Volume 64, Issue 1, 2020
Journal of Scientific Research
Institute of Science,
Banaras Hindu University, Varanasi, India.

Screening for Endophytic Fungi with Antibacterial Efficiency from *Moringa Oleifera* and *Withania Somnifera*

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Abstract: **Plants have a great potential to grow while carrying inside them fungi and bacteria known as endophytes. These endophytes have a potential to produce bioactive molecules which are of immense pharmaceutical importance. In the current study, plants having medicinal properties such as *Moringa oleifera* and *Withania somnifera* were explored for studying endophytic fungi associated with them. *Moringa oleifera* and *Withania somnifera* are widely known for their nutritional and medicinal values. The endophytic fungal associations of these plants have revealed their efficacy in therapeutic field. In the current study, endophytic fungal strains were extracted by incubating the dried plant samples on potato dextrose agar media, supplemented with streptomycin at 28 °C for 5-10 days. A total of 21 and 24 endophytes were isolated from herbal plant *Moringa oleifera* and *Withania somnifera,* respectively. Microscopic study revealed four active strains of *M. oleifera* i.e. *Colletotrichum sp.* (MO-S2), *Cladosporium sp.* (MO-S4, MO-L3) and *Fusarium sp.* (MO-R1), and two active strains of *W. somnifera* i.e. *Alternaria sp.* (WS-S8) and *Fusarium sp.* (WS-R5). Active strains of endophytic fungi were further screened for their antibacterial activity against pathogenic gram negative bacterial strain of *Escherichia coli* (ATCC 25922) and gram positive strain of *Staphylococcus aureus* (ATCC25323). Our results showed that fungal isolates of *M. oleifera* (MO-S2) and *W. somnifera* (WS-S8) had antibacterial activity against both the bacterial strains. However, three fungal strains of *M. oleifera* (MO-L3, MO-S4, and MO-R1) and one of *W. somnifera* (WS-R5) showed antibacterial activity against gram negative bacterial strain of *E. coli*.**

Keywords: **Antibacterial activity, Endophytes, *Moringa oleifera*, Phytochemicals, *Withania somnifera***

I. INTRODUCTION

Microorganisms live in association with almost all plants either in symbiotic or in mutualistic form that can be categorized as endophytes. Endophytes, either fungi or bacteria survive in living plant tissues without affecting the host, similar to the gut microbiome of humans. However, initial studies have reported that only fungi living in plant tissue are referred as endophytes (Bary, 1866; Link, 1809). But later on it was also found that the colonized form of bacteria inside the plant tissues (either inter or intracellular spaces) live as endophytes and spend part of their life cycle (Chanway, 1996). Endophytes are the source of various bioactive compounds which could be used in the area of drug discovery and development.

Traditional medicines utilizes diverse range of plants for therapeutic purposes with less or almost no side effects, due to the presence of the natural bioactive substances or secondary metabolites produced from the endophytic strains (Kaul, Gupta, Ahmed, & Dhar, 2012). Fungal endophyte plays a crucial role in association with plants where it produces number of bioactive compound that promotes growth, competitiveness and protection against pathogens (Porras-Alfaro & Bayman, 2011). Wide range of adaptive ability of endophytic fungi to sustain under uneven and diverse environmental condition have attracted the attention of scientific community to explore for agricultural uses, drug development and industrial purposes (Mapperson, Kotiw, Davis, & Dearnaley, 2014; Teiten et al., 2013).

Isolation and characterization of bioactive metabolites from endophytes may have a potential effect as immunosuppressive, anticancer, antidiabetic, antioxidant, anti-inflammatory and insecticidal (Joseph & Priya, 2011). Beside these medicinal properties, endophytes have also been reported for their environment friendly behaviour. Two groups of studies have concluded that endophytes have a great capacity to degrade plastics (Abdel-Motaal, El-Sayed, El-Zayat, & Ito, 2014; Russell et al., 2011). Medicinal plants such as *Moringa oleifera* (Family: Moringaceae) and *Withania somnifera* (Family: Solanaceae) are identified with repository of endophytes with novel metabolites of immense pharmaceutical importance.

Moringa oleifera is an edible medium sized tree (height 5 to 10 m) popularly known as "sahjan" or "drumstick tree" or "horse radish tree". In African and Asian countries, it is widely used as edible plant; as the leaves, immature pods, fruits and flowers are highly nutritive (Anwar, Latif, Ashraf, & Gilani, 2007; Priyanka & Shashi, 2011; Ramachandran, Peter, & Gopalakrishnan, 1980). Besides the nutritional value, it is also known that almost all part of the plant possess numerous medicinal values. The antioxidant potential of leaves of this plant makes it unique as it contains protein, vitamin C, β-carotene, calcium (Ca^{++}) and potassium (Sreelatha & Padma, 2009). Root of this plant is very much effective in constipation, articular pains, rheumatism, lower back or kidney pain and also has a potential to act as cardiac/circulatory tonic, antilithic, vesicant, carminative, anti-inflammatory, anti -infertility, and rubefacient. Likewise, other parts of the plant such as; flower, gum, seed and stem bark also have medicinal properties. This plant is widely used in pharmaceutical industries for therapeutic purposes as anti-hypertensive, antitumor and anticancer activities, antidiabetic activity, anti-asthmatic and antipyretic, (Farooq, Rai, Tiwari, Khan, & Farooq, 2012; Igado & Olopade, 2017; Khalil, Ghaly, Diab, & El makawy, 2014; Mbikay, 2012; Onsare, Kaur, & Arora, 2013).

Similarly, *Withania somnifera* is an important tropical medicinal plant, also known as Ashwagandha (English name: winter cherry) or Indian Ginseng (Yang, Shi, & Dou, 2007) grows in sub-tropical regions. The Indian Ginseng name was given due to the broad spectrum of therapeutic uses in traditional medicine and in *Ayurveda*. The major states of India which are known to produce Ashwagandha at large scales are Punjab, Gujarat, Rajasthan, Maharashtra, Haryana, Madhya Pradesh and Uttar Pradesh. It is a perennial plant species, having height about 30-150 cm, with stout and paunchy tap-roots. It produces more than 91 pharmaceutical products along with withanoid and alkaloid which are present primarily in fleshy root and in leaves (Rai, Acharya, Singh, & Varma, 2001). *Withania somnifera* shows anticancer, antistress, antiparkinsonian, anti-inflammatory, antioxidant property (Agarwal, Diwanay, Patki, & Patwardhan, 1999; Gupta & Rana, 2007; Rasool & Varalakshmi, 2006; Umadevi et al., 2012; Yang et al., 2007). Medicinal potential of this plant is due to the presence of endophytes. Hence, it is important to explore more on these plants for endophytic fungi and their metabolites. Here, we have screened for the endophytic fungi from *Moringa oleifera* and *Withania somnifera* and later on examined the antibacterial activities of natural bioactive metabolite produced from these isolated endophytic fungi.

II. MATERIAL AND METHODS

Healthy herbal plants *Moringa oleifera* and *Withania somnifera* were randomly collected from different areas near Varanasi (25.5 °N, 82.9 °E; elevation, 279ft per 85m) during rainy, winter and summer season. Collected plant samples were properly verified and tagged. Healthy aerial tissues (leaves and stem) and underground root tissues were harvested. Selected tissues were sealed with parafilm and were kept in the sterile polythene bags within ice box followed by trimming of the tissues. Samples were brought to the laboratory for further study.

A. Preparation and Sterilization of Plant Samples

Plant tissue samples (roots, stems and leaves) apparently having no disease were cut into small pieces of approximately 1.0 cm^2 with sterile pinch cutter to fit into the containers which were later used during the sterilization procedure. Initially samples were washed in running tap water to remove large debris, and were immersed in 70% ethanol for 2-3 minutes. Surface of the samples were disinfected with 1% sodium hypochlorite (NaOCl) for 1 minute, rinsed thrice with autoclaved distilled water. Later on, samples were allowed to dry under aseptic condition.

B. Screening and Identification of the Endophytic Fungal Strains

Dried samples were placed on potato dextrose agar (PDA) plate having antibiotic streptomycin (100µg/ml) to prevent the bacterial contamination and were further incubated at 28 °C for 5-10 days until the mycelia of endophytes emerged. Samples were further processed in the lab as described previously (Ranjan, Tripathi, & Singh, 2016). A total of 21 isolates from *Moringa oleifera* and 24 isolates from *Withania somnifera* were isolated from collected sample tissues.

Identification was carried out according to the culture characteristics, spore morphology and microscopic studies of endophytic fungi. We have examined the fungal spores through inverted microscope (Dewinter: Victory FL) by oil emersion lens at 100X magnification. The specific code number was assigned to all isolates of endophytic fungi. Isolates were later preserved at -20 °C in a lyophilized form.

C. Fungal Fermentation and Metabolites Extraction

Samples were further processed in liquid state fermentation for metabolite production. Isolated fungi were grown in 100 ml Potato Dextrose broth in 250 ml flasks. Further, flasks were incubated at 28±2 °C for 3 weeks, at 150 rpm in periodical shaking BOD incubator. After 3 weeks of culture of endophytic fungi, samples were processed for the isolation of secondary metabolites using the method described by Li et al. (Li, Qing, Zhang, & Zhao, 2005) with some modifications.

D. Antibacterial Assay

Antibacterial activity of endophytic fungal extracts isolated from both *Moringa oleifera* and *Withania somnifera* were examined through disc diffusion plate method. Further, tested strains of bacteria were maintained in nutrient agar media

followed by sub-culturing. To compare the antibacterial activity in fungal extract, streptomycin (0.1µg/ml) was used as described by Devi et al. (Devi, D'Souza, Kamat, Rodrigues, & Naik, 2009).

E. Qualitative Screening of Phytochemicals

Samples of medicinal plants *M. oleifera* and *W. somnifera* were tested for several phytochemicals including alkaloids (Handunnetti, Kumara, Deraniyagala, & Ratnasooriya, 2009; Yadav & Agarwala, 2011), flavonoids (Makris, Boskou, & Andrikopoulos, 2007), polyphenols (Lapornik, Prošek, & Wondra, 2005), steroids (Harborne, 1998) and tannins (Yadav & Agarwala, 2011). Methods for their analysis have been described as follows:

1) Alkaloids

Fungal extracts were dried in boiling water bath. Dried residues were further dissolved in 2N Hydrochloric Acid (HCl). After filtration of the mixture, filtrate was divided into 3 parts with equal volumes. First part was added with few drops of Mayer's reagent while equal volumes of Wagner's reagent and Dragondroffs reagent were added to the other two parts, respectively. Appearance of creamish precipitate, brown precipitate and orange precipitate, respectively showed positive result for the presence of alkaloid.

2) Flavonoids

4 ml of deionized water and 0.3 ml of 5% Sodium Nitrite ($NaNO_2$) was added to 1 ml of extracted aliquot and was left for 5 minutes. Later on, 0.2 ml of 10% Aluminum Chloride ($AlCl_3$) was poured into the mixture and left for 5 minutes. Finally, 2 ml of 1M Sodium Carbonate (Na_2CO_3) and 2.5 ml of deionized water were added to the reaction mixture. The appearance of yellow precipitate confirmed the presence of flavonoids.

3) Polyphenols

The presence of polyphenols was confirmed by spectrophotometric Folin-Ciocalteau method. 0.2 ml crude extract was added to 1.8 ml of deionized water. Later on, 8 ml of 7.5% sodium carbonate was added followed by 10 ml of Folin-Ciocalteau reagent. The mixture was placed in water bath at 45 °C for 15 minutes. Appearance of intense blue colour indicated the presence of polyphenols.

4) Steroids

Crude extract was dissolved with chloroform in test tube, and then equal volume of concentrated Sulphuric Acid was slowly added to the mixture with the help of test tube. Due to the chemical reaction, two distinct layers appeared; upper layer appeared red while lower layer appeared as yellow with green fluorescence, indicating the presence of steroid.

5) Tannins

Treatment of fungal crude extract with alcoholic Ferric Chloride ($FeCl_3$) changed the mixture colour to bluish black. However, the colour disappeared after the addition of little diluted Sulphuric Acid with yellowish brown precipitate, confirming the presence of tannins.

III. Result and Discussion

A. Screening of Endophytic Fungal Strains

The ethanobotanical properties and the importance in Ayurvedic literature for *Moringa oleifera* and *Withania somnifera* were kept in consideration during the selection of plants. During rainy season, a total of 21 endophytes and 24 endophytes were isolated from *Moringa oleifera* and *Withania somnifera*, respectively. From *Moringa oleifera* out of 21 endophyte isolates, 8 were isolated from leaf **(38.09%)**, 6 from stem **(28.61%)** and 7 from root **(33.3%)**. From *Withania somnifera*, out of 24 isolates, 5 isolates were from leaf **(20.83%)**, 10 from stem **(41.66%)** and 9 from root **(37.50%) (Fig. 1)**.

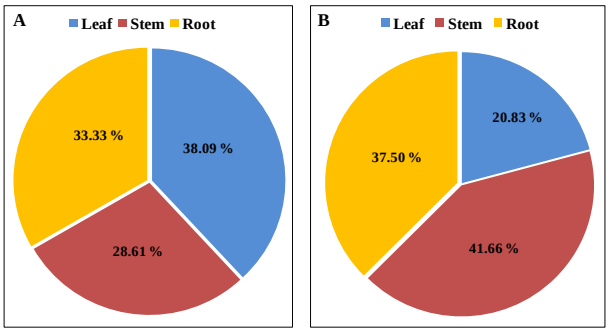

Fig. 1. Pie chart representing the percentage value of biological distribution of endophytes on aerial (stem and leaf) and underground part (root) of plants *M. oleifera* (A) and *W. somnifera* (B).

Out of 21 isolates of *M. oleifera*, only 4 isolates were showing antibacterial activity against the tested strains of pathogenic bacteria. Similarly, out of 24 isolates of *W. somnifera*, 2 isolates were showing antibacterial activity. Therefore, 4 isolates of *M. oleifera* and 2 isolates of *W. somnifera* were selected for further exploration of antibacterial activity **(Table I)**.

Table I. Details of the isolated active strains

S. No	Name of medicinal plant	No. of endophytic isolates				Name of the active isolates
		Stem	Leaf	Root	Total	
1.	*Moringa oleifera*	6	8	7	**21**	MO-L3, MO-S4, MO-S2, MO-R1
2.	*Withania somnifera*	10	5	9	**24**	WS-S8, WS-R5

The above fungal strains **(Table I)** were characterised and classified using light microscope on the basis of colony morphology. On the basis of the morphological characters three

genera in *Moringa oleifera* and two genera in *Withania somnifera* were identified. Isolated Fungal strains from *Moringa oleifera* were of *Cladosporium sp.* (MO-L3, MO-S4), *Colletotrichum sp.* (MO-S2), *Fusarium sp.* (MO-R1) **(Table IIA)**. While the fungal strains of *Withania somnifera* were of *Alternaria sp.* (WS-S8) and *Fusarium sp.* (WS-R5) **(Table IIB)**. Nomenclature of each fungal strain was done in a specific manner, e.g. '**MO-L3**' encodes for 3rd isolates of *Moringa oleifera* from leaf and **'WS-S8'** was named as 8th isolate of *Withania somnifera* from stem.

Table IIA. Endophytic fungi isolated from leaf, stem and root of *M. oleifera*

Number of isolates				
Fungal group	**Sample tissue from *M. oleifera***			
	Stem	**Root**	**Leaf**	**Total**
Alternaria sp.	-	-	-	
Cladosporium sp.	2	2	3	7
Colletotrichum sp.	3	3	2	8
Fusarium sp.	1	2	3	6
Phomopsis sp.	-	-	-	
Xylaria sp.	-	-	-	
Total	6	7	8	21

Table IIB. Endophytic fungi isolates recovered from leaf, stem and root of *W. somnifera*

Number of isolates				
Fungal group	**Sample tissue from *W. somnifera***			
	Stem	**Root**	**Leaf**	**Total**
Alternaria sp.	4	5	2	11
Cladosporium sp.	-	-	-	
Colletotrichum sp.	-	-	-	
Fusarium sp.	6	4	3	13
Phomopsis sp.	-	-	-	
Xylaria sp.	-	-	-	
Total	10	9	5	24

Further, we performed the liquid state fermentation of isolates, using potato dextrose broth medium and carried out secondary metabolites extraction using ethyl acetate. Extracts were further used for performing antibacterial activity tests.

B. Determination of Antibacterial Activity

Antibacterial activity test was performed for both the plant extracts separately and was evaluated against pathogenic gram negative bacterial strains *E. coli* and gram positive strain *S. aureus.* Out of 21 fungal isolates of *Moringa oleifera*, only one fungal isolates (MO-S2) showed antibacterial activity against both gram positive *S. aureus* and gram negative bacterial strain *E. coli*. However, other three fungal strains (MO-L3, MO-S4, and MO-R1) showed antibacterial activity against gram negative bacterial strain of *E. coli* **(Table IIIA)**.

Table IIIA. Antibacterial activity test of *M. oleifera* fungal extract against gram negative *E. coli* and gram positive *S. aureus*

S.No	**Isolates**	**Fungal groups**	***E. coli***	***S. aureus***
1.		*Alternaria sp.*		
2.	MO-L3	*Cladosporium sp.*	+	-
3.	MO-S4		+	-
4.	MO-S2	*Colletotrichum sp.*	+	+
5.	MO-R1	*Fusarium sp.*	+	-

MO: *Moringa oleifera*; R: root; S: stem; L: leaf; +: Zone of inhibition; –: No inhibition zone.

While in 24 fungal isolates of *Withania somnifera*, one fungal isolates (WS-S8) showed antibacterial activity against both gram positive *S. aureus* and gram negative bacterial strain of *E. coli*. Second fungal strain (WS-R5) showed antibacterial activity against gram negative bacterial strain of *E. coli* **(Table IIIB)**.

Table IIIB. Antibacterial activity of *W. somnifera* fungal extract against gram negative *E. coli* and gram positive *S. aureus*

S.No	**Isolates**	**Fungal groups**	***E. coli***	***S. aureus***
1.	WS-S8	*Alternaria sp.*	+	+
2.		*Cladosporium sp.*		
3.		*Colletotrichum sp.*		
4.	WS-R5	*Fusarium sp.*	+	-

WS: *Withania somnifera*; R: root; S: stem; L: leaf; '**+**': Zone of inhibition; '**–**': No inhibition zone.

In the current study, we have found some new strains of endophytic fungi such as *Colletotrichum sp.*, *Fusarium sp.* and *Alternaria sp.* from *Moringa oleifera* and *Withania somnifera*

tested against *E. coli* and *S. aureus* as compared to some of the previously published studies (Madki, Manzoor, Powar, & Patil, 2010; Mahdi, Mohamed, & Yagi, 2014).

C. Qualitative Analysis of Phytochemicals

The primary screening of active strains of fungal endophytes having antibacterial activity from *M. oleifera* and *W. somnifera* was analyzed for phytochemicals including alkaloids, flavonoids, polyphenols, steroids and tannins **(Table IV)**. Our results showed that alkaloids were present in all active fungal strains isolated from *M. oleifera* and *W. somnifera*. Flavonoids were present in all the active strains isolated from both the plants except MO-L3 strain of *M. oleifera*. Analysis for polyphenol revealed that only MO-R1 strain of *M. oleifera* was negative and rest were positive for polyphenols. Analysis for steroids was similar to that the flavonoids; present in all the active strains isolated from both the plants except MO-L3 strain of *M. oleifera*. Tannins were absent in all active strains of fungal endophytes isolated from the plants *M. oleifera* and *W. somnifera*.

Table IV. Details of the antimicrobial activity and phytoactive metabolites from the various isolates of the *M. oleifera* and *W. somnifera*

A: Alkaloids; **F:** Flavonoids; **P:** Polyphenols; **S:** Steroids; **T:** Tannins.

Name of the isolates	Fungal group	Metabolites				
		A	**F**	**P**	**S**	**T**
MO-L3	*Cladosporium sp.*	+	-	+	-	-
MO-S4	*Cladosporium sp.*	++	++	++	+	-
MO-S2	*Colletotrichum sp.*	++	+	+	+	-
MO-R1	*Fusarium sp.*	+	+	-	+	-
WS-S8	*Alternaria sp.*	++	+	++	++	-
WS-R5	*Fusarium sp.*	+	+	+	+	-

D. Morphological Identification of Active Fungal Strains

Morphology of four active strains of *M. oleifera* **(Fig. 2 & 3)** and two active strains of *W. somnifera* **(Fig. 4)** were identified using light microscope. Identification was done on the basis of morphology of spores and hyphae. On the basis of morphology active strain '**MO-S2**' was identified as of *Colletotrichum sp.* due to the presence of white copious cinnamon conidial masses usually elliptical, fast growing sparse aerial mycelium and absence of setae **(Fig. 2A & B)**. Two isolated strains, one from stem (MO-S4) **(Fig. 2C & D)** and other from leaf (MO-L3) **(Fig. 3A & B)** were of *Cladosporium sp.* because of the conidiophores having two to four celled conidia with terminal and intercalary swellings. Morphology of spore of **'MO-R1'** of *M. oleifera* was fusiform (half-moon shaped), septed and pointed at tip resembling to *Fusarium sp.* **(Fig. 3C & D)**.

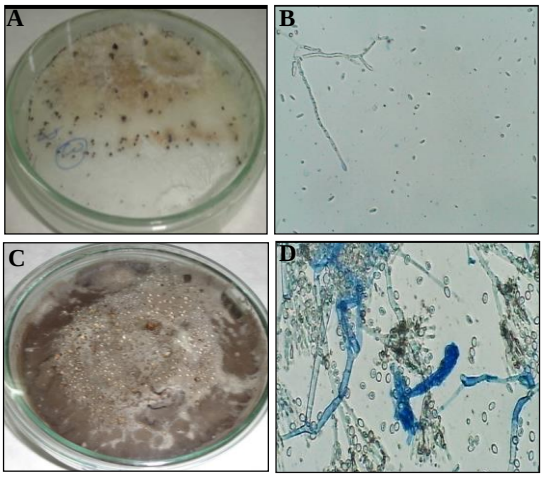

Fig. 2. Photomicrograph showing the morphology of fungal isolates of *M. oleifera* having antibacterial activity. Morphological studies revealed the identification of four fungal strains of *M. oleifera*. Two out of four fungal strains are shown in Figure 2. On the basis of the physical appearance of endophytic fungal strains, grown on PDA plate and microscopic studies of spores and hyphae, species identified were *Colletotrichum sp.* **(A&B)** and *Cladosporium sp.* **(C&D)**. **A** and **C** represents the PDA plate colony containing antibiotic streptomycin, incubated at 28 °C for 5-10 days. **B** and **D** represent the light microscopy images of the respective fungal endophytes.

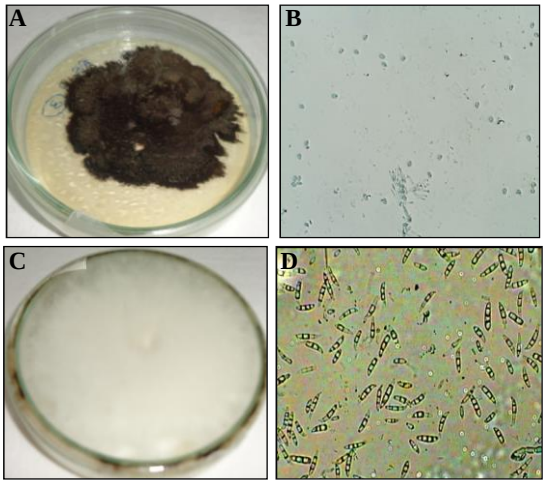

Fig. 3. Photomicrograph showing the morphology of fungal isolates of *M. oleifera* having antibacterial activity. Image is representing the morphological studies of remaining two fungal strains of *M. oleifera*. On the basis of the physical appearance of endophytic fungal strains, grown on PDA plate and microscopic studies of spores and hyphae, species identified were *Cladosporium sp.* **(A&B)** and *Fusarium sp.* **(C&D)**. **A** and **C** represents the PDA plate colony containing antibiotic streptomycin, incubated at 28 °C for 5-10 days. **B** and **D** represent light microscopy images of the respective fungal endophytes.

On the basis of the morphology of the isolated strains '**WS-S8**' from *W. somnifera* was identified as *Alternaria* species. Colonies of *Alternaria sp.* are fast growing with multicellular obclavate, obpyriform, sometime with ellipsoidal conidia and darkly pigmented hyphomycete **(Fig. 4A & B)**. The second isolate '**WS-R5'** was recognized as of *Fusarium sp.* with half septed, moon shaped and pointed spores at tip, similar to the isolates of *M. oleifera* (MO-R1) **(Fig. 4C & D)**.

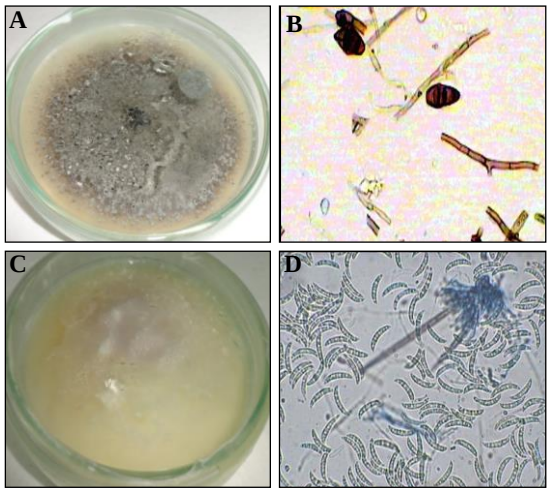

Fig. 4. Photomicrograph showing the morphology of fungal isolates of *W. somnifera* having antibacterial activity. The morphological studies revealed the identification of two fungal strains of *W. somnifera*. On the basis of the physical appearance of endophytic fungal strains grown on PDA plate and microscopic studies of spores and hyphae; species identified were *Alternaria sp.* **(A&B)** and *Fusarium sp.* **(C&D)**. **A** and **C** represents the PDA plate colony containing antibiotic streptomycin, incubated at 28 °C for 5-10 days. **B** and **D** represent the light microscopy images of the respective fungal endophytes.

Our results revealed that the isolated endophytes produce bioactive molecules which might be a good source of broad range antibacterial molecules. Our results further supported that the endophytic extract of both the plants might be a potent source with antibacterial activity.

Conclusion

In the present study, we have isolated the active strains from *M. oleifera* which are of *Colletotrichum sp.* (MO-S2), *Cladosporium sp.* (MO-S4, MO-L3) and *Fusarium sp.* (MO-R1) with antibacterial activity. Active strains isolated from *W. somnifera* are of *Alternaria sp.* (WS-S8) and *Fusarium sp.* (WS-R5) with antibacterial activity. Biochemical compounds produced from these endophytic fungi will be further tested in future using suitable *in vitro* and *in vivo* models for establishing their role as a probable drug molecule.

Acknowledgments

The authors express their gratitude towards Professor-In charge, CEMS, Institute of Medical Sciences, BHU, Varanasi for providing facilities and continuous support. The authors are also very thankful to the Science and Engineering Research Board, DST, Govt. of India for furnishing financial support (File. No. SERB/SR/SO/PS/22/2012).

Conflict of interest

The authors declare that they have no conflict of interest.

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