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Isola tion and molecular identification of fungi present in soil samples of chick Pea (*Cicer areitinum*) crop in Allahabad region

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Abstract
Chick pea is an important pulses crop in India. It is grown mostly during rainy seasons; however development of short duration and disease resistant varieties led its cultivation during spring season in almost all parts of country. The worldwide number of pathogens increases from 49-172 nearly, of which 35 have been recorded in India. Chickpea is reported to be attacked by about 67 fungi, 3 bacteria, 22 viruses and Mycoplasma and 80 nematodes. These pathogens originate from the groups of bacteria, fungi, viruses, Mycoplasma and nematodes. Among, fungal incidence causes more than 35-55% yield loss on Chick pea plant. More emphasis is needed on disease management of this crop as; this field lacks attention in spite of the importance it renders to the masses. To combat such problems, accurate and robust detection and quantification of fungi is essential for diagnosis, modeling and surveillance. Also direct detection of fungi enables a deeper understanding of natural microbial communities, particularly as a great many fungi are difficult or impossible to cultivate. In the last decade, effective amplification platforms, various quantitative PCR technologies have revolutionized research on fungal detection and identification.

Keywords: chick pea cultures, isolates, PCR, phylogenetic tree, data base sequences and fungal pathogens

Introduction
Gram or Chickpea (*Cicer arietinum Linnaeus*), a member of family Fabaceae, is an ancient self pollinated leguminous crop, diploid annual (2N=16 chromosomes) grown since 7000BC, in different area of the world but its cultivation is mainly concentrated in semi-arid environments of different areas of the world. It is ranked 3rd after common bean (*Phaseolus vulgaris L.)* and pea (*Pisum sativum L*.) and known with different regional names like Bengal Gram (English), and locally 'chana' [11].

Area, production and productivity of chickpea crop in India is about 73.7lakhs/ha in area.88.9 lakh tonnes in production, 799.19kg/ha in productivity. In utter Pradesh (2012-2013) area, production and productivity of chickpea are 495051(ha), 440572mt, 890(q/ha).grain legumes are members of subfamily papillionoidae, and chick pea was placed in the tribe Vicieae that includes vetch, lentil and faba bean mainly due to the pollen morphology and vascular anatomy. After this the tribe Ciceraceae was subsequently classified separately from the members of the vicieae. There are 43 species so far reported for the genus *Cicer*, 9 annual (including the cultivated Cicer arietinum), 33 perennial and 1 unspecified. Out of these, three species of *Cicer*, viz *Cicer bijugum, Reck. Cicer echinospermum* Davis and *Cicer reticulatum* are closely related to chickpea that grow wild in nature [6].

There are two types of gram, one is the 'Kabuli' white and other is 'desi' brown. Kabuli type is grown in temperate regions while the 'desi' type chickpea is grown in the semi-arid tropics.

Pulses have occupied a focal attention in recent years due to increasing awareness and concern for sustainable production, food and nutritional security. Pathogens in chick peas are the cause for yield loss (up to 90%).example like fungus *Fusarium oxiysporium* f. sp. *Cicero*, present in most of the major pulse crop growing areas and causing regular yield damages between 10-15% [5]. The worldwide number of pathogens increases from 49-172 nearly, of which 35 have been recorded in India. These pathogens originate from the groups of bacteria, fungi, viruses, Mycoplasma and nematodes [3]. Chickpea is reported to be attacked by about 67 fungi, 3 bacteria, 22 viruses and mycoplasma and 80 nematodes [8]. Among, fungal incidence causes more than 35-55% yield loss on Chick pea plant.

More emphasis is needed on disease management of this crop as; this field lacks attention in spite of the importance it renders to the masses. To combat such problems, there is a need of proper fungal diagnosis program. Conventional methods to identify fungi have often relied on identification of disease symptoms, isolation and culturing of environmental organisms, and laboratory identification by morphology and biochemical tests. Although these methods are still fundamental, there is an increasing move towards molecular diagnostics of fungi in all fields.

Materials and Methods

1. **Collection of soil sample:** Total Fifty soil samples were collected from rhizosphere of Chick pea crop from the growing areas of Ganga par and Jamuna par in Allahabad region. Soil samples (50g each) were collected from four corners and the center of the field. The samples were collected in polythene bags with the help of auger and samples were sun dried. Before the collection of samples auger was cleaned with sterilized water.

2. Isolation of fungal pathogens

Soil Dilution Plate Method [12]: soil dilutions were made by suspending 1g of soil of each sample in 10ml of sterile distilled water. Dilutions of 10-3, 10-4 and 10-5 were used to isolate fungi in order to avoid over-crowding of the fungal colonies. 1ml of the suspension of each concentration was added to sterile Petri dishes, in triplicates of each dilution, containing sterile Potato Dextrose Agar medium. 1% streptomycin solution was added to the medium for preventing bacterial growth, before pouring into Petri plates. The plates were then incubated at 28+2°C for 4-7 days. Organisms were easily isolated because they formed surface colonies that were well dispersed, particularly at higher dilutions. Pure culture of these fungi was prepared and maintained on PDA slants for further study.

3. Molecular Identification

DNA extraction: For DNA extraction, the pathogens were cultured on potato dextrose agar media at 25oC for 5-8 days. By using whatsman filter paper no.1 mycelia were harvested by filtering and with distilled Sterilize water washed repeatedly to remove excess salts adhering to it. DNA extraction was based on the CTAB (cetrimide tetradecyl trimethyl ammonium bromide) method. One gram of Mycelium was crushed in liquid nitrogen and transferred in to pre- warmed DNA extraction buffer, then mixed well and incubated in a water bath at 65Oc with gentle shaking for 45 minutes. Equal quantity of chloroform: isoamyl alcohol was added and mixed gently to denature proteins and centrifuged at 10,000rpm for 10 minutes (Sorvall SS 34 rotor).The pellets were washed twice with chilled 70 % ethanol and dried at room temperature. They were re-suspended in 1 mm TE buffer (10 mM Tris-HC1, 1mM EDTA -pH 8.0) and stored at -20oC. Isolated DNA was electrophoreses in 1.0% agarose gel to check the quality and concentration.

PCR amplification

An 18S r DNA was carried out using the primer pairs. TTAGCATGGAATAATRRAATAGGA and ATTGCAATGCYCTATCCCCA [4]; and GGAAGGGRTGTATTTATTAG and TCCTCTAAATGACCAAGTTTG [9].

Cycling parameters were

1. 94oC for 5 min, followed by
2. 35 cycles of 94oC for 30 seconds,
3. 55oC for 30 seconds and
4. 72oC for 30 seconds with a final extension at 72oC for 5 minutes for each primer pair.

Reactions were performed in duplicate, and negative controls (containing no DNA) were included in each PCR. Amplification products were electrophoresis in 1.5 % Agarose gels stained with Ethidium Bromide and visualized under UV light.

Purification PCR product: The purification was done by gel elution. In this, the DNA fragments of interest were excised using a wide, clean scalpel. It was then transferred into the micro centrifuge tube and equal volume of Binding Buffer (BD) was added the mixture was kept at 550-600C for 10 minutes or until the gel has completely melted. Mix by shaking or inverting the tube every 2-3 minutes. The samples were bind by applying quick column. To increase the concentration of DNA the column was left for 1minute and then was being centrifuge for 1minute the amplified DNA was then analyzed for confirmatory identification of bacterial species isolates.

Construction of Phylogentic Tree

After purification the sequences were analyzed by BLAST on National Center for Biotechnology Information(NCBI) web pages (http//www.ncbi.nim.nihgov/BLAST) and EZ taxon server version 2.1(www.eztaxone.org) to examine EMBL/ Genbank/DDBJ databases for sequences with high similarity to the 18S r DNA sequences. The sequences identity, sequence coverage and E value were considered to be match the sequence with the standard database sequences, the first 18-20 matching sequences were retrieved according to sequencing identity from the BLAST result and multiple sequence alignment was performed along with the test strains. Further, Phylogenetic relationships were inferred by preferential alignments of the soil fungal sequences obtained from Gen Bank. Based on the multiple sequence alignment of the selected sequence set, an evolutionary distance matrix and a phylogenetic tree were then computed using the Neighbor-Joining method. MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 was used for phylogenetic and molecular evolutionary analyses [10].

Results and Discussion

PDA medium is the most commonly used culture media and was stated to be the best media for mycelia growth by several workers worked with it earlier. Large number of colonies grown after 4-5 days of culture on each dilution plates. There were almost uncountable colonies in plates. After all the sub-culturing procedure, some pure colonies were obtained from these plates and the identification of these colonies was done. Among them only four pure cultures responded to molecular identification, they were *Chaetomium globosum* causes Soft-rot, *Fusarium oxysporium* causes wilt diseases, which are important in yield loss and low productivity along with *Aspergillus niger c*auses a disease called black mold and *spergillus terreus*.

The sequence of the conventionally identified fungal isolates was subjected to 18S rDNA was analyzed. During the BLAST search against ref seq rna database, the maximum sequence identified for Chaetomium globosum, Fusarium oxysporium, Aspergillus niger and Aspergillus terreus (95-100%) with E value 0.0 and 99% sequence coverage with 1600 query length. Fig: 1 shows that phylogenetic tree was generated

based on the similarity values of the strains as well as references strains obtained from Gene bank.

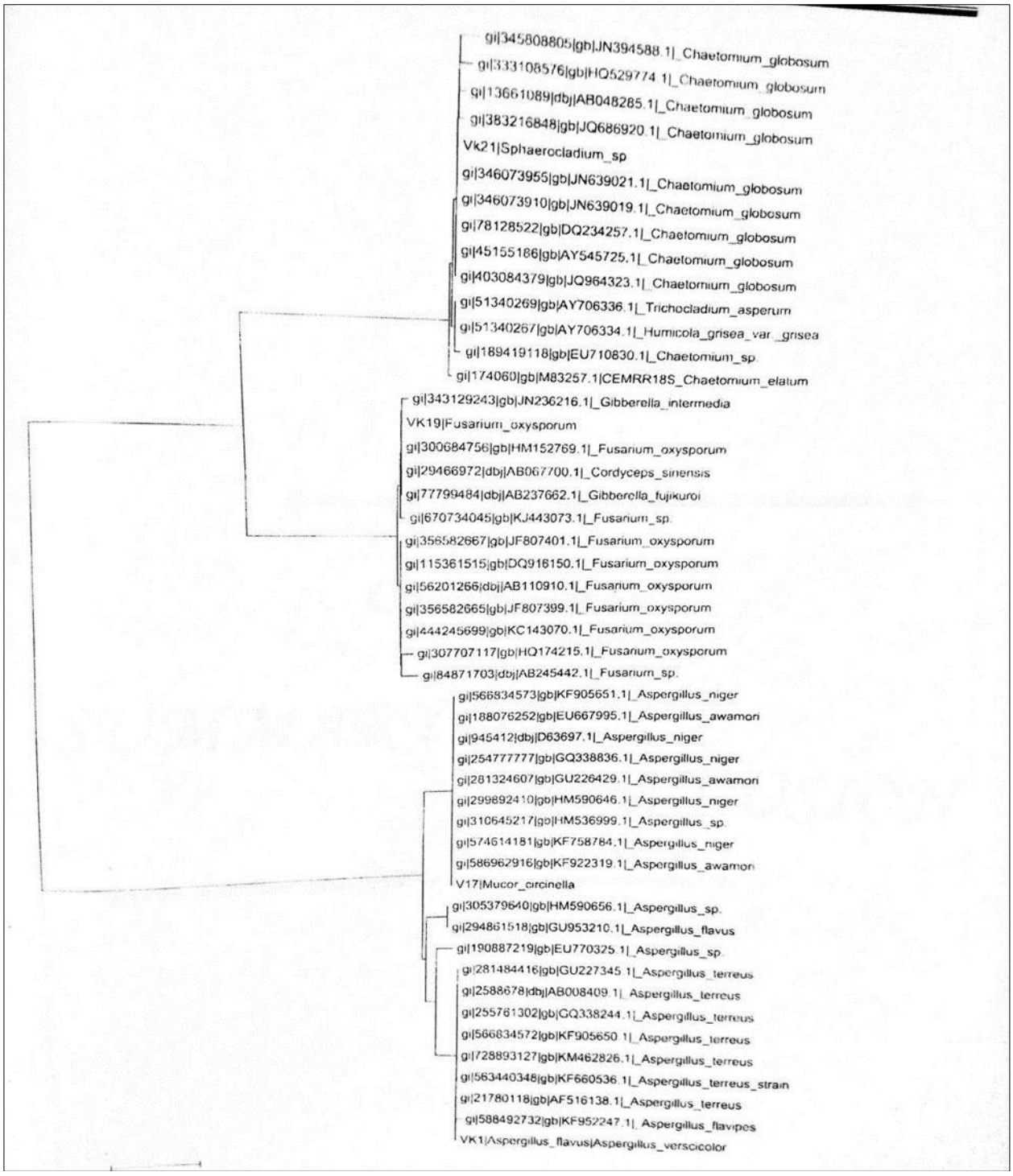

Fig 1

Phylogenetic tree shows distant relation with the remaining sequence retrieved from NCBI and used for alignment with the test sequences. In the present study, the importance of 18 S rDNA and PCR methods for assessing fungal species was alreadyreported in the studies of [1]. four fungal 18 S rDNA and internal transcribed spacer (ITS) polymerase chain reaction (PCR) primer pairs were tested for their specificity towards target fungal DNA in soil.DNA extracts and their ability to assess the diversity of fungal communities in soil was compared. The work demonstrates the usefulness of 18SDNA and ITS PCR primers for assessing fungal diversity in environmental samples and it also highlights some potential limitations of the approach with respect to PCR primer specificity and bias. Fungal constitute an important part of the soil ecosystem, in decomposition, cycling processes, and biotic interactions as reported by [7]. Molecular methods.

The work demonstrates the usefulness of 18SDNA and ITS PCR primers for assessing fungal diversity in environmental samples and it also highlights some potential limitations of the approach with respect to PCR primer specificity and bias. Fungal constitute an important part of the soil ecosystem, in decomposition, cycling processes, and biotic interactions as reported by [7]. Molecular methods have been used to assess fungal communities diversity and also by [2] reported that conventional methods to identify fungal have often relied on identification of disease symptoms, isolation and culturing of

environmental organisms, and laboratory identification by morphology and biochemical test. PCR has provided many molecular diagnostic tools and with the advances in micro-array technology and real –time PCR methods the future is bright for the development of accurate, quantitative diagnostic tools that can provide information not only on individual fungal species but also on whole communities.

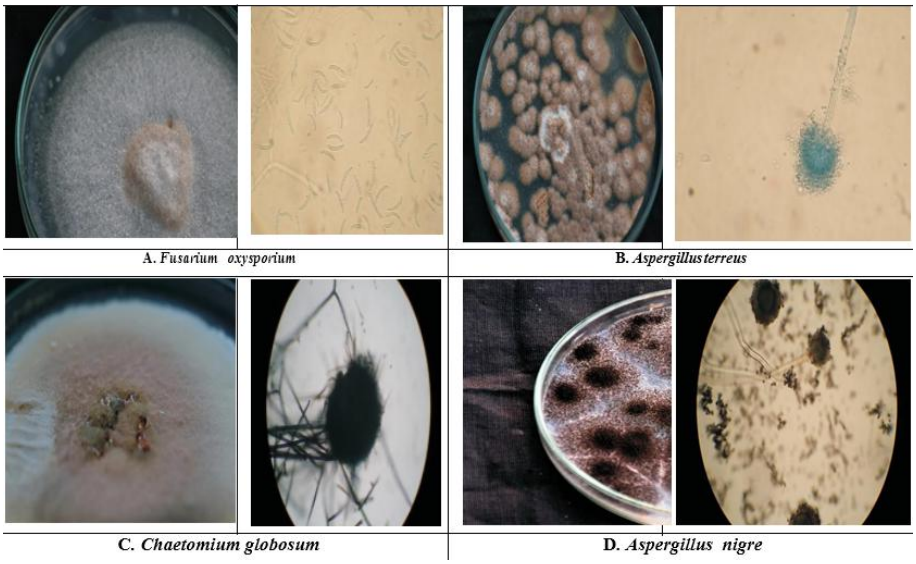

Table 1: A, B, C & D Pure culture on PDA media plates

Conclusion

In conclusion, the sequence of the conventionally identified fungal isolates subjected to 18S rDNA was Analysis. The dendrogram generated based on the similarity values of the strains as well as reference strains obtained from Gene bank. The generated phylogenetic tree shows that the studied isolates were Clustered in groups; phylogenetic tree shows the evolutionary relationship among the group of organisms.

Chaetomium globosum causes Soft-rot, *Fusarium oxysporium* causes wilt diseases, which are important in yield loss and low productivity along with *Aspergillus niger c*auses a disease called black mold and *Aspergillus terreus*. Fungal incidence causes more than 35-55% yield loss on Chick pea plant. More emphasis is needed on disease management of this crop as this field lacks attention in spite of the importance it renders to the masses. To combat such problems, there is a need of proper fungal diagnosis program.

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