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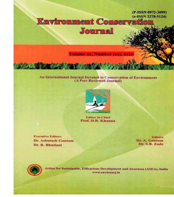

Molecular characterization of selected fungal and bacterial endophytes in acid lime

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ABSTRACT

Endophytes are the microorganisms that are present in living tissue of various plant parts (roots, fruits, stem, seed, leaf *etc*,). Endophytic microorganisms are good source of antibiotics. Endophytic antagonists were isolated from the roots of healthy acid lime plants collected from major acid lime growing areas of Andhra Pradesh. A total of 8 fungal and 10 bacterial endophytic antagonists were isolated. The antagonists were further subjected to preliminary screening, out of which only 6 endophytic fungal antagonists (EFA 1-6) and 8 endophytic bacterial antagonists (EBA 1-8) isolates showed good inhibitory effect on radial growth of *Fusarium solani* causing dry root rot in acid lime *in vitro*. Among them the one of the best fungal and bacterial antagonists that were found to be extremely efficient against *Fusarium solani* in dual culture assay were selected for further molecular identification. The BLAST results revealed that one of the fungal isolate had shown 100% similarity with *Aspergillus fumigatus* and one of the bacterial isolate had shown 95.56% similarity with *Pseudomonas aeruginosa*.

Introduction

Acid lime (*Citrus aurantifolia* Swingle) is one of the largest and most important fruits of tropical and subtropical regions. India is the largest producer of acid lime in the world. Fungi and bacteria are two types of beneficial endophytic microbes that invade internal plant tissues without harming their hosts visibly (Petrini, 1991 and Gouda *et al.,* 2016). They differ from epiphytic microorganisms, which reside on the surface of plant organs and also within the plant tissues, like which they are not harmful, do not infect plants, and do not cause diseases (Hallmann *et al*., 1997). Endophytic microbes are also capable to produce antimicrobial metabolites and several antimicrobial products were extracted from various plants for various pathogens. These microorganisms were found to be effective, environmentally safe and promising biotic tools in plant disease management. In our study, the endophytes were tested against dry-root rot pathogen *Fusarium solani* in acid lime. A roving survey was conducted to isolate endophytic antagonists from roots of healthy acid lime plants. Isolation of endophytic microorganisms needs the elimination of epiphytic contaminants present on the roots' outer surface. Hence, first the roots were surface sterilized followed by isolation (Araujo *et al.,* 2002). In the present investigation, the sterilization was done using two per cent sodium hypochlorite solution for 5 min with slight changes from the method followed by Saini *et al.,* (2016). The surface sterilized samples were blot dried after washing thrice in sterile water. The sterilized healthy roots were triturated with 8 ml of sterile 0.1M Potassium phosphate buffer (pH -7.0) using a

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sterile mortar and pestle. The triturate was serially diluted in sterile water blanks up to 10^{-7}. One ml of the final buffer wash was pipetted out onto a sterile petri plate with a specified growth medium.

A total of 18 endophytic antagonists were isolated, among which 8 were fungal and 10 were bacterial. On further *in vitro* evaluation, 6 fungal and 8 bacterial endophytic antagonists showed inhibitory effect on the radial growth of the *Fusarium solani* causing dry root rot in acid lime. The isolates EFA 4 and EBA 7 were found to be highly efficient against *Fusarium solani* in dual culture with 66.92% and 63.42% inhibition over control and these isolates were selected for further molecular identification.

Material and Methods

The effective endophytic antagonists EFA 4 and EBA 7 were selected for molecular identification after proper *in vitro* antagonistic assays.

Molecular identification of endophytic fungal and bacterial antagonists

DNA extraction from endophytic fungal and bacterial antagonists

Genomic DNA was isolated from mycelial mat of fungus and single colony of bacterial culture following CTAB method (Li and Yao, 2005; William *et al*., 2012).

PCR amplification and sequencing for endophytic fungal antagonists

The isolated DNA was amplified with universal primers ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) in PCR (White *et al.,* 1990). The PCR initial step was kept at 94°C for five minutes, a denaturation step at 94°C for 60 seconds, an annealing step at 55°C for one minute, an extension step at 72°C for 1.5 minutes and a final extension at 72°C for 5 minutes followed by cooling at 4°C for 30 seconds and repeated for 34 cycles.

PCR amplification and sequencing for endophytic bacterial antagonists

The universal primers 27F (AGAGTTTGATC CTGGCTCAG) and 1492R (GGTTACCTTG TTACGACTT) were used for the amplification of bacterial DNA in PCR (Lane, 1991; Stackebrandt and Liesack, 1993). The PCR was done as an initial step for 5 minutes at a temperature of 95°C, a denaturation step at 95°C for 60 seconds, an annealing step for 1 minute at 56°C, an extension step at 72°C for 1.5 minutes and a final extension for 10 minutes at 72°C followed by cooling at 4°C for 30 seconds and repeated for 30 cycles.

Quantification of Genomic DNA

The total obtained genomic DNA concentration was measured using U.V. Spectrophotometer Nanodrop (ND-1000). Blank was kept against millique water. The optical density was measured at 260 nm to determine the DNA concentration. DNA concentration and optical density were related as follows.To figure out the ratio OD260/OD280, the optical density (O.D.) will be measured at 280 nm. The ratio is thought to be optimal around 1.8, which indicates ideal DNA preparation. A score above 1.8 indicates that the sample contains more RNA, whereas a ratio below 1.8 suggests the presence of proteins in the preparation (Moges *et al*., 2017; Ratanacherdchai *et al*., 2007).

Loading of agarose gel

Gel plates were carefully cleaned using a cleaning agent, then rinsed with distilled water and dried. The plates were sealed with cellophane tape at the two open sides. Then ethidium bromide (1.5 µl) was added to the gel at hand tolerable heat. After that, the solution was put into the gel plate (with a comb) and left to polymerize.

Loading and gel electrophoresis

The inserted comb was delicately removed from the gel after polymerization. The tank of the horizontal electrophoretic apparatus was filled with 1X TBE buffer and the gel plate was set within. With the help of micropipettes, the samples were loaded in the wells. Loading dye of 5 µl was added with the help of micropipettes into each DNA sample and mixed well. After loading, a power pack with a 100V regulated electric power source was connected to the electrophoretic unit. After the gel run was completed, the gel was gently removed, and the gel image was examined on a U.V. transilluminator Gel doc (Alpha Innotech Multi image light cabinet filter positions) and stored in gel documentation system. Gene Ruler100-bp plus DNA ladder (© 2012 Thermo Fisher Scientific Inc.) was used as a molecular weight marker.

Results and Discussion

PCR amplification

In molecular characterization, the DNA obtained from the effective endophytic fungal antagonists EFA 4, was amplified with universal primers ITS 1 and ITS 4, which resulted an amplicon size of 540 to 580 bp fragment of DNA. Further confirmation of pathogen was done by DNA sequencing. The molecular characterization of effective endophytic bacterial antagonists EBA 7 was done using universal primers, 27 F and 1492 R and the DNA was amplified using the universal primers. This resulted an amplicon size of 1000 to 1160 bp, fragment of DNA. The band of DNA pertaining to effective endophytic microbes formed during the gel electrophoresis were displayed in Fig. 1.

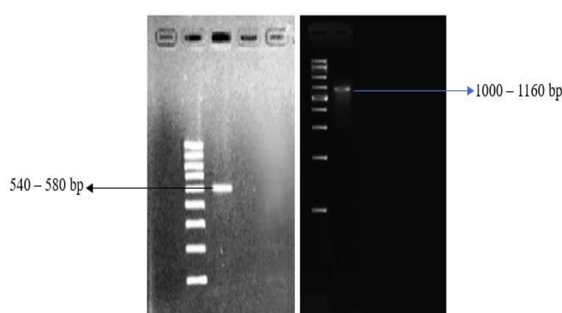

Fig 1. Amplification of DNA of Endophytic Fungal Antagonist (EFA 4) at 540 to 580 bp (left) and Endophytic Bacterial Antagonist (EBA 7) at 1000 to 1160 bp (right)

DNA sequencing

Aspergillus fumigatus (EFA 4)

The BLAST results showed 100% similarity with *Aspergillus fumigatus*. The nucleotide sequence of ITS region of isolate *Aspergillus fumigatus* were submitted to Gen bank under accession number - MN209960. Based on nucleotides homology and phylogenetic analysis the endophytic microbe EFA 4 has shown maximum identity with *Aspergillus fumigatus* strain ZC-2 (Gen Bank Accession Number: MK630344.1). *Aspergillus fumigatus* was reported as an endophytic fungus earlier in *Juniperus communis* L. Horstmann (Kusari *et al*., 2009), *Cynodon dactylon* (Liua *et al*., 2004), *Moringa oleifera* (Abonyi *et al*., 2018). Kumar *et al.* (2012), Savitha and Sriram (2015) and Kannangara *et al*. (2017) also characterized the *Trichoderma* spp. by using universal primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and in order to know their antagonistic activity against root rot and foliar pathogens. Zihad *et a.* (2022) identified five *Aspergillus* spp. from Sundarbans forest trees - *Ceriops decandra* and *Avicennia officinalis* using ITS1 and ITS4 primers. Similar findings were done by Singh *et al.* (2020) where they isolated and identified 20 types of fungal endophytes from *Argemone Mexicana* using ITS1 and ITS4 primers. They identified that the endophytes belonged to *Aspergillus* and *Penicillium* spp. Also, Schoch *et al.,* (2012) stated that ITS regions were used frequently as phylogenetic markers for identifying fungi. There have been numerous molecular characterization studies conducted to identify the fungal endophytes from various medicinal plants (Chen *et al.,* 2011; Bhagat *et al.,* 2012 and Yoo and Eom, 2012). Recently, Al-badi *et al.* (2020) characterized five fungal endophytes isolated from Shirazi Thyme using the primers ITS5 (5’-GGAAGTAAAAGTCGTAACAAGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) and identified them as *Nigrospora sphaerica* (E1 and E6 isolates), *Polycephalomyces sinensis* (E8 and E10 isolates), and *Subramaniula cristata* (E7 isolate).

Pseudomonas aeruginosa (EBA 7)

The BLAST results showed 95.56% similarity of the isolate with *Pseudomonas aeruginosa*. Based on nucleotides homology and phylogenetic analysis the endophytic microbe EBA 7 has shown maximum identity with *Pseudomonas aeruginosa* strain GIMC5015 (Gen Bank Accession Number: CP034429.1). Besides them, based on morphological and physiological characteristics, as well as 16S rRNA gene sequence analysis, the plant growth-promoting bacterial endophyte AL2-14B that was isolated from the leaves of *Achyranthes aspera* L. was identified as *Pseudomonas aeruginosa* (Khaidem, A.D. *et al*., 2017). *Pseudomonas aeruginosa* was identified as the endophytic phosphate-solubilizing bacteria EPR13 that was isolated from the aerial tissues of *Achyranthes aspera* L. (Misra *et al.,* 2012). Similarly, Hassan *et al.* (2016). Amaresan *et al.* (2014) isolated and characterized the beneficial bacteria associated with chilli at molecular level by 16 s rDNA sequencing. The similar line of work was done by Singh *et al*. (2015) on molecular identification and characterization of rhizospheric bacteria, by PCR based 16S rRNA gene

sequencing. Also, Uzair *et al*. (2018) isolated a *Pseudomonas* strain PS24 from soil samples of Balochistan coastline and identified it as *Pseudomonas aeruginosa* by 16srRNA sequence analysis.

Conclusion
In the study, we identified a fungal and a bacterial endophytic antagonist as *Aspergillus fumigatus* and *Pseudomonas aeruginosa,* respectively which were found to be effective in controlling *Fusarium solani* (a dry-root rot causing pathogen in acid lime) under *in vitro*. Further investigations determine their effectiveness under *in vivo* conditions.

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Conflict of interest
The authors declare that they have no conflict of interest.

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