they were transferred to 4 % sodium hypochlorite for 90 seconds and finally rinsed in sterile distilled water for 10 seconds and then removed excess moisture (Amirita *et al.*, 2012). Surface sterilization of stem and root parts were carried out by (Fisher *et al.*, 1993, 1994) method. The segments were immersed first in 75% ethanol for 60 seconds, followed by 4% sodium hypochlorite for 180 seconds, and then again in 75 % ethanol for 30 seconds and finally rinsed in sterile distilled water for 10 seconds. The flower samples were dipped in 30% ethanol for 5 seconds, and then they were immersed in 2% sodium hypochlorite for 60 seconds and rinsed in sterile distilled water for 10 seconds. The samples thus prepared were kept in sterile dry plate to remove excess moisture. The externally sterilized segments were placed on agar plates (Asthana and Hawkers medium and potato dextrose agar medium) supplemented with 150 mg of streptomycin per liter.

Each Petri dish containing 5 segments was incubated at 27 ± 2 °C at 12-h light/dark cycle (Suryanarayana *et al.*, 2003). After 10-15 days of incubation the fungal colonies developing from the sample fragments were isolated and transferred to fresh tubes. Colony morphology of each fungus was recorded. Slides of fungal colonies were made with lactophenol cotton blue and observed under microscope. Mycelia, spore characteristics were recorded. Photomicrographs were taken under fluorescent microscope and fungi were identified with the help of standard manuals (Barnett and Hunter, 1998, Singh *et al.*, 1991). Colonization Frequency (CF), Dominant endophytic fungi (DF) and ecological variation in incidence of different groups of fungi were calculated with the help of following formulae.

Colonization Frequency (CF)

$$CF (\%) = \frac{\text{Number of species isolated}}{\text{Number segments screened}} \times 100$$

Dominant Frequency (DF)

$$DF (\%) = \frac{\text{Number of individual isolates}}{\text{Total number of isolates}} \times 100$$

RESULTS AND DISCUSSION

Endophytic fungi isolated from different parts of *Calotropis gigantea* growing in three different ecological regions viz Warangal, Karimnagar and Khammam are presented in table 1 and (Fig.1). In Warangal region a total of 215 samples (root - 40; stem - 65; leaf - 91 and flower -19) were selected. A total of 24 fungal endophytes were isolated from these samples. However the number and diversity of fungal species varied with the plant part.

In general leaf (9) followed by stem (7) harbored more number of species. Least number of species were recorded in flowers. In Karimnagar region 54 samples (root-22; stem-10; leaf-10; flower-12) representing different parts of the plant were screened. Root samples yielded three species whereas stem and leaf were found to be colonized by one species each. Twelve samples of flower yielded 3 endophytic fungal species. The third region surveyed, in Khammam a total of 64 samples representing stem, leaf and flower were screened. From these samples, seven fungal species were isolated. Thirty five samples of stem yielded three species only, whereas the numbers of fungal endophytes were isolated from 25 samples of leaf. *Penicillium glabrum*, a lone fungal species was isolated from all 4 samples of flower. It is evident from the present data that the incidence and diversity of endophytic fungi associated with *Calotropis gigantea* varied with geographical distribution of the plant. Data pertaining to seasonal variation in colonization frequency of endophytic fungi associated with *Calotropis gigantea* is presented in table -2. (Fig.2). It is evident from the table that the incidence of endophytic fungi varied with the seasons. In 360 plant samples only five endophytic fungal species were isolated. In winter season, maximum number of fungal species were isolated, whereas eight species were recorded in rainy season.

The diversity of fungal species also varied with the seasons, as species recorded in each season were quite different. However, sterile mycelium - 2 was isolated in all the three seasons, whereas sterile mycelium -1 was isolated in rainy and winter seasons. Colonization frequency of endophytic fungi with different plant parts also varied with the seasons. In summer, root and flowers were less colonized than the stem and leaf. *Aspergillus niger* has colonized the root, leaf and flower. However, the four other species were associated with only one part of the plant. Leaf, stem and flower were colonized by three fungal species. Interestingly, colonization frequency of different plant parts was more in rainy and winter seasons. In rainy season colonization frequency, though varied for different species, was same for root, stem and leaf. In case of flower it was less. In winter season colonization frequency for leaf was highest followed by stem and root. Least frequency was observed with flower. Overall, it evident that the diversity of endophytic fungi and colonization frequency of different plant parts varied with the seasons with least in summer and highest in winter. A Preference of colonization of different fungal species is evident with the seasons. Season wise dominant fungi in terms of percentage for different parts of plant were assessed and precised in table -3 (Fig.3). A critical perusal of the table reveals that the percentage of dominant fungi in different parts of *Calotropis gigantea* varied with the seasons. Domination of individual fungal varied with the plant parts also. In summer season, *Aspergillus niger* dominated (14.2 %) in root, leaf and flower, whereas *Aspergillus flavus* (17.6%) and *Penicillium citrinum* (14.2 %) dominated in stem. In rainy season, *Alternaria solani* was the predominant species in leaf segments. Similarly, sterile mycelium I dominated the root segments. In root, stem and leaf segments *Curvularia lunata*, *Dipolodia calotropidis* and *Fusarium graminearum* dominated respectively. In winter season such variation in dominance of different fungi and different parts of the plant could be observed. More number of fungal endophytes dominated in leaves followed by stem. In root and flower only three fungal species were observed. Thus it is evident that the dominance of fungal species varied both with seasons and plant parts.

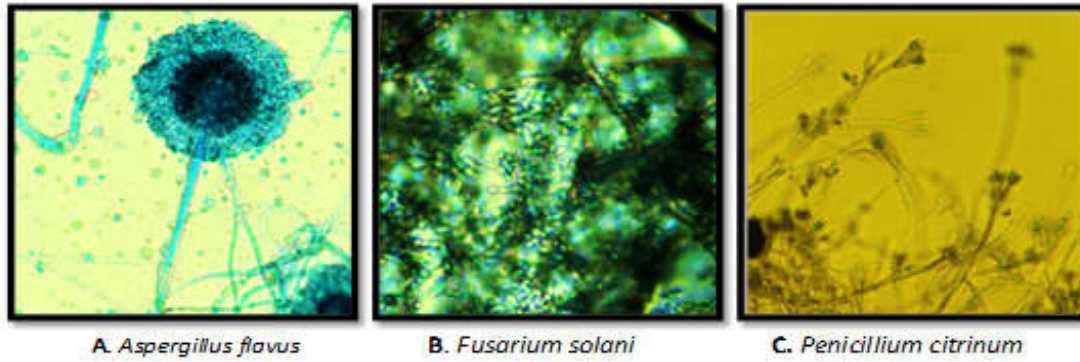
Table 1. Ecological variation in the incidence of endophytic fungi of *Calotropis gigantea* in different localities of Telangana state

Area of collection	S.No.	Plant parts	No of samples	Endophytic fungi isolated
Warangal region (215)	1	Root	5	<i>Aspergillus niger</i>
	2	Root	10	<i>Aspergillus nidulans</i>
	3	Root	12	Sterile mycelium
	4	Root	5	Sterile mycelium
	5	Root	8	<i>Colletotrichum dematium</i>
	6	Stem	10	<i>Aspergillus flavus</i>
	7	Stem	15	<i>Aspergillus nidulans</i>
	8	Stem	5	<i>Fusarium graminearum</i>
	9	Stem	10	<i>Aspergillus terreus</i>
	10	Stem	5	<i>Colletotrichum dematium</i>
	11	Stem	10	<i>Diplodia andamanensis</i>
	12	Stem	10	Sterile mycelium
	13	Leaf	5	Sterile mycelium
	14	Leaf	5	<i>Aspergillus nidulans</i>
	15	Leaf	10	<i>Diplodia calotropidis</i>
	16	Leaf	10	Sterile mycelium
	17	Leaf	15	<i>Aspergillus stellatus</i>
	18	Leaf	12	<i>Aspergillus.oryzae</i>
	19	Leaf	12	<i>Cladosporium cladosporioides</i>
	20	Leaf	12	<i>Verticillium dahliae</i>
	21	Leaf	10	Sterile mycelium
	22	Flower	4	<i>Alternaria solani</i>
	23	Flower	5	<i>Aspergillus oryzae</i>
Karimnagar region (54)	25	Root	12	<i>Curvularia lunata</i>
	26	Root	5	<i>Aspergillus oryzae</i>
	27	Root	5	Sterile mycelium
	28	Stem	10	<i>Penicillium rubrum</i>
	29	Leaf	10	<i>Aspergillus niger</i>
	30	Flower	5	<i>Aspergillus niger</i>
	31	Flower	2	<i>Fusarium graminearum</i>
	32	Flower	5	Sterile mycelium
	Khammam region (64)	33	Stem	15
34		Stem	10	Sterile mycelium 1
35		Stem	10	Sterile mycelium 2
36		Leaf	12	<i>Drechslera spicifera</i>
37		Leaf	8	<i>Alternaria solani</i>
38		Leaf	5	<i>Aspergillus terreus</i>
39		Flower	4	<i>Penicillium glabrum</i>

Table 2. Seasonal variation in colonization frequency of endophytic fungi in *Calotropis gigantea* during the year 2016 – 2017

Seasons	Segments	S.No	Endophytic fungi	% colonization frequency			
				Root	Stem	Leaf	Flower
Season - I Summer (March - June)	{360 segments}	1	<i>Aspergillus flavus</i>	-	4.1	-	-
		2	<i>A. niger</i>	3.3	-	5.2	3.3
		3	<i>Drechslera spicifera</i>	-	-	2.7	-
		4	<i>Penicillium citrinum</i>	-	3.3	-	-
		5	Sterile mycelium 2	-	-	1.3	-
Season -II Rainy season (July - October)	{360 segments}	1	<i>Alternaria solani</i>	-	-	5.5	1.3
		2	<i>Aspergillus nidulans</i>	1.3	2.5	2.5	-
		3	<i>Curvularia lunata</i>	4.1	-	-	-
		4	<i>Diplodia calotropidis</i>	-	-	4.1	-
		5	<i>Fusarium graminearum</i>	-	4.2	-	3.8
		6	<i>Penicillium glabrum</i>	-	-	-	1.2
		7	Sterile mycelium 1	6.1	3.6	1.3	-
		8	Sterile mycelium 2	3.0	0.8	-	-
Season -III Winter season (November -February)	{360 segments}	1	<i>Aspergillus stellatus</i>	-	-	6.1	-
		2	<i>A.terreus</i>	-	4.2	3.1	-
		3	<i>A.oryzae</i>	2.5	-	3.5	2.1
		4	<i>Penicillium rubrum</i>	-	6.9	-	-
		5	<i>Cladosporium cladosporioides</i>	-	-	4.7	0.5
		6	<i>Colletotrichum dematium</i>	3.5	2.5	-	-
		7	<i>Diplodia andamanensis</i>	-	2.5	-	-
		8	<i>Verticillium dahliae</i>	-	-	3.0	-
		9	Sterile mycelium 1	-	2.8	4.1	-
		10	Sterile mycelium 2	3.3	-	-	1.9

A. Photo micrographs of Endophytic fungi isolated from *Calotropis gigantea*



B. Colonies of endophytic fungi isolated from different parts of *Calotropis gigantean*

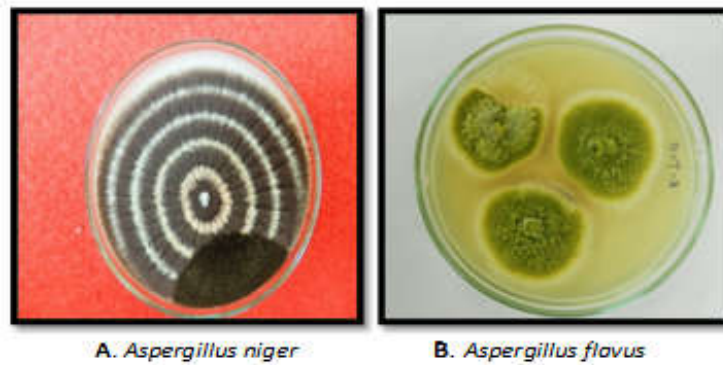


Fig.1. Colonies and photo micrographs of different parts of endophytic fungi in *Calotropis gigantean*

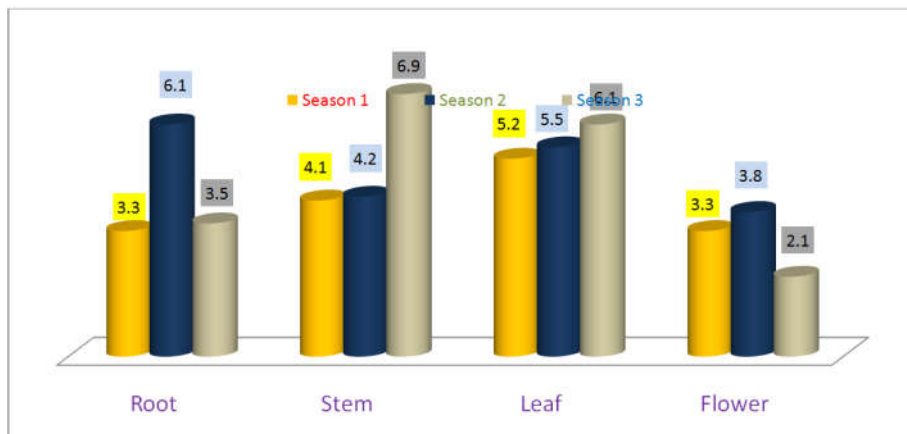


Fig.2. Seasonal variation in colonisation frequency of endophytic fungi in *Calotropis gigantea* (L.) during the year 2016 -2017

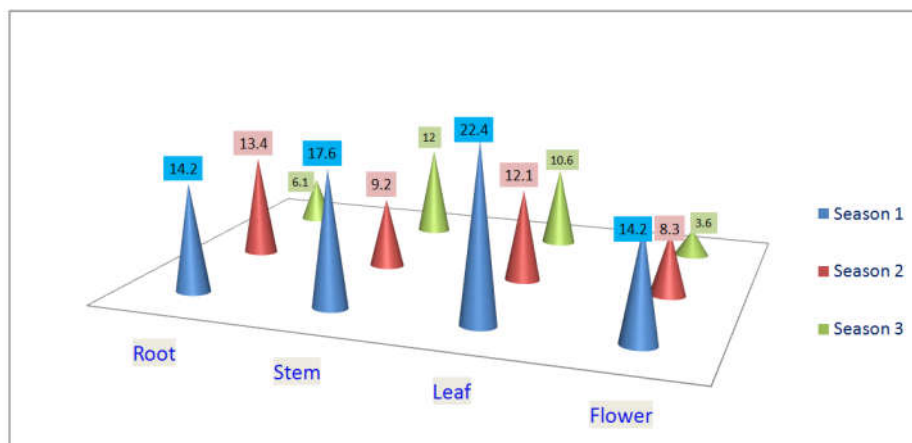


Fig.3. Highest Dominant fungi % of *Calotropis gigantea* during the three seasons of the year 2016-2017

Table 3. Dominant endophytic fungi (%) of *Calotropis gigantea* during the three seasons of the year 2016 – 2017

S.No	Endophytic fungi	% Dominant fungi			
		Root	Stem	Leaf	Flower
		Summer (May - June)			
A					
1	<i>Aspergillus flavus</i>	-	17.6	-	-
2	<i>A. niger</i>	14.2	-	22.4	14.2
3	<i>Drechslera spicifera</i>	-	-	11.6	-
4	<i>Penicillium citrinum</i>	-	14.2	-	-
5	Sterile mycelium 2	-	-	5.6	-
		Rainy season (July-October)			
B					
1	<i>Alternaria solani</i>	-	-	12.1	2.8
2	<i>Aspergillus nidulans</i>	2.8	5.5	5.5	-
3	<i>Curvularia lunata</i>	9.0	-	-	-
4	<i>Diplodia calotropidis</i>	-	-	9.0	-
5	<i>Fusarium graminearum</i>	-	9.2	-	8.3
6	<i>Penicillium glabrum</i>	-	-	-	2.6
7	Sterile mycelium 1	13.4	7.9	2.8	-
8	Sterile mycelium 2	6.6	1.7	-	-
		Winter season (November- February)			
C					
1	<i>Aspergillus stellatus</i>	-	-	10.6	-
2	<i>A. terreus</i>	-	7.3	5.4	-
3	<i>A. oryzae</i>	4.3	-	6.1	3.6
4	<i>Penicillium rubrum</i>	-	12.0	-	-
5	<i>Cladosporium cladosporioides</i>	-	-	8.2	0.8
6	<i>Colletotrichum dematium</i>	6.1	4.3	-	-
7	<i>Diplodia andamanensis</i>	-	4.3	-	-
8	<i>Verticillium dahliae</i>	-	-	5.2	-
9	Sterile mycelium 1	-	4.8	7.1	-
10	Sterile mycelium 2	5.7	-	-	3.3

DISCUSSION

Environmental factors play a crucial role in distribution and diversity of both host and fungal endophytes. Environmentally, the endophyte may be metabolically aggressive by affecting host defense chemicals (Cabral *et al* 1993; Peters *et al* 1998 and Schulz *et al* 1999). Such a hostile environment may account for the evolution of the potentially increased synthetic ability of the endophytes. The environmental conditions which affect on host plant growth, influence the number and variety of endophytic populations, and affect on metabolites produced by endophytes. The difference in the metabolic profile and their biological activity might be related to the chemical difference of host plants (Paulus *et al.*, 2006). This clearly depends on the environment, and shows the importance of studying host endophyte relationship, and the effect of host plants on endophytic metabolic production. Hence, the importance of the host plant as well as the ecosystem were influencing endophytes metabolite production, and affect on biological activities of endophytes (Selim *et al.*, 2012). The variation in the distribution and diversity of endophytes vis - a - vis ecological and seasonal variations observed in the present investigations can be attributed to their effect on host that in turn affected the endophytes.

Conclusion

The result of the present investigations indicates that more attention should be paid to studying the endophytic biodiversity in host plants growing under varied environmental conditions which is likely reveal the diversity of metabolites produced by them. It may also reveal a useful information on how do the endophytes behave in establishing the biological relationship with host plant under diverse conditions.

Acknowledgement

Thanks are due to Dr. M. Surekha Head, Department of Botany, Kakatiya University for encouragement and facilities.

Two of us (A.A & M.A) are also indebted to UGC for providing financial assistance in the form of Rajiv Gandhi National Fellowship. A part of this was also carried out with the financial support provided under UGC-SAP (DRS-III)

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