Isolation, Morphological And Biochemical Identification Of Endophytic Fungi From *Moringa oleifera* And Its Antimicrobial Potential

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Abstract
The present study was aimed at isolating endophytic fungi from the leaves of *M. oleifera* and analyzing its antimicrobial potential. In this study, the fungal endophyte *Acremonium* sp. was isolated identified through morphological and biochemical analysis. In IAA the strain produced 55.27 ± 3.00 µgml^{-1} IAA without exogenous tryptophan application (control) in the culture medium. The addition of 1mgml-^{1} tryptophan to the culture medium increased the IAA production to 76 ± 2.6 µgml-^{1} The production of siderophore was performed in the absence and presence of an iron source in the medium. The strain was cultured in the minimal media supplemented with different Fe(III) citrate concentrations, i.e., 0 μM, 0.25 μM, 2.0 μM, and 4.0 μM. The isolated fungi showed high siderophore production when cultured in a medium without Fe(III) citrate. Siderophore production found to be 40.00 ± 0.4 (psu) in the culture medium without the addition of Fe(III) citrate.. Siderophore production was slightly reduced , i.e., 33 ± 0.3 (psu) at 0.25 μM Fe(III) citrate in the culture medium. However, a significant reduction in the production of the siderophore was observed when Fe (III) citrate content was raised to 2.0 μM and 4.0 μM. In case of bacterial strains it was observed that maximum activity was observed against *P. aeruginosa* at 80 µg/ml (IZ-20mm) while minimum at 20 µg/ml against *E. coli* (10mm) but at 40 and 60 µg/ml activity was at par (IZ-14mm) while *S. aureus* was found to be partially resistant. In case of antifungal it was observed that when extract of PD 20 was tested maximum activity was observed at 80 µg/ml against *T. reesei* (IZ-22mm) while *C. albicans* was found to be partially resistant as initially no activity was observed against it. Against *P. funiculosum* no activity was observed at 20 µg/ml but with increase dose level activity increased.Therefore, we conclude that the isolated *Acremonium* sp. should be further investigated before utilization as a biocontrol agent and plant growth stimulator.

Keywords: Endophytic fungi; *Moringa oleifera*; IAA; Siderophore; Antimicrobial

Introduction

Plant endophytes have benn considered as vital crucial resource for the invention of novel microbes having therapeutic potential (Cabral et al., 1999). In current decades, several reports reported that a prominent varieties of endophytic fungi have been accumulated in plants, possessing a mutually valuable symbiotic rapport with their hosts (Collinge et al., 2022). Further , some endophytes have potential to assist the host to fight against the various types of biotic and abiotic stress (Manzur et al., 2022), enhance physiological development of plants (Abdalla and Matasyoh, 2014), and accumulate various natural products to defence the host plants (Mattoo and Nonzom, 2021). There are many reports which proved or demonstrated the interaction between endophytes and host plants, efficient research on the some medicinal plants and its endophytes are still to be investigated.

The endophytic fungus has now be considered auspiciously deliberate natural bioresource for the innovations of novel phytochemicals with diverse chemical moieties and flexible therapeutic applications (Fan et al., 2022). Further , it has been reported that it is rich deposits of house for discovering novel antibacterial compounds (Cruz et al., 2020).
The prerequisite of endophytic fungi extracted from medicinal plants as antimicrobial agents has proved that these plants possess prominent amount of various natural products accredited for these properties (Afolayan, 2003). In recent decades due to prototype mutants there has been noteworthy disparity in multiple resistances disease causing microbes. Due to wide spread reliance on synthetic drugs based on chemical assisted pathways the treatment of infectious diseases, there has been enormous elevation in toxicity and side effects in body metabolism. This has caused keen interest of scientific researchers towards invention of novel antimicrobial agents extracted from medicinal plants (Mahajan and Das, 2003).

Materials and Methods

Sample collection: Fresh leaves of ***Moringa oleifera*** were collected from Jaipur region (Rajasthan) in months of July, August 2017. The samples were carried in sterile polythene bags and carried for isolation of endophytic fungi under sterile conditions.

a) Isolation of Endophytic fungi: The fresh leaves from the healthy plants were subjected to endophytic isolation soon after the collection according protocol of Araújo et al (2002) The fresh leaves collected were thoroughly washed with running tap water for 45 minutes to remove any dust or soil particles and further air dried.

All the work was performed under laminar air flow hood maintaining sterile conditions. The cleaned leaves after air drying were surface sterilized by dipping in 75% ethanol for 60 sec , followed by 5% sodium hypochlorite solution for 10 minutes and then washed thrice (1minute each time) with sterile distilled water and left for drying under sterilized condition. Both the borders of the sterilized leaf segments were cut off with the help of sterile blade and about 1cm of the plant material (leaf segment) was subjected to endophytic isolation.
The small leaf segments were placed on potato dextrose agar (PDA) media plates supplemented with antibiotic gentamycin (100mg/ml) so as to avoid bacterial growth. Each plate was inoculated with 5 leaf segments. All the plates were sealed and packed with parafilm and were incubated at 25-28^0 C along with control and last rinse plates. The plates were observed daily for 15-20 days for initial growth of endophytes.
The emerging fungal hyphal tips from the plant leaf segments were picked and transferred on PDA plates to check purity of the culture. The pure cultures were maintained on PDA slants at 4^{0}C.

Submerged Fermentation and Screening of Endophytic Isolates for antioxidant potential:
All 20 fungal isolates were screened for total antioxidant potential as per the method given by Guha *et al.,* (2010). The isolates were grown in 2 different media viz., Potato dextrose broth and Malt extract broth. Their composition is as follows:

i. PDB Media
CONSTITUENTS g/litre
Potatoes-200g/L (sliced washed unpeeled Dextrose 20g /L

ii. MEB Media
Malt extract 20g /L
pH 5.8

Five discs of 6mm from a 9 day old culture of each fungal isolate were inoculated in 250 ml flasks containing 100 ml of media. The flasks were incubated 40 days at 27±2^0 C under static condition. After incubation, the mycelial growth was separated by filtration through Whatmann no.1 filter paper and the filtrate was subjected to total antioxidant assay (TAA) assay as per the method given by Guha *et al.,* (2010).

The sample tubes were prepared using various concentrations (100, 200 and 300µl) of each fungal filtrate with distil water (to make 1ml of the volume). To this 3.3 ml reaction mixture containing Sodium phosphate monobasic (28mM), Ammonium molybdate (4mM) and concentrated hydrochloric acid (0.6M) was added. All the tubes were incubated for 1hr in water bath at 95^0 C and absorbance was taken at 695nm with blank containing 1ml distilled water and 3.3ml reaction mixture.

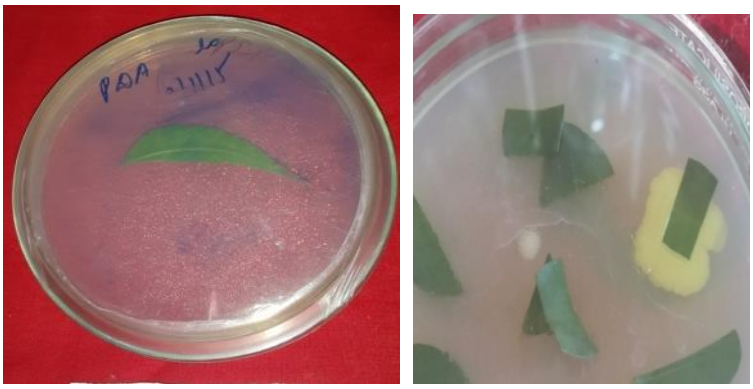
Indole Acetic Acid (IAA) Test
Indole acetic acid (IAA) in isolated endophytic fungi as been performed as per protocol of (Gordon & Weber, 1951) with slight variations (Patten & Glick, 2002) . Potato dextrose broth (PDB) was prepared with 10% tartaric acid inorder to avoid bacterial contamination. The PDB was treated with different dose levels of exogenous tryptophan, i.e., 0 mgml^{-1}, 1mgml^{-1}, 2mgml^{-1}, and 4mgml^{-1}. A fungal disc (5mm) was inoculated into 10 ml PDB, supplemented with the above-mentioned tryptophan concentrations in 50 ml flasks. Samples were incubated for 7 days at 25°C ± 1°C under dark conditions on a shaker with rotation of 120 rpm. Fungal cultures were then centrifuged at 12000 rpm for 10 min at 4°C. Further , 1 ml supernatant from each sample was amalgated with 2 ml Salkowski reagent (98 ml 35% HClO4, 2 ml 0.5M FeCl3) and was incubated in the dark for half an hour . The change of color from yellow to pink was considered as positive for IAA production. The IAA components were estimated by taking absorbance at 530nm in a spectrophotometer. The IAA quantities in samples were measured based on a standard curve of known values.

Siderophore Detection
The capacity of the endophytic fungal strain to produce siderophore was performed by Chrome Azurol S (CAS) assay, (Schwyn and Neilands , 1987).. The fungal mycelia were grown in a PDB for 7-10 days at 24°C and rotation of 120 rpm. After incubation, a fungal sample was added to Minimal Media 9 (MM9) with CAS solution and treated with different doses of iron, i.e., 0 µM, 0.25 µM, 2 µM, and 4 µM Fe(III) citrate. Samples were again incubated at 24°C for 6 days at speed of 120 rpm. After incubation, 100 µl of the blue Chromium Azurol S (CAS) reagent was added to samples followed by incubation for 4 h at room temperature. The change of color from blue to orange/yellow was recommended as positive. The amount of concentrations in all samples were further measured at 630 nm. The siderophore quantities were measured as % of siderophore units by the formula: % of siderophore units = Ar − As/Ar ∗ 100, where "Ar" is the absorbance of

reference (CAS reagent) and "As" is the absorbance of the sample at 630 nm. Qualitative detection was done on Chrome Azurol S (CAS) blue agar. The isolated strain was inoculated on CAS agar plates and incubated at 24°C under dark condition for 14 days. The appearance of yellow/orange color around the colonies confirmed siderophore production. The complete experiment was carried out in triplicates.

Results and Discussion

In the present study a total of 20 morphologically different fungal isolates were obtained from 150 leaf segments of experimental plants **(Fig. 1)**



Isolation and identification of Endophytic fungi from Leaves of *M. oleifera*

Fig. 2 Fungal growth and microscopic observation of the isolated fungal strain Ld-03. Colony cultures on potato dextrose agar incubated at 25 ± 1°C for 7-10 days

Endophytic fungal strain was identified as *Acremonium* sp. which was isolated from leaves of *M. oleifera* . The isolated strain was identified using biochemical, microscopic and molecular techniques. The isolated fungi formed compact and moist colonies with loose and cottony hyphae with white color on PDA plates at 24°C ± 1°C.

IAA test

Quantification of IAA was confirmed through a change of color from yellow to pink (Fig. S4c). The IAA quantities confirmed in the presence and absence of exogenous tryptophan **(Table 1).** The strain produced 55.27 ± 3.00 μgml^{-1} IAA without exogenous tryptophan application (control) in the culture medium. When tryptophan was supplemented to the medium, the IAA content enhanced, thus confirming that tryptophan could enhance the IAA production. The addition of $1mgml^{-1}$ tryptophan to the culture medium increased the IAA production to 76 ± 2.6 μgml^{-1}. However, the concentration and amount increased in ratio of each other thus both were found to be dependent. The strain accumulated 125 ± 5.00 and 170.5± 6.12 μgml^{-1} IAA at $2mgml^{-1}$ and $4mgml^{-1}$ of tryptophan respectively.

Table 1. Quantification of IAA Production

Tryptophan concentration (in mg/ml)	Quantity of IAA (µg/ml)
0	55.27 ± 3.00
1	76 ± 2.6
2	125 ± 5.00
4	170.5± 6.12

Siderophore Production

The isolated endophytic fungal isolated were screened for its potential to produce siderophore **(Table 2).** The production of siderophore was performed in the absence and presence of an iron source in the medium. The strain was cultured in the minimal media supplemented with different Fe(III) citrate concentrations, i.e., 0 μM, 0.25 μM, 2.0 μM, and 4.0 μM. The isolated fungi showed high siderophore production when cultured in a medium without Fe(III) citrate. Siderophore production found to be 40.00 ± 0.4 (psu) in the culture medium without the addition of Fe(III) citrate. However, the production of the siderophore in the culture medium reduced tremendously with the supplementation of various

quantities of Fe(III) citrate. Siderophore production was slightly reduced , i.e., 33 ± 0.3 (psu) at 0.25 μM Fe(III) citrate in the culture medium. However, a significant reduction in the production of the siderophore was observed when Fe (III) citrate content was raised to 2.0 μM and 4.0 μM.. These results revealed the fact that the isolated fungi can produce siderophore in limited iron source in the medium. A qualitative test further confirmed the production of siderophore by forming a yellow/orange color surrounding the fungal colonies. On Chrome Azurol S (CAS) agar plates, the fungi produced a visible yelloworange color that averaged about 1.7cm in radius . The ability of siderophore production is confirmation that the isolated fungi may enhance and promote plant growth partly by providing the rarely available iron sources in the soil

Table 2 . Quantification of Siderophore Production (psu)

Fe (III) Citrate concentrations (in µM)	**Siderophores (psu)**
0	40.00 ± 4
0.25	33 ± 0.3
2	19± 0.21
4	0.8± 0.08

Antimicrobial activity

It was observed that when PD isolate was tested against various clinically important microbes it showed potent activity against both bacterial and fungal strains.

In case of bacterial strains it was observed that maximum activity was observed against *P. aeruginosa* at 80 µg/ml (IZ-20mm) while minimum at 20 µg/ml against *E. coli* (10mm) but at 40 and 60 µg/ml activity was at par (IZ-14mm) while *S. aureus* was found to be partially resistant (**Table. 3 and 4**).

Similar observations were also observed in case of fungal strains. It was observed that when extract of PD 20 was tested maximum activity was observed at 80 µg/ml against *T. reesei* (IZ-22mm) while *C. albicans* was found to be partially resistant as initially no activity was observed against it. Against *P. funiculosum* no activity was observed at 20 µg/ml but with increase dose level activity increased.

Table 3. Antibacterial activity of PD20 (1mg/ml) fungi extract

Concentration (in µg/ml)	***E.coli***	***Pseudomonas eruginosa***	***Staphylococcus aureus***
20	IZ-10±0.009	IZ- 12±0.009	Nil
40	14±0.01	IZ-14±0.01	Nil
60	14±0.01	IZ-16±0.012	10 ±0.01
80	18±0.01	IZ-20±0.03	12 ±0.03
Standard (Gentamycin)	IZ -22	IZ -22	IZ -22

Table 4. Antifungal activity of PD20 (1mg/ml) extracts

Concentration (in µg/ml)	***Penicillium funiculosum***	***Candida albicans***	***Trichoderma reesei***
20	Nil	Nil	16±0.04
40	IZ-6±0.05	Nil	18±0.04
60	IZ-10±0.1	IZ-12±0.04	20±0.04
80	IZ-12±0.2	IZ-16±0.06	22±0.04
Standard (Ketokenazole)	IZ -20	IZ-20	IZ -20

The physiological role of endophytic fungi isolated from plant and their mechanism of communication with the host and other endophytes and organisms associated with the plant flora is yet to be elucidated (Strobel, 2018). Further , the microbial diversity which are having their habitat in different plant species, along with the variety of the natural products that endophytic fungi synthesize, creates the opportunity for the innovation of new natural products with various biotechnological roles (Fadiji and Babalola, 2020). Further , various reports have been proved the importance of endophytic microorganisms in host endurance, since endophytes directly control bioactive compounds like , resist extreme temperatures and scarcity of water , along with occurrence of phytopathogens (Xia et al., 2022). Therefore, the conventional application of the plant and the region in which it harbor are crucial criteria for reproduction of endophytes (Santos et al., 2015).

In the present study a total of 20 morphologically different fungal isolates were obtained from 150 leaf segments of experimental plants *M. oleifera*. Based on biochemical, morphological and molecular sequencing endophytic fungal strain was identified as *Acremonium* sp. which was isolated from leaves of *M. oleifera* . The isolated strain was identified using biochemical, microscopic and molecular techniques. The isolated fungi formed compact and moist colonies with loose and cottony hyphae with white color on PDA plates at 24°C ± 1°C. The fungus produced cylindrical-shaped conidia.

In biochemical test The IAA quantities confirmed in the presence and absence of exogenous tryptophan. The strain produced 55.27 ± 3.00 µgml^{-1} IAA without exogenous tryptophan application (control) in the culture medium. When

tryptophan was supplemented to the medium, the IAA content enhanced, thus confirming that tryptophan could enhance the IAA production. The addition of 1mgml-^{1} tryptophan to the culture medium increased the IAA production to 76 ± 2.6 µgml-^{1}. However, the concentration and amount increased in ratio of each other thus both were found to be dependent. The strain accumulated 125 ± 5.00 and 170.5± 6.12 µgml^{-1} IAA at 2mgml^{-1} and 4mgml-^{1} of tryptophan respectively.

In another test the isolated endophytic fungal isolated were screened for its potential to produce siderophore. The production of siderophore was performed in the absence and presence of an iron source in the medium. The strain was cultured in the minimal media supplemented with different Fe(III) citrate concentrations, i.e., 0 μM, 0.25 μM, 2.0 μM, and 4.0 μM. The isolated fungi showed high siderophore production when cultured in a medium without Fe(III) citrate. Siderophore production found to be 40.00 ± 0.4 (psu) in the culture medium without the addition of Fe(III) citrate. However, the production of the siderophore in the culture medium reduced tremendously with the supplementation of various quantities of Fe(III) citrate. Siderophore production was slightly reduced , i.e., 33 ± 0.3 (psu) at 0.25 μM Fe(III) citrate in the culture medium. However, a significant reduction in the production of the siderophore was observed when Fe (III) citrate content was raised to 2.0 μM and 4.0 μM.. These results revealed the fact that the isolated fungi can produce siderophore in limited iron source in the medium. A qualitative test further confirmed the production of siderophore by forming a yellow/orange color surrounding the fungal colonies. On Chrome Azurol S (CAS) agar plates, the fungi produced a visible yellow orange color that averaged about 1.7cm in radius . The ability of siderophore production is confirmation that the isolated fungi may enhance and promote plant growth partly by providing the rarely available iron sources in the soil.

The antimicrobial potency of EtOAc extracts of each isolated fungal extracts were screened out . The results of antibacterial efficacy after analyzing of fungus extract with dose level of 5% against *S. aureus*, *E. coli*, and *C albicans* have been reported . The EtOAc extract possessed maximum antibacterial efficacy against the growth of *S. aureus* and *E. coli* has been identified as RTKB7 possessing average IZ of 19.90 ± 0.34 and 18.61 ± 0.87 mm, respectively. The RTKB3 isolate had an average IZ of 11.29 ± 1.17 and 10.69 ± 0.56 mm. In disparity, the RTKB4 fungi had an average IZ of 9.71 ± 0.56 and 10.15 ± 0.61 mm.

In the present investigation it was observed that when PD isolate was tested against various clinically important microbes it showed potent activity against both bacterial and fungal strains. In case of bacterial strains it was observed that maximum activity was observed against *P. aeruginosa* at 80 µg/ml (IZ-20mm) while minimum at 20 µg/ml against *E. coli* (10mm) but at 40 and 60 µg/ml activity was at par (IZ-14mm) while *S. aureus* was found to be partially resistant. Similar observations were also observed in case of fungal strains. It was observed that when extract of PD 20 was tested maximum activity was observed at 80 µg/ml against *T. reesei* (IZ-22mm) while *C. albicans* was found to be partially resistant as initially no activity was observed against it. Against *P. funiculosum* no activity was observed at 20 µg/ml but with increase dose level activity increased.

Wang et al ., 2023 studied isolation, classification, and antimicrobial efficacy of endophytic fungi from Gannan navel orange. They reported a total of 54 strains of endophytic fungi extracted from the pulp, peel, twig, and leaf of Gannan navel orange; tgus on the based on biochemical test they were identified and classified as 17 species of 12 genera. All these strains were fermented in potato-dextrose agar (PDA) medium, and their bioactive compounds were then isolated and purified with ethyl acetate (EtOAc). The antibacterial efficacy of *E. coli*, methicillin-resistant *S. aureus* (MRSA), and *Xanthomonas citri* subsp. citri (Xcc) were also screened of the EtOAc extracts of these strains.

Conflict of interest

Authors declare that there is no conflict of interest

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