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First Report Of *Pythium Ultimum* From Soil Sample JMBL -10 Gorakhpur Uttar Pradesh India: Morphology Taxonomy Dna Barcoding and Phylogenetic Analysis

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Abstract

Isolate JMBL-10 of Oomycete was taken from soil samples in the residential campus of DDU Gorakhpur University, Gorakhpur, Uttar Pradesh, India. The oomycete grows well on hemp seed halves as well as on solid media like PCA, CMA and PDA producing a chrysanthemum pattern. It also grows luxuriantly on hemp seed halves in water. The daily growth rate of this oomycete on PCA at 25 °C was 35 mm. On hemp seed halves in water, the oomycete produces whitish colonies. The oomycete JMBL-10 does not produce zoospores nor sporangia. However, it produces hyphal bodies of different dimensions. These hyphal bodies germinate by producing germ tubes to give rise to new mycelium. The oomycete JMBL-10 produces male gametangia (antheridia) and female (oogonia) gametangia plentifully in water on hemp seeds and on solid media. The antheridia are usually monoclines, 1 to 3 per oogonium At times the antheridia are sessile. The female gametangia are smooth walled, mostly terminal, at times intercalary, globose, measuring between 17 to 22 µm in diameter (average 18 µm). A fertilization tube is developed between the antheridia and oogonia and after fertilization the antheridia quickly disappear. At times the remains of the antheridial cell was visible even after fertilization and the formation of zygote (oospore which are mostlyaplerotic). These morphological characteristics of our isolate JMBL-10 comes very close to *Pythium ultimum* Trow. which war a highly parasitic oomycete. The amplified product of PCR amplicons with COX II primers were subjected to BLASTn. By this, the amplified products (Query sequences) shown 94% identity with the *Pythium* species. The BLAST analysis shows 94% identity with the maximum score of 861 with 97% Query coverage for the species *Pythium ultimum* KJ639192.1. The T-COFFEE algorithm alignment showed very good score of 99%. This region showed 97% consensus region with *Pythium ultimum* therefore, the fungal isolate belongs to the *Pythium* spp. The strong homology revealed the isolate was *Pythium ultimum*. The amplified COX II of our isolate JMBL-10 was also subjected to MSA through CLCbio Main Workbench while constructing the phylogenetic tree by NEJ method our isolate has showed close relation with *Pythium ultimum* KJ639199.1 and *Pythium ultimum* KJ639196.1 that are aligned followed by *Pythium ultimum* AF196641.1, *Pythium ultimum* KJ639175.1 and *Pythium ultimum* KJ639192.1. The results of phylogenetic analysis using COXII indicated our isolate JMBL-10 is *Pythium ultimum* which is close to *Pythium ultimum* KJ639199.1 and *Pythium ultimum* KJ639196.1, than *Pythium ultimum* AF196641.1, *Pythium ultimum* KJ639175.1 and *Pythium ultimum* KJ639192.1

Keywords: *Pythium;* morphology; taxonomy; DNA barcoding; phylogeny; India

1. INTRODUCTION

The Oomycetes are microscopic Stremenopiles. Oomycetes are also known as "Water molds". Many year ago, the Oomycetes were defined as "Aquatic phycomycetes". In the latest edition of the classification of fungi, Oomycetes are defined as – *"A class within the kingdom Chromista*". The Oomycetes *Pythium* and *Phytophthora* are two of the harmful genera of plant pathogens worldwide. They are known to cause seed rot, root rot, seedling, damping off, rots of lower stems, tubers and soft rots of fleshy fruits in contact with the soil. Most of the Oomycetes are important sources of both bioactive compounds and mycotoxins. So, that they are very ubiquitous in our environments. Oomycete's identification has been primarily based on their phenotypic and physiological characteristics.

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Pythium is a genus with over 200 species found worldwide, some of which are residents of terrestrial habitats while others are aquatic. In terms of nutrient acquisition, species within the genus may be saprophytes, plant or animal parasites, or mycoparasites. *Pythium* is a closely related to *Phytophthora*, famous for causing the ill fated "Famine of Ireland" in the years 1845 to 1849 in which 1 million people died. Schoch et.al. observed DNA phylogenetic comparisons have shown that morphology-based species recognition often underestimates fungal diversity[1]. Therefore, the need for accurate DNA sequence data, tied to both correct taxonomic names and clearly annotated specimen data, has never been greater. Furthermore, the growing number of molecular ecology and micro-biome projects using high-throughput sequencing require fast and effective methods for enmasse species assignments. The present study therefore, is aimed to isolate and purify Oomycetes belonging to the Stramenopiles of order peronosporales. This method is free from harmful chemical pesticides and eco-friendly approach to identify the species and further its Barcoding and phylogenetic position so as to its use in biological diversity. It's impact in our opinion, has never been studied in Eastern U.P. in particular and in India in general. Our Oomycete isolate JMBL-10 was isolated from a soil sample taken in DDU Gorakhpur University Residential campus in the city of Gorakhpur, Uttar Pradesh India.

2. MATERIALS AND METHODS

2.1 Soil samples

Samples of brown decaying twigs, leaves and woods of the local dominant vegetation were collected from Gorakhpur, India and it was brought to the laboratory in separate sterile polyethylene bags. Oomycetes was isolated from these samples by the usual baiting techniques.

2.2 Morphological characterization in water and PCA cultures

Water culture of the JMBL-10 fungal was isolated, prepared and morphological identification for the microscopic method.

2.3 Growth rate measurements

The JMBL-10 fungal strain was inoculated on PCA, incubated at 25^0C and growth measurements in mm was recorded every 24 hours until the isolate covered the entire plate.

2.4 Maintenance of culture

The purified cultures was inoculated in 15ml culture tubes containing PCB liquid broth which was placed on rotatory shaker incubated at 25ºC. The growth of pure mycelia JMBL - 10 was recorded at the interval of 24 hr. for 96hr. (1-4). After, 72-96 hours of growth, the culture media was transferred at 4ºC in refrigerator. Mycelial biomass (of JMBL-10) formed after three to five days of inoculation was collected on a muslin cloth by discarding the liquid media. Biomass was washed 5-6 times with deionised water carefully and mycelia was air dried and same was used for further characterization.

2.5 Extraction of Fungal Genomic DNA

0.1g of dried JMBL-10 mycelium was ground in liquid nitrogen into a fine powder in a pre-chilled mortar and pestle. And extracted with 600µl of extraction buffer and crushed it well using tissue grinder. Then again extracted with 50µl of 10% SDS and incubated at 65˚C for 30 minutes. After incubation added 800µl of Phenol:chloroform:Isoamylalchol (25:24:1) and centrifuged at 10000rpm for 15 minutes. The upper aquous layer was transferred into fresh eppendorf tubes and added 10µl RNAs and incubated it 37˚C for 30 min. Then added equal volume of isopropanol and again incubated at -20˚C for 20 min. After incubation centrifuged at 10000rpm for 20min. Discarded the supernatant and added 500µl of 70% ethanol, vortexed and again centrifuged at 10000rpm for 2 min. Finally added 200µl of pre warmed nuclease free water to dissolve the pellet.

2.6 Characterization of JMBL-10

The JMBL-10 isolate was characterized by morphological as well as molecular biology tools. For their identification by molecular tools, DNA was extracted by our own protocol but basic tenets prescribed by Saghai-Maroof et.al. followed by PCR amplification of the ITS region of ribosomal DNA and COX II gene for oomycetes[2]. The amplicons were sequenced by Pyrosequence analyzer and the resultant sequence were subjected to BLASTn, T-COFFEE and CLC Biomain Workbench analyses for identify the specific identification of oomycete.

2.7 PCR Amplification

The ITS region of JMBL-10 isolate was PCR amplified and sequenced using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [3]. PCR reaction consisted of 5.0µl of 10X amplification buffer (100mM Tris-HCl, pH 8.3; 500mM KCl, 2.5mM $MgCl_2$), 3.0µl of 10mM dNTPs, 1µl each of 10µM ITS1 primer and ITS4 primer, 1µl of Tag Polymerase , about 100ng of sample DNA and nuclease free water to the final volume of 50µl. Prior to thermocycling, samples were heated at 94˚C for 5min., this was followed by 40 cycles of 94˚C for 1min., 55˚C for 1min. and 72˚C for 1min. using Mastercycler personal (Eppendorf) the final extension was performed at 72˚C for 7 minutes. The successful amplification of the ITS region was checked by running 20µl of reaction mixture 1.5 % agarose gel in 1X Tris acetate EDTA buffer (TAE) at 100 volts for 60 minutes.

2.8 Pyrosequencing

Apical Scientific Sequencing Division Bhd , Malaysia has used ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA, for sequencing.

2.9 Phylogenetic tree

A phylogenetic tree or evolutionary tree is a branching diagram or " tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and /or genetic characteristics .the taxa joined together in the tree are implied to have descended represents

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the inferred most recent common ancestor.in a rooted phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants, and the edge lengths in some trees may be interpreted as time estimates . Each node is called a taxonomic unit. Internal nodes are generally called hypothetical taxonomic unit (HTUs)as they cannot be directly observed. trees are useful in field of biuologyun such as bioinformatics , systematics and comparative phylogenetics.

3. RESULTS AND DISCUSSION

The oomycete (JMBL-10) grows well on hemp seed halves as well as on solid media like PCA, CMA and PDA. On hemp seed halves in water, the oomycete produces whitish colonies. JMBL6 fungal isolate of oomycete grows well on solid media like PCA, CMA and PDA producing a chrysanthemum pattern. It also grows luxuriantly on hemp seed halves in water. The daily growth rate of this oomycete on PCA at 25 °C is 35 mm. The mycelium is well branched, non- septate (coenocytic) having dense granular cytoplasm and diploid nuclei. The colonies in water are hyaline, whitish while on the solid media are submerged. The oomycete JMBL-10 does not produce zoospores nor sporangia. However, it produces hyphal bodies of different dimensions. These hyphal bodies germinate by producing germ tubes to give rise to new mycelium.

Fig.1: Chrysanthemum growth pattern on PCA

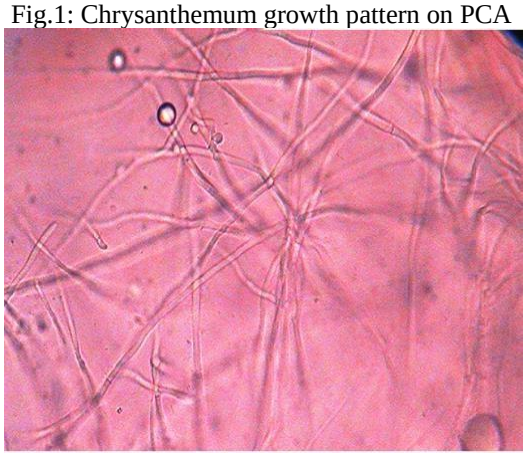

Fig.2: Vegetative hyaline mycelium

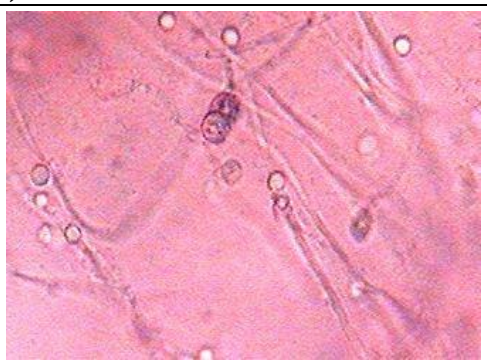

Fig.3: Young Oogonium with Monoclinous Antheridium

Fig.4: Aplerotic Oospores

3.1 Molecular characterization of soil oomycete JMBL-10 Genomic DNA extraction

Prior to extraction, pure isolate of JMBL-10 was reactivated by sub-culturing growth medium and inoculated at 25 ^{0}C for 3-7 days massive production of mycelia. DNA was extracted from resulted mycelia JMBL -10 using our own protocol developed but following the basic tenets of Saghai-Maroof et.al.[2]. Agarose gel electrophoretic analysis of the purified DNA of JMBL-10 showed approximate molecular weight of 40 kb.

M 1 2 3 4 5 6 7

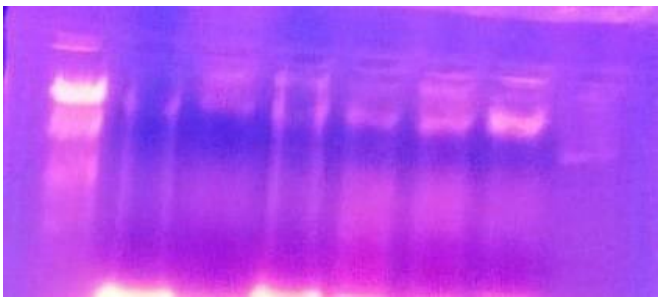

Fig.5: Agarose gel electrophoresis of purified genomic DNA of JMBL-10.
M, 50bp DNA Marker; Lane 6, Isolate JMBL-10.

3.2 PCR amplification

The cytochrome oxidase II (COX II) gene of JMBL-10 was PCR amplified using forward FM 58 (5′-CCACAAATTTCACTACATTGA-3′) and Reverse FM 66(5′-TAGGATTTCAAGATCCTGC 3′)[4]. The amplified

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COX II gene was approximately 600 bp. It is in consonance with the results of previous authors [4-7].

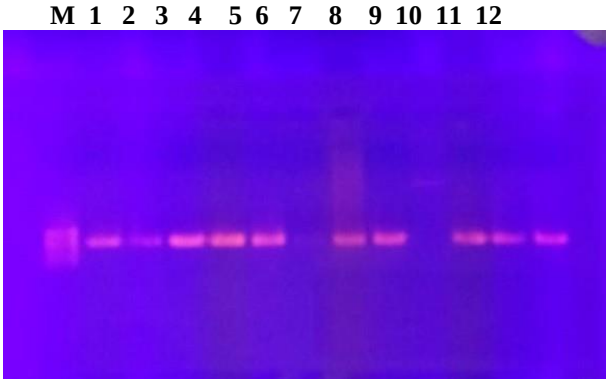

Fig.6: Band pattern of PCR amplified COX II region
M, 1Kb DNA ladder Marker; Lane 10, Isolate JMBL-10

3.3 Pyrosequencing

The PCR products from the gel were cut and was submitted to the Apical Scientific Sequencing Division Bhd., Malaysia for sequencing using Applied Biosystems (as mentioned under the pyrosequencing of materials and methods) and the same Primers use it for amplification.

3.4 Analysis of Sequences

The amplified product of PCR amplicons with COX II primers were subjected to BLASTn. By this, amplified products (Query sequence) shown 99% identity with the *Pythium* species. The BLAST analysis shows 99% identity with maximum score of 861 with 97% query coverage for the species *Pythium ultimum* KJ639192.1. Then we did sequence homology with the wild type *Pythium ultimum* COX II primer with our amplified product (Query sequence) through T-COFFEE algorithm alignment. The alignment shows very good score of 99%. This region shows 97% consensus region with *Pythium ultimum* therefore, the fungal isolate belongs to the Pythium species. The strong homology reveals the isolate belong to the *Pythium ultimum.*

3.5 Phylogenetic tree

The amplified COX II of our isolate JMBL-10 was also subjected to multiple sequence alignment through CLC bio-Main Workbench while constructing the phylogenetic tree using NEJ method. According to the tree our sequence has showed close relation with *Pythium ultimum* with the different isolates sequences (accessions) that are aligned. Further studied MSA using NEJ method with different other species COX II and it was found our query sequence JMBL-10 showing identity with *P. ultimum KJ639199.1 and Pythium ultimum* KJ639196.1 that are aligned followed by Pythium ultimum AF196641.1, Pythium ultimum KJ639175.1 and Pythium ultimum KJ639192.1. The results of phylogenetic analysis using COX II indicated our isolate JMBL-10 is *Pythium ultimum*, which is close to *Pythium ultimum* KJ639199.1 and *Pythium ultimum* KJ639196.1, than *Pythium ultimum* AF196641.1, *Pythium ultimum* KJ639175.1 and *Pythium ultimum* KJ639192.1.

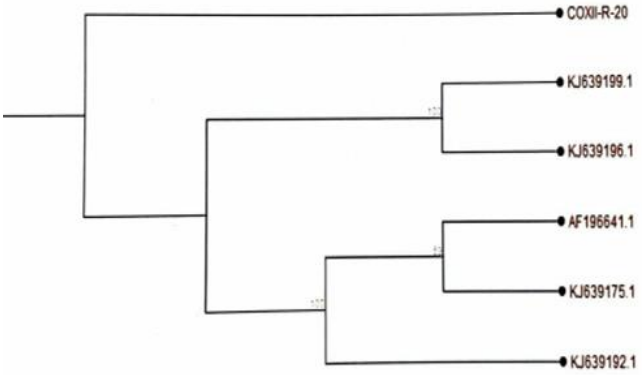

Fig.7: Phylogenetic tree

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