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RESEARCH PAPER

Colonization frequency, diversity and siderophore producing ability of endophytic fungi isolated from *Prosopis* species

SATHWIK M. N. RAJ AND G. P. SANTOSH

Department of Agricultural Microbiology, College of Agriculture, Dharwad
University of Agricultural Sciences, Dharwad - 580 005, Karnataka, India
E-mails: sathwik080996@gmail.com, sgp2222@rediffmail.com

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Abstract: A total of 46 endophytic fungi were isolated from 270 segments of leaf and stem tissues of *Prosopis juliflora* and *P. cineraria* that were naturally found in semi-arid habitat of Vijayapur and Bagalkot districts of Karnataka (India). Twenty six endophytic fungi were obtained from stem, while twenty were obtained from leaf. The diversity and species richness were found higher in stem tissues than in leaf. The average colonization frequency of *P. juliflora* was 27.69 per cent, while *P. cineraria* recorded average colonization frequency of 36.42 per cent. Eleven samples from *P. juliflora* and sixteen samples from *P. cineraria* did not yield any endophyte irrespective of the tissue material used. The endophytes belonging to genera *Aspergillus, Alternaria, Fusarium, Penicillium* and *Colletotrichum* were recovered. *Aspergillus* sp. had the highest colonization frequency of 28.26 per cent followed by *Alternaria* sp. and *Colletotrichum* sp. with each of them showing 8.69 per cent colonization frequency. Fungal endophytes PJB-1 and PJV-6 were found to be positive for siderophore activity.

Key words: Colonization, Endophytic fungi, Frequency, Siderophore

Introduction

Endophytes are the plant-associated microorganisms that colonize and live part of their life cycle within a plant without causing harm or disease (Bamisile *et al*., 2018). This is a topographical term and includes bacteria, fungi, actinomycetes, and algae, which spend their whole life or a period of life cycle in the symplast or apoplast region of healthy plant tissues without producing any disease or clinical symptoms.

A single plant part (leaf, stem, or root) can contain different endophyte species (Fürnkranz *et al.,* 2012). Plants studied till date has at least one endophytic fungus species, while several woody plants can have tens of species (Arnold *et al*., 2000). These fungi play a significant role in evolving the plant populations and influencing processes like colonisation, competitiveness, co-existence, soil nutrient management and dynamics (Schulz *et al*., 2002).

Endophytes play a major role in plant community health by providing resistance to hosts against different biotic and abiotic stresses (Kharwar *et al*., 2008; Gond *et al*., 2010). Endophytes are viewed as an outstanding source of novel bioactive natural products because many of them occupy literally millions of unique biological niches (higher plants) growing in a variety of unusual environments (Verma *et al*., 2009).

Prosopis is a genus of flowering plants in the pea family, Fabaceae. It is locally called Bellary jaali (Jaali), whose population is abundant in and around Bellary district of Karnataka (India). It contains around 45 species of spiny trees and shrubs found in subtropical and tropical regions of the America, Africa, Western Asia and South Asia. They often thrive in arid and semi-arid soil and are resistant to drought, on occasion of developing extremely deep root systems. Their wood is usually hard, dense and durable. Their fruits are pods and may contain large amounts of sugar.

Prosopis cineraria is a small tree, ranging in height from 3-5 m (9.8-16.4 ft). Leaves are bipinnate, with seven to fourteen leaflets on each of one to three pinnae. Branches are thorned along the internodes. Flowers are small and creamy-yellow. The tree is found in extremely arid and semi-arid conditions, with rainfall as low as 15 cm (5.9 in) annually, but it is indicative of the presence of a deep water table. *Prosopis juliflora* grow to a height of up to 12 m (39 ft), has a trunk diameter of up to 1.2 m (3.9 ft). Its leaves are deciduous, geminate-pinnate, light green, with 12 to 20 leaflets. Flowers appear shortly after leaf development. The flowers are in 5-10 cm long green-yellow cylindrical spikes, which occur in clusters of 2 to 5 at the ends of branches (Anon., 2020).

Keeping in view the drought tolerant ability of *Prosopis* spp., the objectives of the work reported were to isolate the endophytic fungi from leaf and stem tissues of *Prosopis* spp. and to study their colonization frequency, siderophore producing ability and diversity.

Material and methods

Plant material and collection site

In this study, the plants growing in the semi-arid habitat of Vijayapur and Bagalkot districts (Bilgi, Bagalkot, Badami, Basavanabagewadi, Vijayapur, Bableshwar, Jamakhandi, Mudhol and Indi taluks) of Karnataka were selected. A total of 54 samples were collected from two *Prosopis* spp. *viz*., *P. juliflora* and *P. cineraria*. Leaf and bark samples were collected, labelled and placed in polypropylene bags and brought to the laboratory.

Procedure for isolation of endophytic fungi from stem and leaf samples

A protocol described by Arnold *et al*. (2000) was used to isolate fungal endophytes from the samples. Plant parts were

brought to the laboratory, washed in running tap water to remove the dust and debris and cut into 1 cm long pieces by a sterilized blade under aseptic conditions. The cut sample was first surface sterilized with 70 per cent ethanol for 1 min, then treated with sodium hypochlorite (NaOCl) solution for 30 sec to 1 min and later treated with 70 per cent ethanol for 1 min. The samples were washed 3-4 times with sterile distilled water for two min to remove the traces of NaOCl and then blot dried. After drying, tissue samples like leaf and stem were inoculated on potato dextrose agar (PDA) (5 explants/plate) medium supplemented with antibiotic (streptomycin; 0.1 g/l) (Chloramphenicol and Ampicillin could also be used). The plates were wrapped carefully with parafilm wrap and kept in an incubator at 28 ± 2°C for endophytic fungi emergence.

Frequency of colonization by endophytic fungi was determined as the total number of segments yielding ≥1 isolate in a host sample divided by total number of segments incubated (Sandhu *et al.,* 2014).

$$\text{Colonization frequency (\%)} = \frac{\text{Total number of segments yielding fungus}}{\text{Total number of segments inoculated}} \times 100$$

Morphological characterization to study the diversity of endophytic fungi

Fungi, a predominantly mycelium producing eukaryotic organisms are much diversified. A detailed study of their morphology is essential for their understanding. Fungal isolates were identified based on the colony character, cultural characteristics and type of spores. In this regard, study of colonies and spores helped to putatively identify these isolates and study their diversity (Domsch and Gams, 1972; Sutton, 1980).

Siderophore production

Siderophore production was detected by using chrome azurol S (CAS) assay developed by Schwyn and Neilands (1987). The medium contains an iron CAS-HDTMA (Hexadecyltrimethyl ammonium bromide) complex which is blue coloured. The presence of iron chelator (siderophore) is indicated by decolourization of the blue coloured ferric-dye complex, resulting in a yellow to orange coloured halo around the colonies. Chrome azurol S agar medium plates were prepared and spot inoculated with endophytic fungi and incubated at 28 °C for 48-72 hours. Development of yellow to orange halos around the compound was considered positive for siderophore production.

Results and discussion

A total of 54 samples were collected from two drought adapted *Prosopis* sp. *viz*., *Prosopis juliflora* (26 samples) and *P. cineraria* (28 samples). Stem and leaf samples of *Prosopis* spp. from semi-arid habitats of Vijayapur and Bagalkot districts of Karnataka were collected and used for the study. The altitude covered for isolation ranged from 490 m to 628 m above MSL.

Forty six endophytic fungal isolates were obtained from 270 segments placed on potato dextrose agar (PDA) media supplemented with streptomycin (0.1g/L). The colonization frequency across the plant species and tissue type ranged between 0 to 100 per cent. Out of 46 endophytic fungal isolates, 26 isolates were obtained from stem and 20 isolates were obtained from leaf samples.

Out of 26 samples of *Prosopis juliflora*, colonization frequency of 20 per cent was observed in four samples, 40 per cent was observed in five samples, 60 per cent was observed in three samples, 80 per cent was observed in two samples while, one sample showed colonization frequency of 100 per cent. Similarly, in *P. cineraria* colonization frequency of 20 per cent was observed in three samples, 40 per cent was observed in thirteen samples, 60 per cent was observed in three samples, 80 per cent was observed in two samples and one sample showed 100 per cent colonization frequency. While eleven samples from *P. juliflora* and sixteen samples from *P. cineraria* did not yield any endophyte irrespective of the tissue material used and thus recording 0 per cent colonization frequency. The average colonization frequency of *P. juliflora* was 27.69 per cent, while *P. cineraria* recorded average colonization frequency of 36.42 per cent (Table 1).

Higher per cent colonization was observed in stem, followed by leaf. Total colonization frequency of 30.77 and 41.43 per cent was observed in stem samples of *P. juliflora* and *P. cineraria*. A decrease in total colonization frequency of 23.08 and 28.57 per cent was observed in leaf samples of *P. juliflora* and *P. cineraria,* respectively.

Morphological and functional characterization

Out of 46 isolates, sixteen isolates showed velvety colony texture, 12 fungal isolates showed cottony texture, 11 isolates showed hairy colony texture, 6 fungal isolates showed granular colony texture and one isolate showed glabrous colony texture. The endophytic fungal isolates PJB-5 and PJB-8, PCB-5 and PCV-4 produced soluble pigments around the colonies on growing media (Table 2).

The morphological studies helped to examine the diversity found in the *Prosopis* spp. The endophyte PJB-1 was putatively identified as *Fusarium* sp. based on sickle shaped conidia. A total of thirteen fungal endophytes *viz.,* PJB-2, PJB-4, PJB-6, PJB-8, PJB-9, PCB-1, PCB-4, PCB-6, PCB-8, PCV-3, PCV-5, PJV-2 and PJV-7 were identified as *Aspergillus* sp. based on dark spherical spores. Similarly, a total of 4 fungal endophytes *viz.,* PJB-5, PJB-15, PJV-1 and PJV-3 were identified as *Alternaria* sp. based on club/clavate shaped conidia. The dumbell shaped conidia helped to identify the four endophytes PCB-3, PCV-7, PCV-8 and PCV-10 as *Colletotrichum* sp. The endophytes PCV-11 and PJV-6 were identified as *Penicillium* sp. based on chain of round spores. The endophyte PJB-14 with sclerotial bodies was putatively identified as *Rhizoctonia* sp. A total of 21 endophytes could not be identified because of their non-sporulating nature (Plate 1).

Microbes release siderophores to scavenge iron from the complex mineral phase by formation of soluble ferric (Fe^{3+}) complexes that can be taken up by active transport mechanisms.

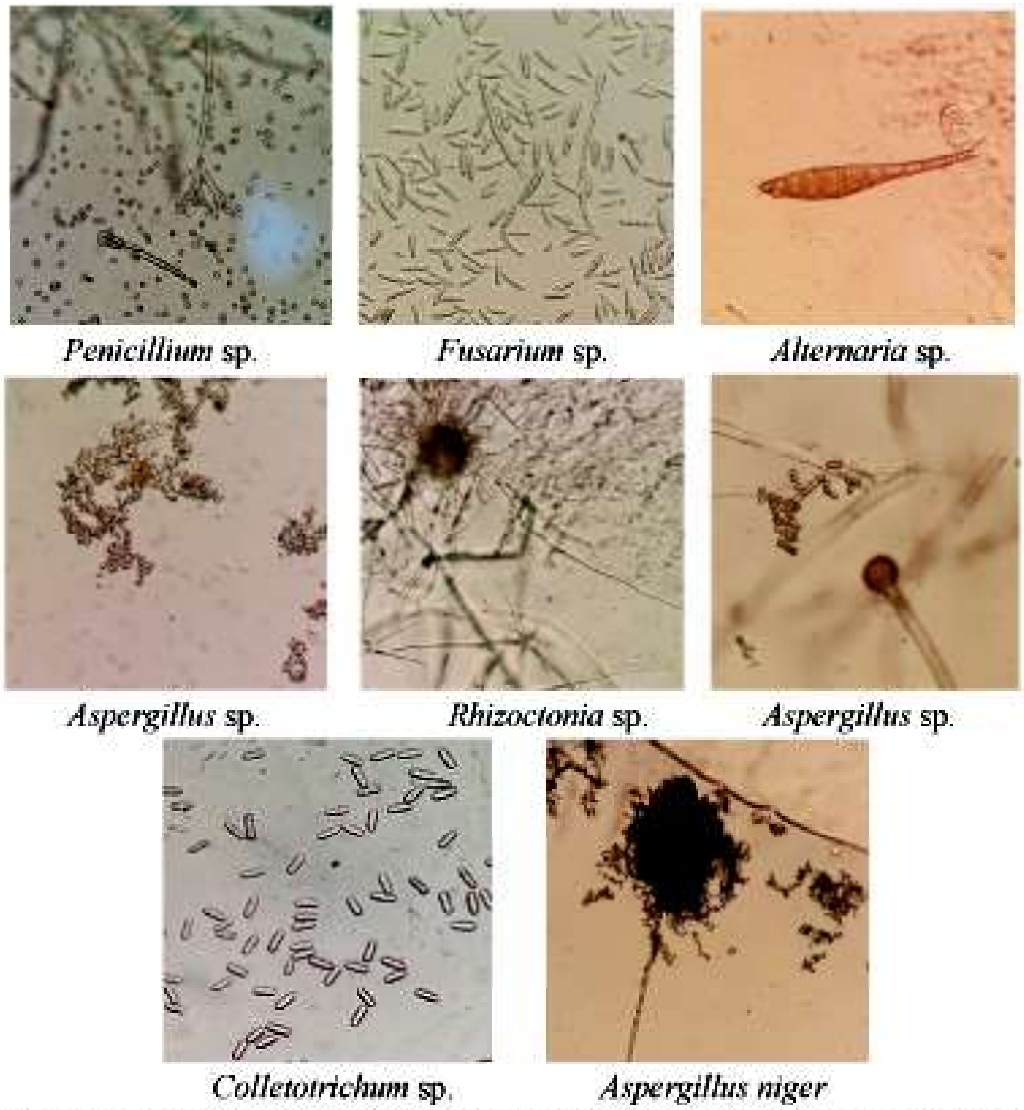

Plate 1. Morphological studies showing hyphae and spores/conidia of endophytic fungi isolated from stem and leaf samples of *Prosopis* sp.

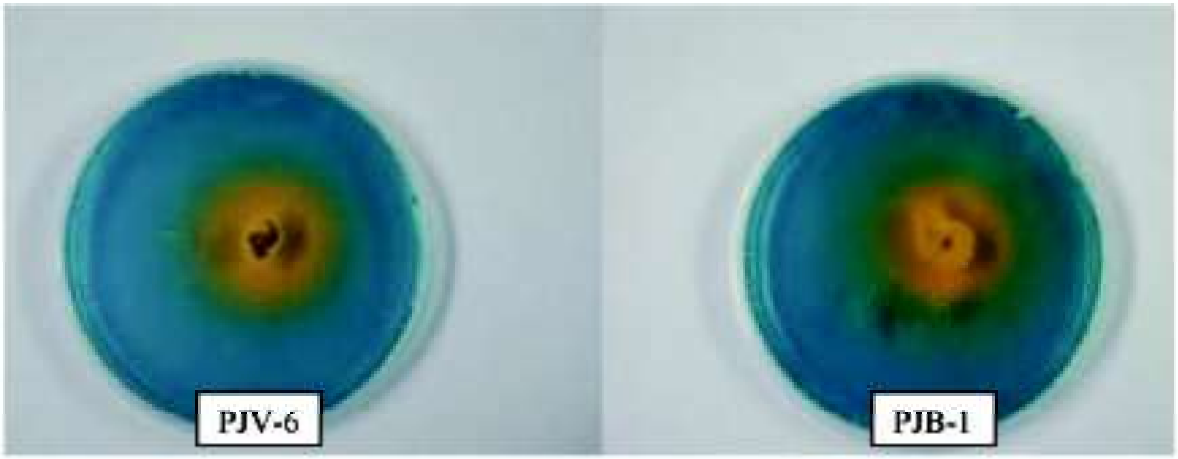

Plate 2: Siderophore production by endophytic fungal isolates

Table 1. Colonization frequency (%) of stem and leaf parts of *Prosopis* spp. collected from semi-arid habitat of Vijayapur and Bagalkot districts

Sample no.	Host	Sample type	Segments yielding endophytes	Total segments	Colonization frequency (%)
1.	*Prosopis juliflora*	Stem	3	5	60
2.		Leaf	2	5	40
3.		Stem	0	5	0
4.		Leaf	0	5	0
5.		Stem	1	5	20
6.		Leaf	2	5	40
7.		Stem	2	5	40
8.		Leaf	2	5	40
9.		Stem	2	5	40
10.		Leaf	1	5	20
11.		Stem	0	5	0
12.		Leaf	0	5	0
13.		Stem	0	5	0
14.		Leaf	1	5	20
15.		Stem	0	5	0
16.		Leaf	1	5	20
17.		Stem	4	5	80
18.		Leaf	0	5	0
19.	*Prosopis juliflora*	Stem	5	5	100
20.		Leaf	4	5	80
21.		Stem	3	5	60
22.		Leaf	0	5	0
23.		Stem	0	5	0
24.		Leaf	3	5	60
25.		Stem	0	5	0
26.		Leaf	0	5	0
27.	*Prosopis cineraria*	Stem	2	5	40
28.		Leaf	3	5	60
29.		Stem	2	5	40
30.		Leaf	0	5	0
31.		Stem	2	5	40
32.		Leaf	0	5	0
33.		Stem	3	5	60
34.		Leaf	2	5	40
35.		Stem	2	5	40
36.		Leaf	2	5	40
37.		Stem	4	5	80
38.		Leaf	2	5	40
39.		Stem	4	5	80
40.		Leaf	2	5	40
41.		Stem	5	5	100
42.		Leaf	1	5	20
43.		Stem	3	5	60
44.		Leaf	1	5	20
45.		Stem	2	5	40
46.		Leaf	0	5	0
47.		Stem	0	5	0
48.		Leaf	2	5	40
49.		Stem	1	5	20
50.		Leaf	0	5	0
51.		Stem	2	5	40
52.		Leaf	2	5	40
53.		Stem	2	5	40
54.		Leaf	1	5	20

Note: Total colonization frequency (%) of leaf and stem parts of *Prosopis* spp.

Plant species	Stem (%)	Leaf (%)
Prosopis juliflora	30.77	23.08
Prosopis cineraria	41.43	28.57

Table 2. List of isolates with morphological characteristics and putative identification

Sl. No.	Isolates	Texture	Colour	Reverse of colony	Margin	Elevation	Sulcation	Exudates	Soluble pigments	Hyphae	Conidia/ Spore	Organism (Putative)
1.	PJB-1	Cottony	Creamy white concentric	White	C	Un	-	-	-	Septate	Sickle shaped conidia	*Fusarium* sp.
2.	PJB-2	Granular	Grey with white margin	Black	C	Fl	-	Yes	-	Septate	Round	*Aspergillus* sp.
3.	PJB-3	Cottony	White	White	F	Fi	-	Yes	-	Septate	No sporulation	-
4.	PJB-4	Granular	Grey	Grey	C	Fi	-	-	-	Septate	Round	*Aspergillus* sp.
5.	PJB-5	Velvety	Olive with white margin	Grey	Ir	Un	-	-	-	Septate	Clavate shaped	*Alternaria* sp.
6.	PJB-6	Hairy	Black with white margin	Black	C	Fl	-	Yes	Yes	Septate	Round	*Aspergillus* sp.
7.	PJB-7	Velvety	Brown with pink margin	Brown	C	Cr	-	-	-	Septate	No sporulation	-
8.	PJB-8	Hairy	Black with white margin	Black	C	Fl	-	Yes	Yes	Septate	Round	*Aspergillus* sp.
9.	PJB-9	Hairy	Grey with white margin	White	Ir	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.
10.	PJB-10	Velvety	Grey with pink margin	Grey	Ir	Ra	Yes	-	-	Septate	Rod shaped/ Cylindrical	-
11.	PJB-11	Velvety	Pink with brown margin	Brown	C	Cu	Yes	Yes	-	Septate	No sporulation	-
12.	PJB-12	Velvety	Olive with white margin	Brown	C	Fl	Yes	-	-	Aseptate	Non sporulating	-
13.	PJB-13	Velvety	Olive with white margin	Brown	C	Fl	Yes	-	-	Aseptate	Non sporulating	-
14.	PJB-14	Cottony	Yellowish concentric	Cream	C	Cu	-	-	-	Septate	Sclerotial body	*Rhizoctonia* sp.
15.	PJB-15	Velvety	Olive with white margin	Grey	Ir	Un	-	-	-	Septate	Club shaped	*Alternaria* sp.
16.	PJB-16	Cottony	Ochre yellow	White	Ir	Fl	-	-	-	Septate	Oval shaped	-
17.	PCB-1	Hairy	Grey with white margin	Black	C	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.
18.	PCB-2	Velvety	Cream	Cream	C	Fl	-	-	-	Septate	No sporulation	-
19.	PCB-3	Cottony	White	White	F	Co	-	-	-	Septate	Dumbell shaped	*Colletotrichum* sp.
20.	PCB-4	Hairy	Grey with white margin	White	Ir	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.
21.	PCB-5	Velvety	Pink	Dark brown	Ir	Um	Yes	-	Yes	Aseptate	Non sporulating	-
22.	PCB-6	Cottony	Grey with white margin	White	Ir	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.

23.	PCB-7	Velvety	Dirty white	Light brown	Ir	Cu	Yes	Yes	-	Septate	No sporulation	-
24.	PCB-8	Hairy	Grey with white margin	Black	F	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.
25.	PCB-9	Granular	Buff	Buff	Ir	Fl	-	-	-	Septate	No sporulation	-
26.	PCB-10	Hairy	Hairy	Grey	C	Fl	-	Yes	-	Septate	No sporulation	-
27.	PCB-11	Hairy	Hairy	White	F	Fi	-	Yes	-	Septate	No sporulation	-
28.	PCV-1	Granular	Grey with white margin	White	Ir	Ra	Yes [base]	-	-	Septate	No sporulation	-
29.	PCV-2	Granular	Black with white margin	White	Ir	Ra	Yes [base]	-	-	Septate	No sporulation	-
30.	PCV-3	Hairy	Greyish white	Black	F	Um	-	-	-	Septate	Round	*Aspergillus* sp.
31.	PCV-4	Cottony	White	Yellowish white		C	Fl	-	-	Yes	Aseptate	Globose -
32.	PCV-5	Hairy	Brown with white margin	Grey	C	Fi	-	Yes	-	Septate	Round	*Aspergillus* sp.
33.	PCV-6	Cottony	Light orange	Orange	F	Ra	-	Yes	-	Septate	No sporulation	-
34.	PCV-7	Hairy	Pinkish white	White	F	Fi	-	-	-	Septate	Dumbell shaped	*Colletotrichum* sp.
35.	PCV-8	Cottony	Pink concentric	Crimson red	F	Ra	-	-	-	Septate	Dumbell shaped	*Colletotrichum* sp.
36.	PCV-9	Glabrous	Cream	Cream	C	Cu	Yes [base]	-	-	Aseptate	Non sporulating	-
37.	PCV-10	Cottony	Pinkish white	White	F	Ra	-	-	-	Septate	Dumbell shaped	*Colletotrichum* sp.
38.	PCV-11	Velvety	Turquoise with white margin	White	Ir	Fl	Yes	-	-	Septate	Round	*Penicillium* sp.
39.	PJV-1	Velvety	Olive with white margin	Grey	Ir	Un	-	Yes	-	Septate	Club shaped	*Alternaria* sp.
40.	PJV-2	Cottony	Grey with white margin	White	Ir	Um	-	Yes	-	Septate	Round	*Aspergillus* sp.
41.	PJV-3	Velvety	Olive with white margin	Grey	Ir	Un	-	-	-	Septate	Club shaped	*Alternaria* sp.
42.	PJV-4	Cottony	Dirty white	Light brown	Ir	Un	Yes [base]	-	-	Septate	No sporulation	-
43.	PJV-5	Granular	Creamy white	White	C	Fl	-	Yes	-	Septate	No sporulation	-
44.	PJV-6	Velvety	Turquoise white conc.	Cream	C	Un	Yes	Yes	-	Septate	Round chain	*Penicillium* sp.
45.	PJV-7	Velvety	Brown with white margin	Dark brown	Ir	Ra	-	Yes	-	Septate	Round	*Aspergillus* sp.
46.	PJV-8	Velvety	Grey with white margin	Black	R	Fl	-	-	-	Septate	Globose	-

Note: Margin: C-Circular, F-Filamentous, Ir-Irregular, R-Regular; Elevation: Un-Undulate, Fl-Flat; Fi-Filiform, Cr-Crateriform, Um-Umbonate, Co-Convex, Ra-Raised, Cu-Curled, Lo-Lobate.

It also elicits plant defence through antagonist mechanism by activating salicylic acid and jasmonic acid as a part of systemic resistance to diseases. This test was conducted to check if fungal endophytes produced siderophore. The endophytic fungal isolates *viz*., PJB-1 and PJV-6 caused the decolourization of the blue coloured ferric-dye complex (CAS agar) resulting in a yellowish orange halo which indicated the presence of iron chelating compound siderophore (Plate 2). The remaining 44 endophytes were found to be negative for siderophore production.

A rich diversity of endophytic fungal biota was obtained from the plant species examined among which fungi belonging to the genera *Aspergillus*, *Alternaria*, *Colletotrichum* and *Fusarium* were found to be dominant. In a previous study, Gehlot *et al*. (2008) reported a rich diversity of endophytes from *Prosopis cineraria*, a tree species of the Indian Thar Desert and suggested that the moisture and organic matter of the inner bark of the tree could help harbour a diverse community of the fungi.

Endophytes colonize inside healthy plant tissues to get nutrition and shelter from the host, and in response produce many functional metabolites. Fungal endophytes are relatively less explored and these are the new addition to the available diversity of fungi (Kharwar *et al*., 2009). Earlier it was thought that one plant can be a habitat of six fungi, but after including fungal endophytes, the ratio of fungal: plant species has now been changed to 33:1 (Hawksworth and Rossman, 1997). The most isolated genera in this study were *Aspergillus* sp. followed by *Alternaria* sp and *Colletotrichum* sp. This result is supported by earlier work done by Kharwar *et al*. (2010), where he isolated 33 fungal endophytes from leaf and stem segments. Nine out of 33 species were found in leaf tissues. These species included *Alternaria alternata*, *Aspergillus fumigatus*, *A. terreus*, *Cladosporium cladosporioides*, *Drechslera rostrata*, *Humicola grisea*, *Nigrospora oryzae*, *Penicillium cristata* and *Pestalotia* sp. Meanwhile, when the fungal endophytes in this study were tested for siderophore production, two isolates *viz*., *Fusarium* sp. (PJB-1) and *Penicillium* sp. (PJV-6) were found to be positive for siderophore activity. These results were similar to study conducted by Prathyusha *et al*. (2015) where an endophytic fungus *Acremonium sclerotigenum* isolated from the leaves of *Terminalia bellerica* was reported to produce siderophore. In addition to this, the endophytic fungi having the ability to produce siderophore was reported by Chowdappa *et al*. (2020) while exploring orchid plant, *Cymbidium aloifolium*.

The mycelia or spore/conidia of a fungal endophytes *viz*., PJB-5 and PJB-8, PCB-5 and PCV-4 isolated in this study produced pigments. As supported by previous studies, pigments produced by the fungi (melanin and carotenoids such as lycopene) could provide protection against UV light, pathogenicity and environmental stresses. Pigmented fungi were isolated from several cactus species and plants of arid habitat (Suryanarayanan *et al*., 2005; Gehlot *et al*., 2008).

In the present study, *Aspergillus* was found to be the dominant genera. It is likely that plants growing in extreme habitats harbour the deeply pigmented *Aspergillus* as an adaptive strategy against the stressful environments. It is well-known that high temperature augments melanin content in microorganisms (Sandra *et al*., 2017) and melanin-containing fungi are tolerant to abiotic stresses such as solar radiation, high temperature, drought, and chemical and radioactive pollution (Gessler *et al*., 2014; Belozerskaya *et al*., 2017).

Plants in harsh environments such as arid and semi-arid habitats experience high temperature, low water availability, high irradiance, variable rainfall patterns and nutrient deprivation and could be expected to harbour a rich diversity of endophytes that might be responsible for alleviating some of these stresses in the plants. However, little is known of the diversity of endophytic fungi biota of semi-arid habitat and the implications they might hold for imparting stress tolerance to their host plants (Suryanarayanan *et al*., 2005; Gehlot *et al*., 2008; Massimo *et al*., 2015).

Conclusion

The present study has attempted to explore the diversity of endophytic fungal biota associated with plants adapted to the semi-arid habitats of Karnataka. In summary, our results open up the prospect to examine the role of these identified fungi, both in their ecological context to the plants in the semi-arid habitat of Vijayapur and Bagalkot (Karnataka), as well as to their role in alleviating abiotic stress in agriculturally important crops.

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