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Molecular Characterization of Effective Biocontrol Agent against Aspergillus Flavus Causing Yellow Mold in Groundnut (Arachis *HYPOGEA* L.)

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Abstract: ***Isolation of the rhizosphere microflora from the groundnut fields was done by serial dilution method and the microorganisms isolated were screened for antagonistic effect against Aspergillus flavus. Two fungal strains namely Penicilliumsp, and Trichodermaasperellum and four bacterial strains namely Pseudomonas fluorescence, Bacillus megaterium, Bacillus subtilis and Bacillus sp. were isolated and identified through morphological and biochemical characteristics from rhizosphere of groundnut. Trichodermaasperellum among fungal isolate and Pseudomonas fluorescence from bacterial isolate showed more antagonistic effect against Aspergillus flavus and thus proved to be potent biocontrol agents .Of the two potent biocontrol agents, Trichodermaasperellum showed maximum inhibitory zone against Aspergillus flavus and hence molecular characterization of isolate of Trichodermaasperellum was carried out. These studies revealed that revealed 99.84% similarity with ITS sequence of T. asperellum (Gen Bank Accession No. KY225658.1) when compared with GenBank Database using DDBJ BLAST search programme.***

Keywords: Groundnut, *Aspergillus flavus*, Yellow mold, Molecular characterization, IT sequences, *Trichodermaasperellum*, antagonism

1. Introduction

Introduction Groundnut (*Arachis hypogea* L.) is one of the oilseed crops cultivated in the tropical and subtropical regions of the world. It is the thirteenth most important food crop of the world and third most important oil seed crop used for vegetable oil production. The groundnut, being a highly nutritive crop is prone to attack by numerous diseases. More than 55 pathogens including viruses affect this crop causing a great loss in the yield. Of the seed borne and soil borne diseases, collar rot, stem rot and dry root rot cause severe seedling mortality resulting in patchy crop stand and reduce the yield from 25-40%. Apart from these, aflatoxin is one of the major problems, produced in the infected groundnut seeds by *Aspergillus flavus* Link ex fries and *Aspergillus parasiticus* Speare, particularly at the end of season under drought conditions (Diener *et al.,* 1987).Groundnut producers in both developing and developed countries with advanced agriculture have found it almost impossible to meet above regulations as Aspergillus spp. can infect and produce aflatoxin at various stages of the cropping period including pre-harvest, post-harvest, and storage**.** Hence, it is necessary to take precautionary measures before sowing until it reaches the end users.

Aid of molecular technique for accurate identification to explore the integrated approach will be a best solution to address this problem. In this context, species specific primers used for accurate. The present paper will highlight the molecular characterization of potential biocontrol agent against *Aspergillus flavus* producing yellow mold disease in groundnut.

2. Materials and Methods

Isolation of microflora

Biocontrol agents were isolated from groundnut seeds by using direct plate technique (Aneja, 2003). Groundnut seeds were obtained from farmers for isolation of seed microflora. The seeds were surface sterilized with 1% sodium hypochlorite for 3 minutes. Then the seeds were rinsed three times with sterile distilled water to remove the disinfectant, dried on sterile paper towels and plated on selective media such as King's B medium and potato dextrose agar (supplemented with streptomycin sulphate) for growth of bacteria and fungi respectively. The nutrient agar containing plates were incubated at 30^0C for 48 h for bacterial growth and PDA containing plates were incubated at 27^0C for 7 days for fungal growth. After incubation, colonies were picked from dilution plates and maintained as pure cultures in their respective agar slants with periodic transfers to fresh medium for further studies.

Identification of Bio-control agents

Identification was done based on cultural and morphological characteristics of pure cultures of the isolates. The visual and microscopic examinations were done to determine the genus and later identification keys and illustrated manuals were used for species separation. For fungal isolates, micro slides of each culture were prepared in lactophenol-cotton blue, examined under the microscope, observed their morphological characters and identified with the help of the keys provided by Ellis (1971), Booth (1971) and Subramanian (1971) in their representative manuals besides consulting relevant published literature. The measurements of the spores and vegetative structures were taken with the help of an ocular micrometer.

Screening of antagonistic effect against the pathogen

All the fungal and bacterial isolates obtained from the rhizosphere samples were initially screened by dual culture technique for antagonism *in vitro* against the pathogen, *A. flavus*. Twenty ml of prepared PDA was poured into petriplates and allowed to solidify. After solidification of PDA media, 5 mm diameter mycelia disc from the margins of 7 day-old culture of pathogen, *A. flavus* and the biocontrol agents were placed on the opposite ends of the plate at equal distance from the periphery on the same day. The plates

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were incubated at 28^{0}C and checked daily until the fungal growth on the control plate (inoculated only with *A. flavus*) reached the edge of the plate. Three replications were maintained in each treatment, and for each replication three plates were used. The *in vitro* assay was repeated twice to short list the biocontrol agent. Based on *in vitro* screening, the potential isolate was selected and maintained on PDA slants for further study.

Molecular identification of isolates of Trichoderma sp.

An efficient biocontrol agent, *Trichoderma*sp obtained from screening was identified by 18S rDNA sequence. The fungal culture was identified to its nearest species based on ITS sequence. Genomic DNA was isolated from the sample provided by the scientist.The ~600bp, ITS fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced bi-directionally. The sequence data was aligned and analyzed to identify the fungi and its closest neighbours.

Genomic DNA extraction

For genomic DNA extraction, a pure fungal culture, growing on PDA, was used as a source of DNA after incubation at 25^{0}C using the BioGene kit method. Squares of the cultured mycelia (0.5 cm^2) were cut from one week old cultures. The agar was scraped from the bottom of the pieces to exclude as much agar as possible. The pieces were ground in the presence of dry ice using a mortar and pestle. The genomic DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in sterile double distilled water and 1 µl samples of this solution were used for PCR amplification. The polymerase chain reaction (PCR) primers ITS-4 and ITS-5, developed by White *et al*. (1990) were used to amplify the internal transcribed spacer regions of ribosomal DNA, which encompass the 18S rDNA gene and both ITS-1 and ITS-2 regions.

The reaction mixture contained 50 µl of 1U Taq DNA polymerase, 5 µl of 10×PCR buffer (10mM Tris HCl, pH 8.3, 500 mMKCl, 15mM MgCl2), 160 µM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 10pmoles of each ITS-5 and ITS-4 primers, 2 µl of 5M betaine and 50 ng of genomic DNA. The final volume (25 µl) was adjusted using PCR-grade double distilled water (Fisher Scientific). The PCR amplification was performed in a thermocycler (eppendorfPvt. Ltd). Cycle parameters consisted of an initial denaturation at 94^{0}C, 55^{0}C and 72^{0}C for 30, 45 and 60 seconds, respectively, and a final extension step of 7 minutes at 72^{0}C was included. The resulting PCR products were checked on 0.8% agarose gel electrophoresis and purified with QIAquick spin column (QIAGEN) following the manufacturer's instructions.

PCR product of ITS-amplified region containing ITS-1, 18S rDNA and ITS-2 was directly sequenced using ITS-5 (forward primer) and ITS-4 (reverse primer) by using the ABI PRISM TM Bigdye Terminator Cycle Sequencing kit, Version 3.1 (Applied Biosystems Inc.) and analysed on an ABI prism 3730XL automated DNA sequencer (Applied Biosystems Inc.). The sequence data obtained from ITS-4 reverse primer was inversed using gene doc software and clubbed with sequence data of ITS-5 to obtain complete sequence of amplified ITS product. The 18S rDNA gene sequence alignments were performed using Clustal × 1.83 software (Thompson *et al*., 1997).Electrophoresis was carried at 70 V for 1.0 hr. The gel was observed under UV light and documented using gel documentation unit.

Sequences obtained were submitted to GenBank on the DNA data base of Japan DDBJ/EMBL/GenBank databases website (https://www.ddbj.nig.ac.jp/). The Sequences obtained in this study were compared to the GenBank data base using BLAST software on ther DNA data base of Japan DDBJ/EMBL/GenBank databases.

3. Results and Discussion

The fungi and bacteria associated with groundnut rhizophere soil were isolated using serial dilution method. From this, two fungal strains and three bacterial strains were obtained. All the isolates were identified based on their morphological characters. The two fungal strains are identified by their morphology as Penicillium sp., and *Trichodermaasperellum* respectively.

Colonies of the fungus grow well to a diameter of 5.0-6.0 cm in 7 days as dull green coloured, powdery masses (Fig. 1). The reverse of the colony appeared purple red colour. Conidiophores arised from substratum, smooth, variable in length, upto 200 µm long, 2.3-3.3 µm wide, biverticillate and symmetrical. Metulae were 5-10, measured 9.3-14.0 x 2.3-1.3 µm in size. Sterigmata were lanceolate and 10.5-14.0 x 1.5-2.3 µm. conidia were smooth, variable in dimensions, elliptical to ovate or sub-globose and 3.0-4.5 x 2.3-3.1 µm in size (Fig.2).

Figure 1

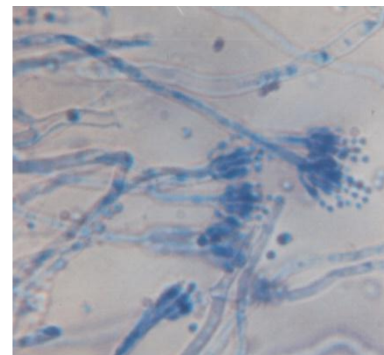

Figure 2

***Trichodermaasperellum*:**

The fungus grows rapidly with immense vegetative mycelium. Colonies were white at first, later turned to light-green to deep green due to conidial mass (Fig. 3).

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Conidiophores were distinct from vegetative hyphae, indefinite in length and dichotomously or trichotomously branched. Conidia were born in groups, green, smooth, thick-walled, globose or ovoid, 3.0-4.0 μm in diameter or 3.0-5.0 x 2.5-3.5 μm in size (Fig. 4).

Figure 3

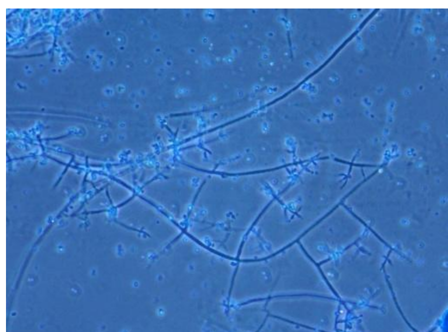

Figure 4

Screening of Antagonistic Effect against the Pathogen: All the fungal and bacterial isolates obtained from the rhizosphere sample were screened by dual culture technique for antagonism i*n vitro* against the pathogen, *A. flavus*. *In vitro* studies on the dual culture in agar plate against *Aspergillus flavus* revealed that *Trichodermaasperellum* inhibited maximum (Fig. 5) followed by *Pseudomonas fluorescence* (Fig. 6).

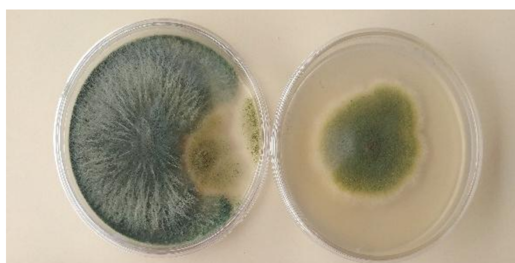

Figure 5

Figure 6

The *Pseudomonas fluorescens* shows inhibitory activity against *Aspergillus flavus*. The inhibition is thought to be because of the production of secondary metabolites or some antimicrobial compounds (Anjaiah*et al*., 2003).

Trichodermaasperellum isolated from rhizosphere soils was screened for antagonistic against *A. flavus* inhibited maximum in dual culture agar plate by observing maximum inhibition and was selected for further studies. Preliminary study on morphology of the fungus suggested that the fungus is from *Trichodermaasperellum*.

The identity of the fungus was confirmed through internal transcribed spacer-polymerase chain reaction (ITS-PCR) technique, where the amplified products yielded around 615-bp (Fig. 7). The fungi was found to be most similar to Trichoderma sp. isolate SDAS203489 small subunit ribosomal RNA gene, partial sequence Sequence ID: MK870960.1.The next closest homologue was found to be Trichoderma sp. isolate yi1447_1 small subunit ribosomal RNA gene Sequence ID: MH284811.1

Subsequently, 18S rRNA gene sequence based phylogenetic tree showing the relationships between the test strain *Trichodermaasperellum* and selected representatives of the genus *Trichoderma* is given (Figure 20). It is evident from phylogenetic analysis of 18S rRNA gene that the isolate represents a genomic species in the genus *Trichoderma*. The resulting sequence was compared to the GenBank database using the DDBJ search program. BLAST analysis of the 615-bp amplicons showed 99.84% similarity with ITS sequence of *Trichodermaasperellum* (GenBank Accession No. KY225658.1). Sequence from this study was submitted to DDBJ GenBank database (**Genbank Accession No. LC500131**). However, Gajera and Vakharia (2010) found a relationship between RAPD polymorphisms of *Trichoderma* isolates and their antagonism against *Aspergillus niger*.

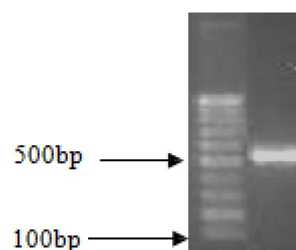

Figure 7: Agarose gel showing a single band about 615 bp.

Molecular characterization of the potent biocontrol agent was studied. Of the two potent biocontrol agents, *Trichodermaasperellum* showed maximum inhibitory zone against *Aspergillus flavus* and hence molecular characterization of isolate of *Trichodermaasperellum* was carried out. These studies revealed that revealed 99.84% similarity with ITS sequence of *T. asperellum* (Gen Bank Accession No. KY225658.1) when compared with GenBank Database using DDBJ BLAST search programme.

4. Acknowledgment

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