Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2014; 2(4): 38-45

Received: 18-05-2014
Accepted: 12-06-2014

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Evaluation of endophytic fungi from *Butea monosperma* for antimicrobial and enzyme activity

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Abstract
Endophytic fungi are likely to produce novel bioactive compounds and enzymes. Bioprospecting of endophytic fungi from medicinal plants is gaining significance. A total of 73 endophytic fungal isolates from medicinal plant *Butea monosperma* were evaluated for their antimicrobial property and enzyme producing potential. Eleven endophytic fungal isolates were found to secrete antifungal compounds inhibiting conidial germination of plant pathogenic fungi. Differences were observed among the endophytic fungal isolates in their antifungal activity. Two isolates of Morphotype-1 (BM 8 and BM 56) showed distinct antifungal activity in conidial germination inhibition assay. Isolates of *Fusarium* spp., *Colletotrichum* sp. and *Sclerotium* sp. were also effective against many target fungi. Three isolates of endophytic fungi showed antifungal activity against human pathogenic fungi. Five isolates of endophytic fungi were found to be antibacterial against Gram positive bacteria in agar well diffusion assay. The isolate of *Aspergillus fumigatus* (BM 6) showed antibacterial activity against both Gram positive and Gram negative bacteria. Ability of endophytic fungal isolates to produce amylase, cellulase and pectinase was assessed in plate assay. Highest amount of all the enzymes were found to be produced by *Cladosporium* sp. as indicated by enzyme index. A range of enzyme activity was shown by isolates of *Fusarium verticillioides*, *Colletotrichum* sp., *Sclerotium* sp., *Pithomyces chartarum*, *Curvularia lunata*, Morphotype-1, Morphotype-2 and Morphotype-3. Considerable variations in enzyme producing ability are indicated clearly by the isolates. Promising endophytic fungal isolates of *B. monosperma* producing both antimicrobial compounds and enzymes were identified. Potential isolates may be exploited biotechnologically.

Keywords: Amylases, *Butea monosperma*, *Candida*, cellulases, *Cryptococcus*, pectinases.

1. Introduction
Endophytes are microorganisms that colonize plants without causing any disease symptoms. Such organisms constitute major component of biodiversity and are also significant in understanding plant biology. Endophytic fungi are known to be associated with most of the plant species studied [1, 2]. Fungi belonging to a wide range of species have been reported as endophytes of several plants [3, 4, 5, 6, 7]. Endophytic fungi are known to produce metabolites that help the host plant to tolerate biotic and abiotic stress, increase growth rate and extent of reproduction [8, 9, 10, 11]. The chemical constituents and the medicinal property of the plant may also be due to the interactions with its endophytes [12, 13].

Continued antibiotic resistance by the pathogenic microorganisms demands constant search for novel antimicrobial compounds [14, 15, 16]. Even for plant pathogen management new antimicrobials are needed. Endophytic fungi from medicinal plants offer a promise to novel metabolites with wide biotechnological applications [17, 18, 19, 20, 21, 22, 23, 24, 25]. Wide range of extracellular enzymes is also produced by endophytic fungi [26, 27, 289, 29, 30, 31, 32]. These enzymes help them for the entry, colonization and obtain nutrition [33, 34]. They also play a key role in decomposition of plant material after tissue death and senescence aiding to carbon cycle [33, 35, 36]. Fungal enzymes have vast biotechnological applications in agriculture, biomedical, food and beverage industries.

Butea monosperma (Lam.) Taub. is a tropical medicinal plant, well known for its various medicinal properties [37]. Medicinal values of *B. monosperma* are evident in the treatment of flatulence, diarrhea, dysentery, rectal diseases, wounds, skin diseases, boils and tumor [38, 39, 40, 41]. In spite of wide medicinal uses of *B. monosperma*, no reports are available on medicinal

property of endophytes associated with it. Occurrence and distribution of endophytic fungi in *B. monosperma* have been reported [42]. Bioprospecting of such fungi for useful compounds is essential in biotechnology. Hence, this study was conducted to evaluate antimicrobial and enzyme potential of endophytic fungi isolated from *B. monosperma*.

2. Materials and Methods

2.1 Endophytic fungi and their culture filtrates

Endophytic fungi were isolated from different parts of *Butea monosperma* tree using standard methods [42, 43]. For the production of endophytic fungal culture filtrate, the inocula from pure cultures of endophytic fungi were inoculated into 10 ml of Richard's solution taken in vials separately. The vials were then incubated in dark at 27±2 °C. The eight-day-old stationary cultures were filtered through sterile filter paper separately followed by centrifugation at 5000 rpm for 10 min. The supernatant collected is used for antimicrobial assays.

2.2 Test organisms

Fungi associated with seeds of sorghum, sunflower and maize were isolated by blotter method [44]. Fungal isolates which inhibited the germination of seeds or inhibited the growth of seedlings were selected as phytopathogenic fungi. The fungal isolates were identified based on colony morphology and microscopic features [45, 46, 47, 48, 49]. *Alternaria alternata* was selected from sunflower seed sample whereas *Curvularia lunata* and *Drechslera hawaiiensis* were isolated from sorghum seeds. *Fusarium acuminatum* was obtained from maize seed samples. Pure cultures of the fungal isolate were established and used as target fungi in the present investigation.

Bacillus subtilis (MTCC 441) and human pathogenic bacteria like *Escherichia coli* (MTCC 1687), *Salmonella typhi* (MTCC 3917), and *Staphylococcus aureus* (MTCC 3160) and human pathogenic yeasts like *Candida albicans* (MTCC 3017) and *Cryptococcus neoformans* (MTCC 4425) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

2.3 Antimicrobial assays

2.3.1 Conidial germination assay

Endophytic fungal culture filtrates were used to determine antifungal activity against selected phytopathogenic fungi. Conidia of target fungi, *A. alternata*, *C. lunata*, *D. hawaiiensis* and *F. acuminatum* were placed in 50 µl culture filtrates of endophytic fungi taken separately in cavity slides. Conidia of target test fungi in 50 µl of sterile Richard's solution served as control. The slides were then incubated in moist chamber at 27±2 °C for 15-20 hrs. Conidia were observed for the production of germ tube in different microscopic fields [50]. For each treatment a minimum of 100 conidia were considered. Three independent trials were conducted. Conidial germination percentage for each treatment was calculated. Statistical analysis was made to calculate standard error considering the mean values. Conidial germination inhibition over control was also calculated using the formula,

$$\text{Conidial germination inhibition (\%) over control} = \frac{\text{Germination in Control (C)} - \text{Germination in Treatment (T)}}{\text{Germination in Control (C)}} \times 100$$

2.3.2 Agar well diffusion assay

Clture filtrates of endophytic fungal isolates were separately tested for antibacterial and antifungal activity by agar well diffusion assay [51]. The cell density of 24–hour–old bacterial and fungal broth cultures were adjusted to 0.5 McF units (approx. 10^6 cfu ml^{-1}) and swab inoculated on Mueller-Hinton agar medium and Yeast extract malt extract glucose agar medium respectively in separate Petri plates. Agar wells were made equidistantly in the inoculated plates using a sterile cork borer (6 mm diameter). Each well was loaded with 100 µl culture filtrate of each endophytic fungus separately. Wells loaded with 100 µl of standard antibacterial chloramphenicol solution (300 µg/ml, w/v) for bacteria or antifungal compound fluconazole solution (100 µg/ml, w/v) for fungi separately served as positive controls. Wells containing sterile distilled water and Richard's solution served as negative controls. The plates were incubated at 37±2 °C in an incubator. The plates were observed at an interval of 24 hours up to 48 hours for growth inhibition around the wells. The experiment was performed twice with duplicates.

2.4 Enzyme assay

Endophytic fungal isolates obtained from *Butea monosperma* were screened for the production of amylases, cellulases and pectinases by plate assay [34]. Modified Czapek Dox agar medium with sole source of carbon as starch or carboxy methyl cellulose or pectin was used respectively. Spore/mycelial suspension of all the endophytic fungi were prepared separately in sterile physiological saline and point inoculated on to respective media. The plates were incubated at 27±2 °C for four days. The experiment was performed in duplicates. The plates were observed for the growth of fungi and the diameter of the fungal colony was measured. The plates were then flooded with 3% iodine solution and allowed for five minutes [27, 34]. Later the plates were observed for the appearance of clear zone around the colony and the diameter of clear zone was measured. Enzyme index was calculated as diameter of clear zone including the fungal colony divided by diameter of the fungal colony. Standard error was calculated considering the mean values.

3. Results

3.1 Conidial germination assay

Conidia of target fungi seeded in sterile Richard's broth germinated well by producing profuse germ tubes. The endophytic fungal culture filtrates showed antifungal activity against conidial germination of target fungi (Table 1). The results revealed that out of 73 endophytic fungal isolates only 11 isolates showed variable antifungal activity in this assay. The conidial germination inhibition (%) over control in endophytic fungal filtrate is depicted in Fig. 1. The Morphotype-1 isolate (BM 56) had highest inhibitory activity on *A. alternata*, *C. lunata* and *D. hawaiiensis*. Morphotype-1 isolate (BM 8) showed moderate effect on *D. hawaiiensis* and *F. acuminatum*. *Fusarium solani* (BM 2) was effective against all the target fungi. Both the isolates of *Fusarium sterilihyphosum* (BM 58 and BM 61), *Colletotrichum* sp. isolate (BM 62) and *Sclerotium* sp. (BM 36 and BM 43) showed similar antifungal effect on all the target fungi except *F. acuminatum*. The isolates of *Pithomyces chartarum* (BM 5 and BM 9) showed moderate activity on *A. alternata* and *F. acuminatum* whereas isolate BM 7 showed antifungal activity against *A. alternata.* Variations in antifungal activity were observed with culture filtrates of *Pithomyces chartarum* isolates (Table 1 and Fig. 1).

Table 1: Antifungal activity of culture filtrates from endophytic fungi of *Butea monosperma* in conidial germination assay.

Source of endophytic fungi	Isolate code No.	Culture filtrate of endophytic fungi	Conidial germination (%) ± standard error			
			Alternaria alternata	***Curvularia lunata***	***Drechslera hawaiiensis***	***Fusarium acuminatum***
-	-	Richard's solution (control)	96.32±0.08	97.95±0.40	94.79±0.26	95.17±0.56
Root	BM 8	Morphotype-1	95.76±0.93	96.21±0.37	67.59±0.57	78.85±2.30
Root	BM 56	Morphotype-1	2.89±1.63	2.75±1.16	8.30±0.77	92.04±1.99
Root	BM 2	*Fusarium solani*	14.01±0.99	4.03 ± 0.19	23.16±1.02	28.74±2.75
Root	BM 58	*Fusarium sterilihyphosum*	2.72 ± 1.28	4.12 ± 1.36	8.8 ±0.62	93.74±1.15
Stem	BM 61	*Fusarium sterilihyphosum*	5.86±0.33	7.45±0.45	4.13±0.47	91.73±1.91
Stem	BM 62	*Colletotrichum* sp.	6.51 ± 0.28	6.74 ± 0.50	5.37±0.94	93.61± 0.57
Stem	BM 5	*Pithomyces chartarum*	73.30±1.18	91.18±2.40	93.32±1.65	72.06±0.92
Flower	BM 9	*Pithomyces chartarum*	73.13±0.36	96.78±1.42	90.66±0.25	56.83±0.94
Flower	BM 7	*Pithomyces chartarum*	65.54±4.38	92.11±0.61	90.00±1.11	92.61±2.06
Lamina	BM 36	*Sclerotium* sp.	4.69±0.46	6.96±1.36	6.81±1.25	92.08±1.56
Lamina	BM 43	*Sclerotium* sp.	2.91±0.60	5.09±0.57	3.86±0.19	91.44±1.81

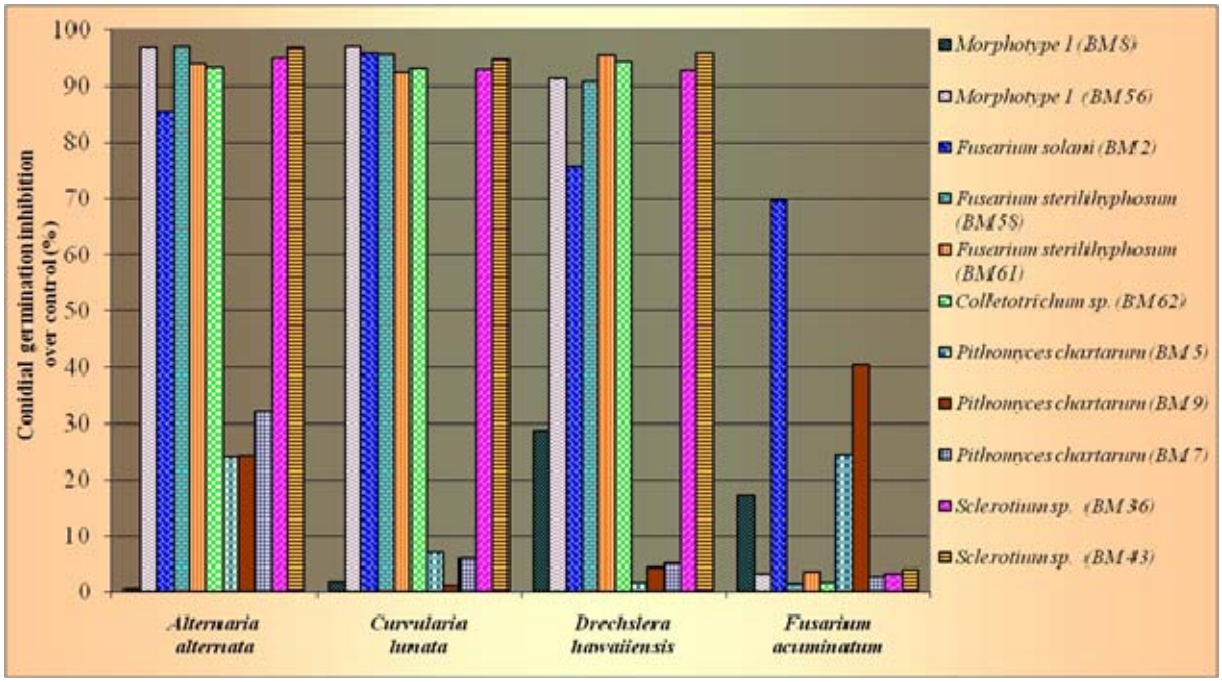

Fig 1: Conidial germination inhibition of target fungi by culture filtrates from endophytic fungi of *Butea monosperma*.

3.2 Agar well diffusion assay

Zone of inhibition was observed around the agar wells loaded with antibiotic solutions whereas no inhibition was found around the wells loaded with distilled water or Richard's solution. Out of 73 endophytic fungal culture filtrates from *B. monosperma*, eight recorded different degrees of antimicrobial activity (Table 2). Only *Aspergillus fumigatus* (BM 6) was as effective as chloramphenicol against *E. coli*, showing inhibition zone of 29 mm (Table 2). Five of the endophytic fungal culture filtrates were effective against *Bacillus subtilis* (Table 2). Four isolates of *F. verticillioides* (BM 13, BM 14, BM 16 and BM 18) were equally effective against *Staphylococcus aureus*. The culture filtrates of *F. solani* (BM 2), *F. sterilihyphosum* (BM 58) and Morphotype-1 (BM 56) showed antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (Table 2, Fig. 2). None of the culture filtrates were effective against *Salmonella typhi*.

Table 2: Antimicrobial activity of endophytic fungal culture filtrates against bacteria and fungi in agar well diffusion assay.

Source of endophytic fungi	Targets → / Treatments ↓	Diameter of inhibition zone (in mm) ± standard error					
		Bacteria				Fungi	
		Escherichia coli	***Salmonella typhi***	***Bacillus subtilis***	***Staphylococcus aureus***	***Candida albicans***	***Cryptococcus neoformans***
	Control *	29±0.25	29±0.41	45±0.21	39 ±0.50	34 ±0.21	33±0.25
	Control †	0	0	0	0	0	0
Root	*Fusarium solani* (BM2)	0	0	0	0	22±0.21	24±0.00
Root	Morphotype-1 (BM56)	0	0	0	0	15±0.21	15±0.35
Root	*Fusarium sterilihyphosum* (BM58)	0	0	0	0	16±0.21	17±0.41
Root	*Aspergillus fumigatus* (BM6)	29±0.41	0	20±0.64	0	0	0
Petiole	*Fusarium verticillioides* (BM13)	0	0	19±0.64	28±1.13	0	0
Stem	*Fusarium verticillioides* (BM14)	0	0	14±0.41	27±0.86	0	0
Stem	*Fusarium verticillioides* (BM16)	0	0	13±0.25	25±0.54	0	0
Stem	*Fusarium verticillioides* (BM18)	0	0	16±0.41	28±0.21	0	0

* Positive control (antibacterial chloramphenicol 30 µg/ well or antifungal fluconazole 10 µg/well)
† Negative control (Sterile distilled H_2O / Sterile Richard's solution)

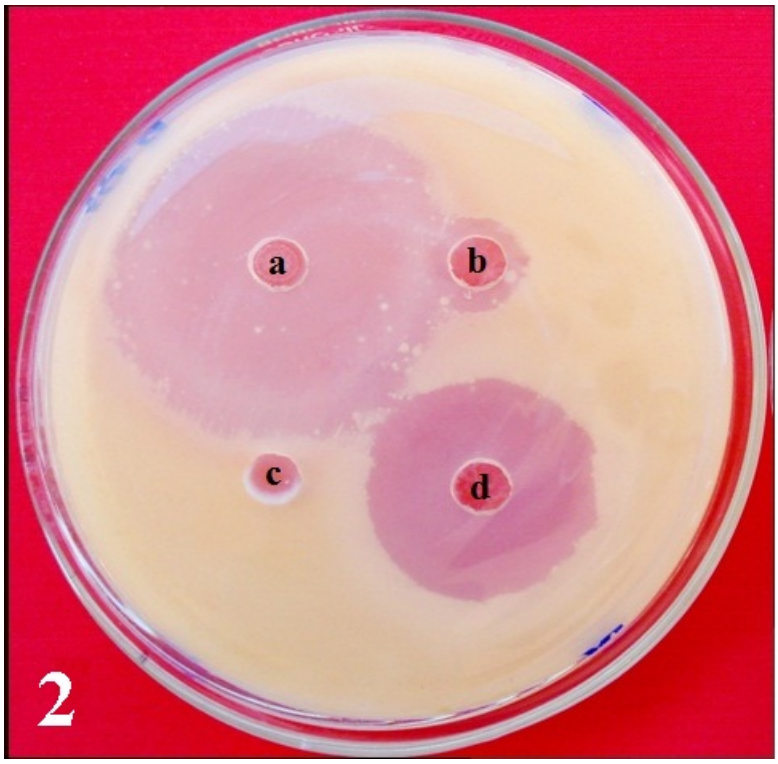

Fig 2: Effect of culture filtrates of endophytic fungi from *Butea monosperma* on growth of *Cryptococcus neoformans*.
a- 10 µg of fluconazole,
b- Culture filtrate of Morphotype-1 (BM 56),
c- Culture filtrate of *Aspergillus fumigatus* (BM 6),
d- Culture filtrate of *Fusarium solani* (BM 2).

3.3 Enzyme assay

Fungal growth was observed in all the inoculated plates with differences in colony diameter. Detection of enzyme activity in plate assay is shown in Fig. 3. The colony diameter and clear zone varied among the endophytic fungi of *B. monosperma*. Variations were observed among 73 endophytic fungal isolates of *B. monosperma* with respect to enzyme production (Table 3). Out of 16 species of endophytic fungi, enzyme index of more than 2.00 was shown by three fungi for amylase and four fungi for cellulases and pectinases. Highest enzyme index was noticed in *Cladosporium* sp. indicating its enzyme potential. Extensive variation with respect to enzyme production was noticed among isolates of *Colletotrichum* sp. One of the isolate *Colletotrichum* sp. (BM 40) from lamina showed pectinase enzyme index of 0.78 whereas other isolate (BM 44) from the same tissue showed enzyme index of 1.61. The other *Colletotrichum* isolate (BM 23) from stem showed pectinase index of 1.87. All the isolates of *A. sydowii* produced higher levels of all the enzymes. Extensive variation among the isolates *Curvularia lunata* was also noticed. *Chaetomium crispatum* was poor producer of amylases recording enzyme index of 0.88. Morphotype-2 was also poor producer of all the enzymes.

Table 3: Enzyme activity of endophytic fungi from *Butea monosperma* in plate assay.

Endophytic fungus	No. of isolates	Enzyme index* ± standard error		
		Amylase	Cellulase	Pectinase
Cladosporium sp.	1	2.23 ± 0.06	2.42 ± 0.00	2.76 ± 0.04
Papulaspora immersa	1	1.19 ± 0.00	1.40 ± 0.01	1.26 ± 0.02
Chaetomium crispatum	1	0.88 ± 0.01	1.15 ± 0.00	1.15 ± 0.00
Fusarium solani	1	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Fusarium verticillioides	5	1.14±0.01–1.63±0.01	1.14±0.00–1.49±0.01	1.09±0.0–1.53±0.02
Fusarium sterilihyphosum	2	1.46±0.02 – 1.48±0.04	1.38±0.00–1.44±0.00	1.50 ± 0.00
Colletotrichum sp.	17	0.82±0.03–1.84±0.00	0.81±0.00–1.97±0.04	0.78±0.00–1.87±0.08
Sclerotium sp.	10	1.00±0.00–1.95±0.03	0.98±0.00–2.45±0.02	0.85±0.00–2.12±0.03
Aspergillus fumigatus	1	1.00 ± 0.00	1.03 ± 0.00	1.03 ± 0.02
Aspergillus sydowi	3	1.93±0.04–2.32±0.06	2.00±0.00–2.72±0.00	2.16±0.02–2.57±0.01
Scopulariopsis canadensis	4	1.69±0.01–2.10±0.02	1.96±0.06–2.35±0.09	1.56±0.02–2.04±0.02
Pithomyces chartarum	6	1.40±0.03–1.63±0.00	1.25±0.01–1.88±0.00	1.16±0.01–1.80±0.00
Curvularia lunata	9	0.70±0.02–1.56±0.01	1.00±0.00–1.46±0.00	0.97±0.01–1.23±0.00
Morphotype-1	5	1.00±0.00–1.71±0.01	1.00±0.00–1.86±0.02	1.00±0.00–1.50±0.00
Morphotype-2	2	0.65±0.00–0.67±0.01	0.83±0.02–0.88±0.03	1.00 ± 0.00
Morphotype-3	5	1.00±0.00–1.80±0.01	0.99±0.01–1.81±0.00	1.00±0.00–1.58±0.00

* Enzyme index = Diameter of the halo zone including fungal colony / Diameter of fungal colony.

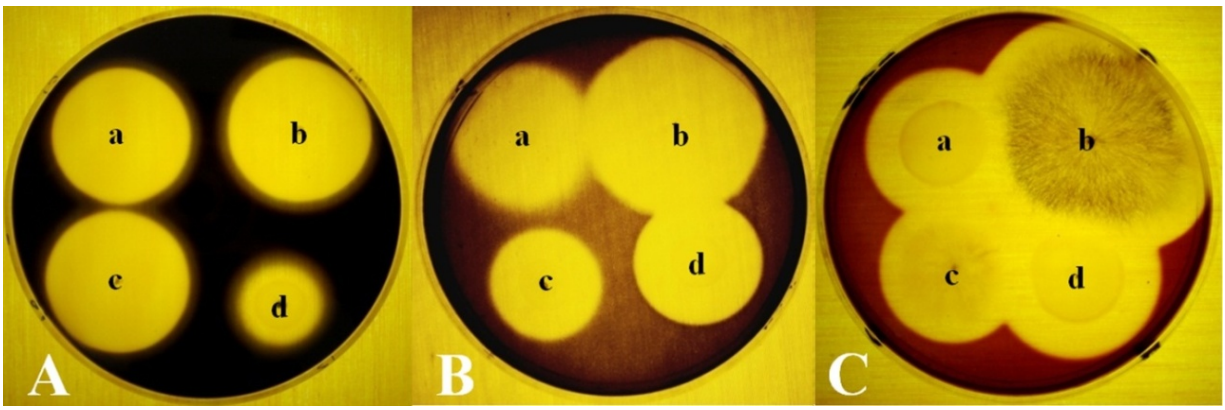

Fig 3: Detection of enzyme production by endophytic fungi of *Butea monosperma*.

A- Amylase production on starch agar medium, B- Cellulase production on Carboxy methyl cellulose agar medium, C- Pectinase production on pectin agar medium.

a) *Scopulariopsis canadensis* (BM 3),
b) *Curvularia lunata* (BM 68),
c) *Colletotrichum* sp. (BM 23),
d) *Aspergillus sydowi* (BM 42).

4. Discussion

Medicinal property of the plant has been attributed to endophytic microorganisms that reside in them [17, 52]. Tropical endophytes have produced more active and significantly higher number of biologically active secondary metabolites [53]. Chemical diversity existing in endophytic fungi with respect to antimicrobial compounds has been reported. Array of both antibacterial and antifungal compounds have been characterized [11]. In the present investigation both plant pathogenic and human pathogenic microorganisms were used as targets. Endophytic fungi from *B. monosperma* have shown great potential by producing different types of antimicrobial compounds. Antimicrobial activity of endophytic fungi from various other plants has been worked out [22, 23, 25, 54, 55, 56]. Among the 11 endophytic fungal culture filtrates, six isolates showed antifungal activity against *A. alternata and D. hawaiiensis*. Only one isolate showed activity against *F. acuminatum* and seven isolates against *C. lunata*. This indicates the extent of variation found in metabolites of endophytic fungi. It is interesting to note that Morphotype-1 (BM 8 and BM 56) isolates are from the root but have distinct antifungal activity. Endophytic *Fusarium* isolates (BM 2 and BM 58) and Morphotype-1 (BM 56) were effective against plant pathogenic and human pathogenic fungi. This is clear indication of chemical diversity existing in endophytic fungi in spite of colonizing same plant part. Such result is also true with *P. chartarum* isolates (BM 7 and BM 9) from flower. Bioactive flavonoids have been detected in the flowers of *B.* monosperma [40]. Differences in degree of antimicrobial activities indicate metabolic diversity existing in these endophytic fungal isolates. Differences in antibacterial and antifungal activity were also noticed in culture filtrates of endophytic fungi. *F. verticillioides* isolates inhibited the growth of Gram positive bacteria only indicating the production of narrow spectrum antibacterial compounds. It is also interesting to note that *A. fumigatus* isolate (BM 6) inhibited Gram negative (*E. coli*) and Gram positive bacteria (*B. subtilis*). On the other hand, *F. solani* isolate produced a broad-spectrum antifungal compound(s) that could inhibit the growth of all the fungi tested. It is evident from the results that various kinds of bioactive metabolites are found in endophytic fungi. Such differences have been noticed earlier [27, 57, 58]. This provides an excellent opportunity to explore novel antimicrobial compounds from endophytic fungi of *B. monosperma*. This may account for medicinal value of the plant.

Growth of endophytic fungi on starch, carboxy methyl cellulose and pectin as sole source of carbon in Czapek Dox agar medium itself is an indication of enzyme production by fungal isolates. Different media are used for enzyme assays [31, 37]. In the present investigation, modified Czapek Dox agar medium was used with specific carbon source for the detection of enzymes. Fungi are known to show differences in colony diameter on different media. The result of present study clearly demonstrates use of specific substrate for the growth in turn indicating the enzyme potential of fungi. The ratio of colony diameter and clear zone is indicative of enzyme activity. In many cases colony diameter and clear zone are being same giving value of 1.00 whereas colony diameter is more than the clear zone the enzyme index was less than one. Enzyme producing capacity varied among isolates of endophytic fungi from *B. monosperma*. Such extensive variations in enzyme production among fungi have been noticed [27, 31, 34, 59]. These enzymes are essential for endophytic fungi to colonize in the plant tissue. Analysis of enzyme index among isolates of same fungus provides interesting insights into the enzyme potential of endophytic fungi. Earlier we had reported tissue specific occurrence of endophytic fungi in *B.* monosperma [42]. The differences in enzyme potential may be due to occurrence of different degrees of pectin and cellulose in different parts of *B. monosperma*. Naturally, stem and leaf tissues show different extent of chemical constituents. Choi et al. [34] reported different levels of enzyme production in endophytic fungi in relation to wood degradation. Isolate variation with respect to enzyme production will provide information on functional diversity. Extracellular enzyme profile has been studied using isozyme polymorphism in fungi [60, 61, 62, 63]. Hence, the isozyme profile of these fungi may also be useful in diversity assessment at species/strain level. The enzyme potential of endophytic fungi may also be exploited biotechnologically. Hence, endophytes of medicinal plants may account for significant sources of useful metabolites.

5. Acknowledgments

Our sincere gratitude to Kuvempu University and Davangere University for providing the facilities. First author is grateful to OBC cell, Kuvempu University and Indian Institute of Science, Bangalore for their financial assistance.

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