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A productive approach towards molecular analysis of black mold rot disease in mango using different bioinformatics tools

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Abstract

Many pathogens especially fungi associated with the spoilage of fruits due to several factors such as pH, temperature, atmosphere, humidity and postharvest factors. These pathogens destroy the fruits or damage the quality level of fruits by causing different diseases. In Pakistan, mango production reaction to agricultural GDP is positive but the loss in fruit's quality also lowers the GDP of the country. *Aspergillus niger* has been originated as an opportunistic cause of human infection and fruit crop diseases. There are multiple molecular and other indirect methods to help identify these pathogenic fruit diseases. *Aspergillus niger* causes a damaging disease in mango that is Black mold rot. Total fifteen diseased mango samples were collected from different cities of Punjab; Faisalabad, Multan and Lahore. Potato dextrose agar and Sabouraud liquid media were used for fungal culture. Genomic DNA was isolated through cultured media from all the samples, purified and subjected to agarose gel electrophoresis to test the integrity of DNA. The basis of specificity by PCR performed by using universal primers to identify fungi and 18S-rRNA primers were designed by using different bioinformatic tools. The amplified products were sequenced, identified, phylogenetic analysis and gene characterized by *in-silico* analysis. Scanning electron microscopy was performed that ideally suited for observation of intact sporing structure over a wide range of magnification, duplicating and supplementing information of *Aspergillus niger*. Molecular parameters were used to study the *Aspergillus niger* that causing black mold rot which is a disastrous disease of mango.

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Introduction

Agriculture is an important sector of Pakistan's economy that directly supports the country's population and accounts for ~26 % of gross domestic product (GDP). Losses linked to foodborne pathogens occur in various ranges of fruit crops and are caused by a wide variety of organisms including many bacteria, viruses, fungi and parasitoids. These pathogens destroy the crops or damage the quality level of crops by causing different diseases. As such food crops are the main source of the GDP, so the loss in fruit quality will also reduce the GDP of the country. So identification and cure for the food crop's disease is a major goal of today. The main goal of the research was to identify the fungus that causing black mold rot, a disastrous disease of mango through molecular parameters. In order to minimize the effects of fungi and to find ways to control the pathogen or there any way to cure the disease. Molecular biology can provide a promising pathway to generate disease-resistant mango varieties through targeted genome editing (Akhtar *et al*., 2009).

Mango (*Mangifera indica* L.) is the second major crop of Pakistan and maintains 6th position in production. Mango has become a world-famous fruit and is appreciated for its delicious taste, special and attractive flavor with strong nutritional, diuretic and therapeutic qualities (Amin *et al*., 2008). The fleshy mesocarp is abundant in organic acids such as oxalic, malic, succinic, pyruvic, glucuronic and galacturonic acids, including citric acid and phytochemicals such as ascorbic acid, carotenoids, polyphenols, mangiferin, coumaric acid, gallic acid and much more. The presence of these antioxidants means that, apart from improving cardiac health and decreasing the risk of heart disease, mango can be included in the daily diet for several health benefits, including anti-activities against cancer and viruses (Sivakumar *et al*., 2011). There are 150 mango cultivars produced from all over the world. For Pakistan, mango is an important commodity for the earning of foreign exchange. Pakistan ranks 4th among the leading mango exporting countries (Maqbool *et al*., 2007). Pakistan has the ability to produce 1.8 million tons per annum and the response of mango production to agricultural GDP is positive (Pawlowska *et al*., 2012).

The Mango industry in Pakistan is facing several challenges, especially in the postharvest loss of mango fruits caused by diseases and insect pests (Ishaq *et al*., 2004). A serious threat to the mango industry is postharvest decay. Postharvest losses may be due to various factors, including physiological changes, physical damage, chemical injury or residues and pathological decay. Postharvest losses of fresh mango are reported to be 25-40% in India and 69% in Pakistan. Microbial decay accounts for 17.0-26.9 % of the total postharvest losses in Asian countries. The incidence of postharvest diseases can also affect the quality of mangos limiting their shelf life up to 3-4 days (Prabakar *et al*., 2005).

Fruits contain high levels of sugar and nutrient elements and their low pH values make them particularly desirable to fungal decayed. Mold growth depends on many factors such as pH, water activity (aw), temperature, atmosphere, time (Gadgile *et al*., 2010). Black mold rot is a disease on mango caused by a fungus that is called *Aspergillus niger*. *Aspergillus* is a genus of *Hyphomycetes* which is the major cause of the deterioration of seeds. *Aspergillus niger* forms easily recognized black or dark brown colonies. *Aspergillus niger* (black mold), a filamentous ascomycete having the ability of fast growth and pH tolerance is the most important cosmopolitan fungi associated with postharvest decay of different substrates (Pitt and Hocking, 2009). The spoiling fungi are generally considered to be toxigenic or pathogenic. Some molds can produce mycotoxins during refrigeration. In comparison, pathogenic fungi may cause infections or allergies. The first symptom observed was a mild lesion of pale-yellow suppression around the stamen region. The scale of the lesion increases and the mesocarp and soft rot decreases. The lesion core had been sunken and coated with brown-black spores (Mons, 2004). The growth temperature for *Aspergillus niger* is 25-30 °C while light intensity has no effect on growth. Such fungus has maximum growth at a pH range of 6-6.5 on PDA

medium (Leong *et al.,* 2004).

Classification and isolation of fungi that are linked to fruit's spoilage need have increased. There are multiple molecular and other indirect methods to help identify these pathogenic fruit diseases. The molecular field includes various methods for genome editing that support us to convert these diseased fruit samples into healthy-one samples by improving the genome and correcting defective genes. Extraction of genomic DNA from plant pathogenic fungi is a common practice in plant pathology laboratories. This method is simple and inexpensive but time-consuming, especially when multiple samples are subject to DNA extraction. A genomic DNA template derived from selected fungus for the isolation from alkaline protease gene is used for the polymerase chain reaction. The polymerase chain reaction is a process of making million to billion copies of specific DNA samples. Phylogenetic relationships provide information on shared ancestry but not necessarily on how organisms are similar or different. A phylogenetic tree is a diagram that represents evolutionary relationships among organisms (Avise and Robinson, 2008).

Material and methods

Isolation

Mango Samples with black mold rot symptoms were collected from different regions of Punjab. After collection, the most common procedure of isolating this fungus from mango is simple PDA prepare which is considered as the best medium for fungal culture. The preparation is done according to standard laboratory protocol (39 gm PDA powder per liter of sterilized distilled water). Autoclaving was carried out at 121°C for 15-20 minutes. This destroys nearly all living cells and spores in the medium. The growth medium was poured into Petri plates and solidified. The samples were inoculated under a laminar airflow hood, decontaminated by a UV lamp for about 15-20 minutes and sprayed with 70% ethanol to prevent contamination. Using the inoculation loop picked a small number of fungal mycelia and streaked slightly on the agar. Flamed the loop after every quadrant. One plate was used as a control without inoculation. Carefully labeled and wrapped the plates with a cling film. Plates were incubated at 30 °C for 3-4 days. Colonies of black spores were developed in the Petri plates.

Sabouraud dextrose media was combined with dextrose to promote fungal growth. Peptone special provides the source of carbon, nitrogen, vitamins, minerals, amino acids, and growth factors. Dextrose provides a source of energy for microorganisms to evolve. The low pH helps fungal growth and prevention of contamination of clinical specimens by bacteria (Ishaq *et al.*, 2004). For the chemical composition of sabouraud dextrose media in which add 3.33 g dextrose, 0.83 g meat peptone, 0.83 g casein peptone dissolved in 150 mL distilled water. Mix well and dispense as appropriate sterilize at 15 IBS and 121 ° C for 15 minutes by autoclaving (Pandey *et al.*, 1997). After autoclaving, maximum time requires for cooling the flasks at room temperature. Using a red hot wire loop, picked a black colony from a solid Petri plate and injected it in sabouraud liquid media very carefully. The cultures were incubated at 25° C in an incubator shaker which was conducted for 48 hours at 150 rpm (Bastos, 2001).

Nucleic Acid Extraction and Purification

Extraction of genomic DNA from plant pathogenic fungi is a common practice in plant pathology laboratories. This method is simple and inexpensive but time-consuming, especially when multiple samples are subject to DNA extraction (Saleem *et al.*, 2010).

Reagents and solution

Extraction buffer (KCL 0.745 g, 1 mL Tris HCl of 1M, 200 µL EDTA (pH:8) by adding distilled water to make 10 mL solution), 0.3 mL isopropanol, 70% ethanol, 90% ethanol, RNase, 1X TE buffer are used for DNA extraction.

Protocol for DNA Extraction

Firstly add 0.5 mL extraction buffer from 10 ml extraction buffer and secondly add 0.3 mL

isopropanol in the Eppendorf. Collect 60 mg of fungal mycelia with a toothpick and carefully put it into the extraction buffer. Pulverize the fungal mass with the machine in the extraction buffer for 1-2 seconds. Push the plastic pestle onto the mycelial tissue. Wash the tip between the samples and soak the pestle into 70% ethanol. Centrifuge cell lysate 10 minutes at a rate of 13,000 rpm then pour supernatant directly into 0.3 mL isopropanol and discard remaining lysate very carefully. Mix isopropanol and lysate with inverting tube multiple times. Centrifugation again for 10 minutes at 12,000 rpm and discard the supernatant. Pellet wash with 0.8 mL of 95% ethanol and 70% ethanol. Evaporate remaining alcohol by incubation at 37°C for 15 minutes and tissue paper used for drying. Add 50 µL of 1X TE buffer and 5 µL of RNase and dissolve DNA pellet by tapping or vortex at low speed (Aras and Duman, 2007).

Polymerase chain reaction

A genomic DNA template is derived from selected fungus for the isolation from alkaline protease gene was used for the polymerase chain reaction. After several attempts, PCR conditions were optimized for amplification. Through polymerase chain reaction which able to obtain the desired fragments of our amplified gene, which have been confirmed by agarose gel electrophoresis. The PCR reaction mixture with an optimizable variable range has been used after the reaction. Reagents used for PCR are *Taq* Buffer (10X), 2 mM of dNTPs, Forward primer (18 S), Reverse primer (18 S), *Taq* DNA polymerase, 25 mM $MgCl_2$, Template DNA and Nuclease free water.

The purity of the DNA was assessed by measuring the absorbance in a spectrophotometer at 260 nm and 280 nm (A260/A280). 0.8 % agarose gel has been tested for DNA integrity and the gel was prepared in buffer 1X TAE. 1 µL 6X DNA loading dye was applied to the 3 µL DNA sample and the gel was run in 1X TAE buffer at 90 V (Photita *et al.*, 2001). It was kept in ethidium bromide for 15-20 minutes and documented after the removal of gel from the UV apparatus. For PCR product purification, the FavorPrepTM gel purification mini kit was used. Kit reagents are FAGP Buffer, wash buffer and elution buffer.

Primer designing

Using online primers designing tools; Primer-3 and integrated DNA technologies (IDT) universal primers for the 18S-rRNA were designed (Irshad *et al.*, 2018). The primers were designed from online bioinformatics software for designing primers with different hosted tools in this software after specific primers have been amplified to PCR. At the first gene sequence, stop codon and initial codon were selected and this data was obtained from the previously reported GenBank (NCBI) sequence. The sequence of unwanted test marks (space/gaps) was initially cleaned using this software with CLEANER hosting software. Primer restriction was calculated by OLIGOCALC. The addition of DNA and cDNA reverse sequence was obtained through the application of the Complementor tool. Primer Anneal tool rapidly investigates and envisage where primer hybridizes on your DNA and cDNA pattern. Forward and reverse primers with unique sequences were specific that designed against *Aspergillus niger*.

18S1F: 5'-GACTCAACACGGGGAAACTC-
18S1R: 5'-AAACCTTGTTACGACTTTTA-

Sequencing

The sequencing was performed from ' Eurofins, USA, ' followed by sequences submitted at GenBank. The results were checked at NCBI-BLAST for phylogenetic studies and gene characterization (Rahman *et al.*, 2003).

Phylogeny analysis

Phylogeny is the evolutionary history and relation of an individual or group of organisms. Phylogeny describes an organism's relationships, such as which organisms it is thought to have evolved from, to which species it is most closely related, and so on. Phylogenetic relationships provide information on shared ancestry but not necessarily about how similar or different organisms. A "tree of life" can be designed to demonstrate the evolution of different organisms,

and to show the relationships between different species. A phylogenetic tree is a diagram representing evolutionary links between organisms (Avise and Robinson, 2008).

Pathogenicity Test

Healthy mango sample surface sterilized with 70% ethanol and pick mycelia from agar containing the culture of fungal mycelia of the isolates. Such fungus was inoculated in a laminar flow chamber in the hole created on the healthy fruit. Petroleum jelly was applied to the inoculated wound. Two controls were developed with an incision but not inoculated. The inoculated fruit and the controls were placed in a clean polythene bag (one fruit per bag) each moistened with absorbent cotton wool wet balls to create a moist environment and incubated at 28°C for 5-7 days of the incubation period. Inoculated fruit was detected for symptom development. The casual agents were re-isolated from the diseased fruit and compared with the original isolates (Akinmusire, 2011).

Aspergillus niger analysis under light microscopy and scanning electron microscopy

Light microscopy and Scanning electron microscopy for *Aspergillus niger* were performed in Central Hi-Tech Laboratory, University of Agriculture Faisalabad.

Light microscopic analysis of *Aspergillus niger* with high magnification of 40X and 100X. Observe the fungal cells by looking for circular structures with an irregularly shaped core or nucleus. Take note of the various structures within the fungal mycelial cell, such as the cell membrane around the cell sample and nucleic structures within the cell's cytoplasm (Parton and Read, 1999).

SEM provides the surface topography, crystalline structure, chemical composition and electrical behavior information for the top 1 μm of the specimen. For scanning electron microscopy (SEM), *Aspergillus niger* was inoculated at 25 °C for 5 days in PDA. The sample disks were submerged in a fixative solution after the incubation period (Modified Karnovsky's fixative 2.5 percent glutaraldehyde 2.5 percent paraformaldehyde, 0.05 M cacodilate buffer, $CaCl_2$ 0.001 M) at pH 7.2. The discs were then washed in cacodilate buffer (three times, for 10 min each wash), post-fixed in 1% osmium tetroxide solution and water for 1 hour and washed three times in distilled water, followed by dehydration in increasingly concentrated acetone solutions (25, 50, 75, 90 and 100%, once for concentrations up to 90% and three times for 100% concentration). Subsequently, the samples were transferred to a silica-containing desiccator for drying completion. The specimens collected were mounted in stubs known as aluminum supports with a double-faced carbon tape placed on a film of aluminum foil, coated with gold in a sputter (BALZERS SCD 050) and viewed in a LEO EVO 40XVP scanning electron microscope. For each sample, a number of images were digitally generated and captured at variable magnifications (Kaplan *et al.*, 2007).

Results and discussion

The first step of the analysis was the cultural growth of fungus on solid media. Therefore, Potato dextrose agar media was used for this purpose (Burki *et al.*, 2005). The plates were examined after 72 hours which showed that there was a presence of fungal mycelia in plates and the control plate was clear without any contamination. Fig 1. Below showing Fungal growth colonies of mango samples.

2-3 colonies were picked from Petri plates and injected in all flasks of sabouraud dextrose medium. After picking colonies, all the flasks were the club with cotton and wrapped with cling film and fitted in a shaker for shaking at 100 rpm. Flasks were observed after 48 hours and clear growth in all flasks was observed. A suitable lysis buffer method was used for genomic DNA extraction and clear bands were observed under agarose gel electrophoresis. Fig .2. below shows DNA isolates from mango samples.

An effective primer designing is important for efficient PCR results. The primers were designed for

18S rRNA against *Aspergillus niger* by using bioinformatics tools (Mayer *et al.*, 2003). Sequence 5'-3' of 18S-F primer for the amplification of *Aspergillus niger* is GACTCAACACGGGGAAACTC with 20 lengths. 18S rRNA gene was amplified through PCR reaction. In PCR, specific primers were used for the amplification of sequences of genomic DNA by a polymerase enzyme that is thermostable which speeds up the buffered reaction (Hoffman *et al.*, 2002). In this process, a large amount of oligonucleotide primer and four dNTPs was used for the formation of million copies of the targeted sequence. Fig .3. below shows PCR amplification from mango samples of 18S rRNA sequence.

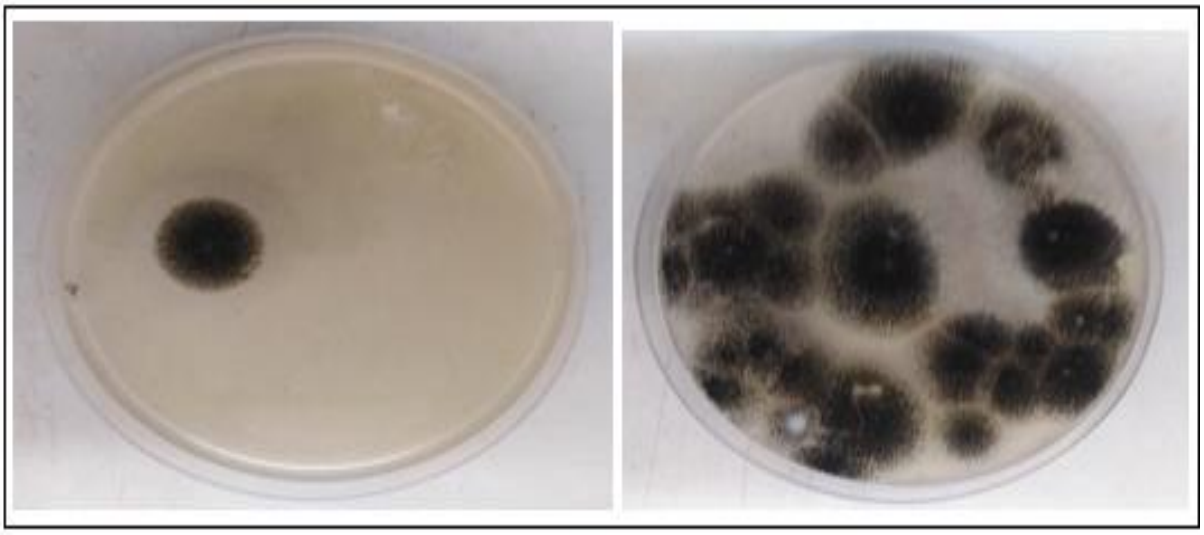

Fig. 1. Fungal colonies of mango samples were observed in Petri plates after 72 hours.

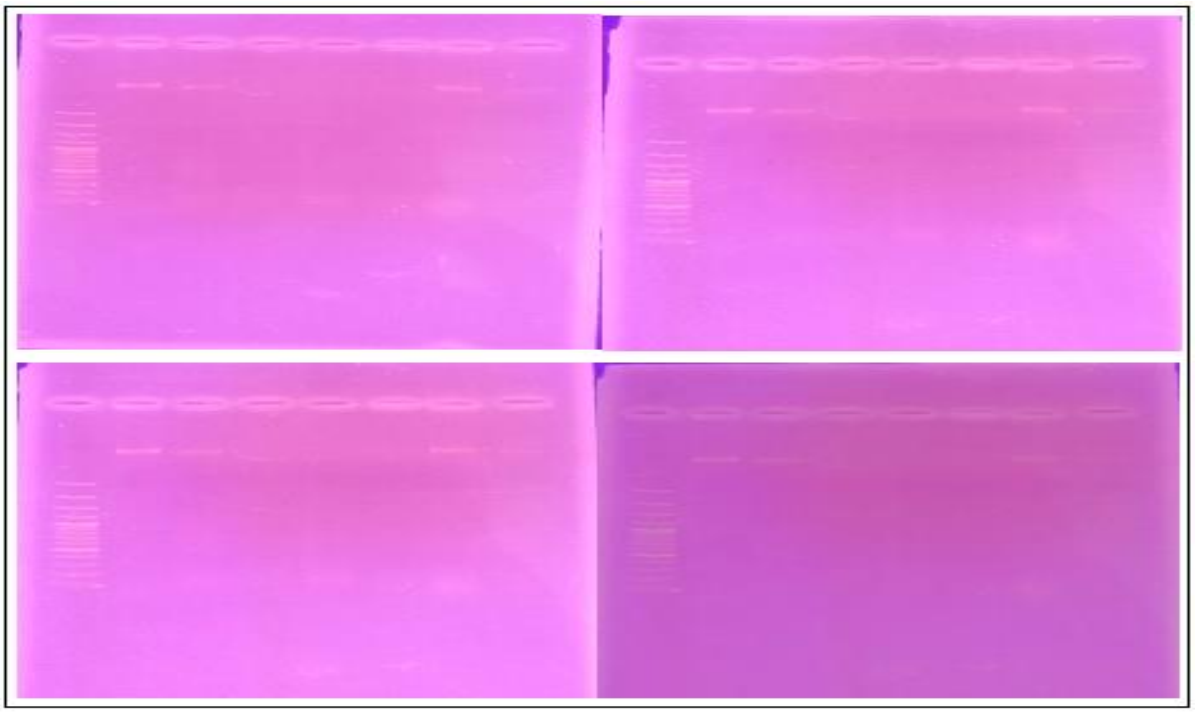

Fig. 2. DNA isolates from mango samples having clear bands visible under AGE.

The DNA purification was performed using the FavorPrepTM Gel purification mini kit and to get the standard results with 1.5% agarose gel electrophoresis for purification of samples. Samples of PCR products after their purification were sent to 'Eurofin – USA' for sequencing with the accession number

MN720150. The results were checked at NCBI-BLAST for phylogenetic studies and gene characterization. Fig .4. below shows NCBI-BLAST results of sequencing against submitted accession number. The gene sequences can be compared among species of *Aspergillus niger* and used for the design of the phylogenetic tree. The phylogenetic tree was drawn for comparison among gene sequences of *Aspergillus niger* species. Fig .5. below showing Phylogenetic tree for comparison among gene sequences of *Aspergillus niger* species.

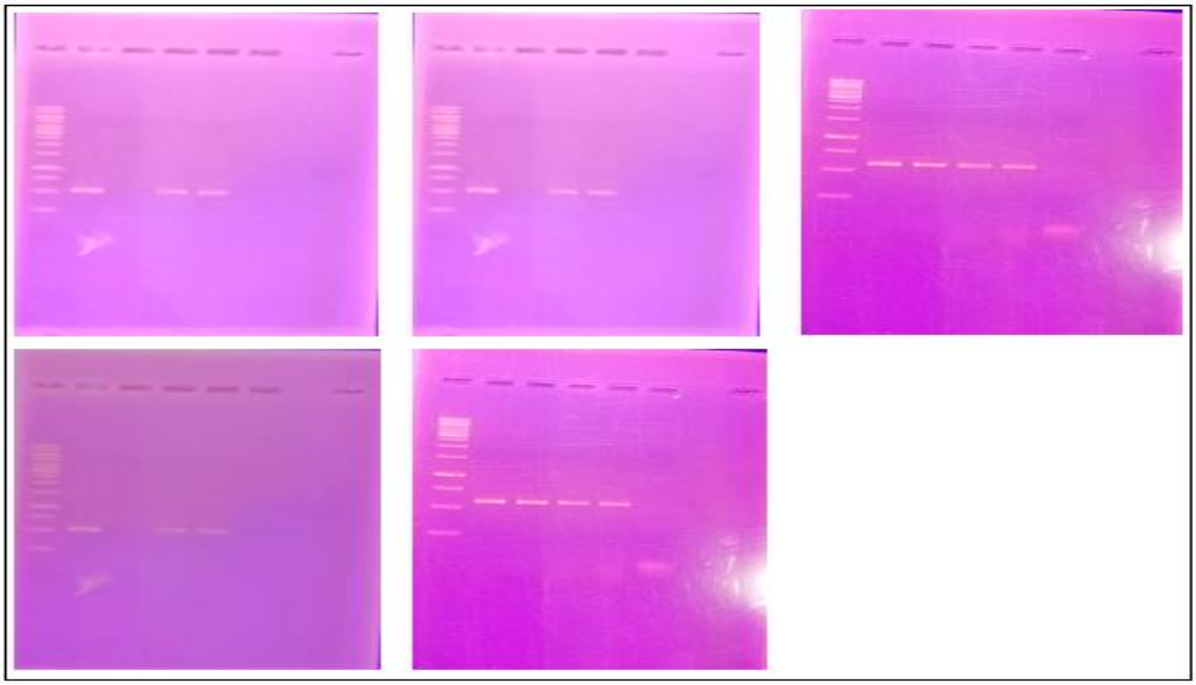

Fig. 3. PCR amplification from mango samples of 18S rRNA sequence visible under AGE.

select all 100 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aspergillus niger strain MBL-02 small subunit ribosomal RNA gene, partial sequence	981	981	100%	0.0	100.00%	MN720150.1
Aspergillus sclerotioniger CBS 115572 hypothetical protein (BO94DRAFT_374370), mRNA	976	976	99%	0.0	100.00%	XM_025607228.1
Uncultured eukaryote clone MEU7-70 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	JQ693066.1
Aspergillus niger strain ETYB-13 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	GQ903338.1
Aspergillus niger strain EHNH-07 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	GQ903337.1
Aspergillus niger strain IMT 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	GQ338836.1
Aspergillus carbonarius strain SIIA 1584 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	EU278604.1
Aspergillus niger contig An03c0110, genomic contig	976	2928	99%	0.0	100.00%	AM270052.1
Aspergillus niger contig An03c0100, genomic contig	976	976	99%	0.0	100.00%	AM270051.1
Aspergillus niger strain YM33182 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	DQ915806.1
Aspergillus niger strain VanTieghem 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence, and	976	976	99%	0.0	100.00%	DQ157694.1
Aspergillus ficuum strain Henninas 3.432 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence;	976	976	99%	0.0	100.00%	DQ157693.1
Aspergillus niger strain UPSC 1769 18S ribosomal RNA gene, partial sequence	976	976	99%	0.0	100.00%	AF548064.1
Chaetosartorya cremea gene for 18S rRNA, partial sequence	976	976	99%	0.0	100.00%	AB008399.1
Aspergillus awamori DNA for 18S rRNA, partial sequence	976	976	99%	0.0	100.00%	D63695.1
Aspergillus neoflavipes CBS 260.73 18S rRNA gene, partial sequence; from TYPE material	970	970	99%	0.0	99.81%	NG_062800.1
Uncultured eukaryote clone MEU7-69 18S ribosomal RNA gene, partial sequence	970	970	98%	0.0	100.00%	JQ693067.1
Aspergillus neoflavipes strain CBS 260.73 18S ribosomal RNA gene, partial sequence	970	970	99%	0.0	99.81%	GU733353.1
Aspergillus sp. enrichment culture clone NJ-F1 18S ribosomal RNA gene, partial sequence	970	970	99%	0.0	99.81%	GU190182.1
Aspergillus sp. HD86-9 18S ribosomal RNA gene, partial sequence	970	970	99%	0.0	99.81%	EU853156.1

Fig. 4. NCBI-BLAST results of sequencing against submitted accession number.

The mango samples were observed daily under laminar fumehood to visualize the appearance of symptoms. Till 3rd-day samples were observed with good conditions but somehow due to some unfavorable conditions the samples got contaminated and some fungus growth was observed which

destroyed the fruit and causes the excretion of fruit juice outside (Arduengo, 2010). And either due to unfavorable temperature or any other conditions sample didn't get the symptoms of black mold rot. Fig . 6. below showing pathogenicity test for *Aspergillus niger* infection on mango.

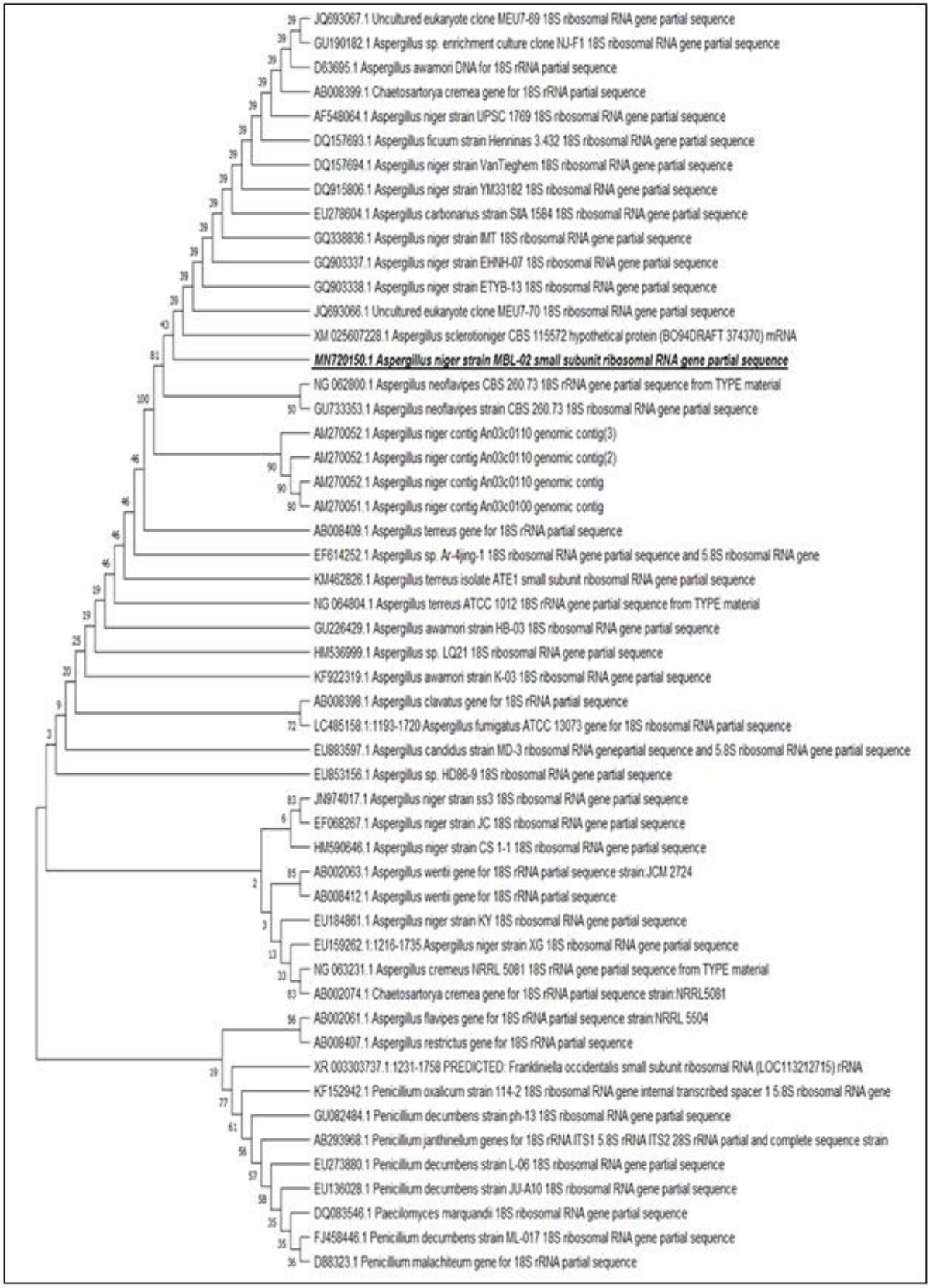

Fig. 5. Phylogenetic tree for comparison among gene sequences of *Aspergillus niger* species.

When the microscope was turned on, choose the 40X magnification level on the light microscope using oil imersion. The total focus was on the viewing lens for viewed the image and turned the focusing dial to change the focus until a clear, crisp image has been seen.

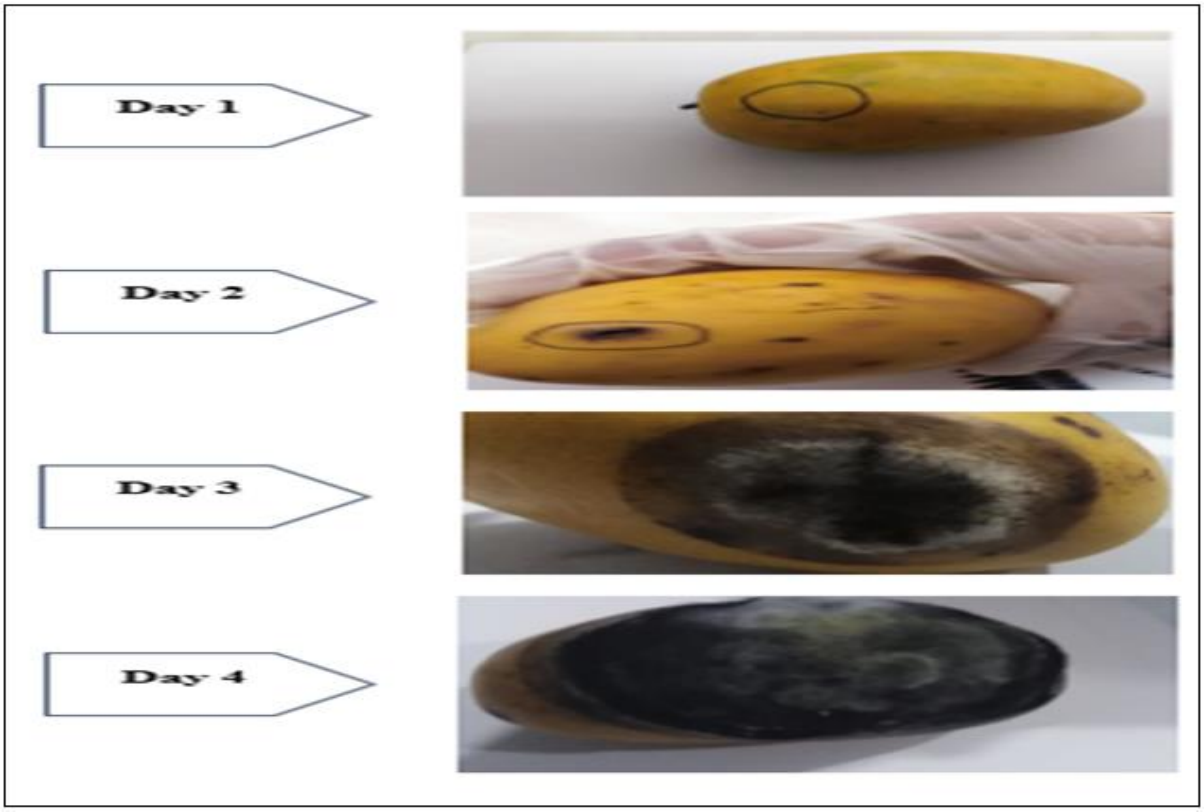

Fig. 6. Pathogenicity test for *Aspergillus niger* infection on mango.

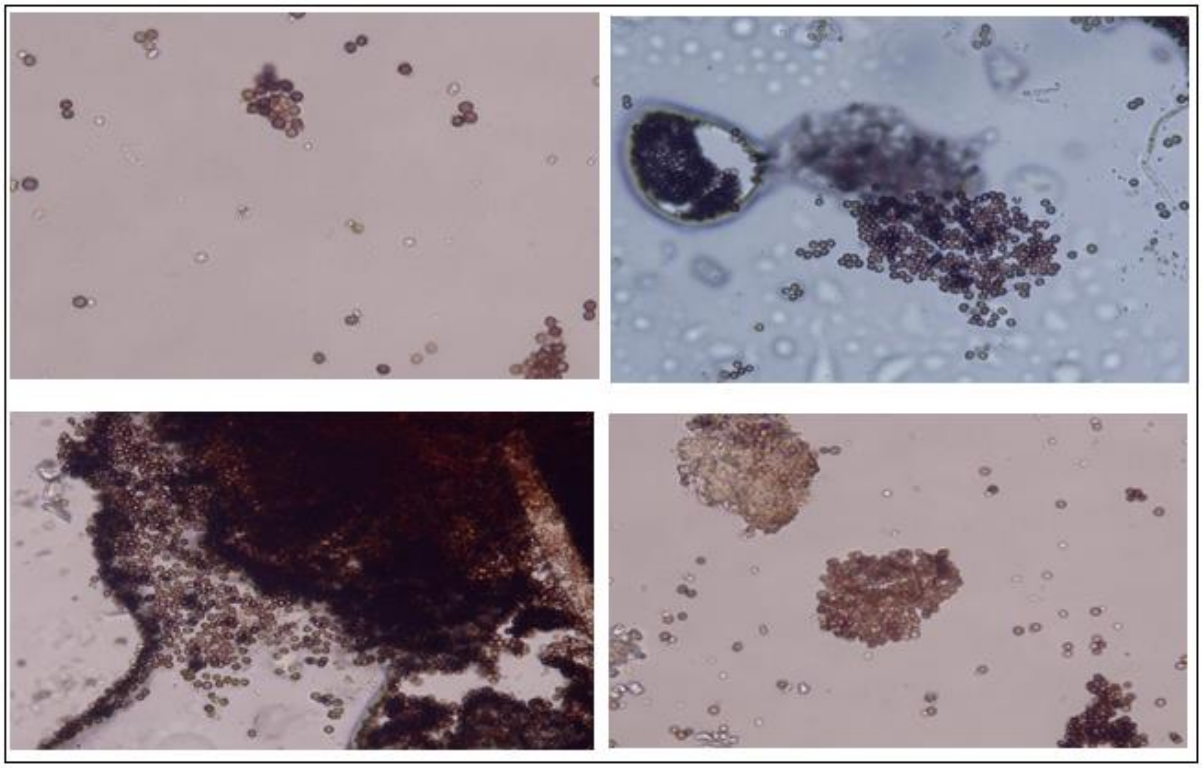

Fig. 7. Light microscopic analysis of *Aspergillus niger* mycelium and spores under 40X magnification.

The *Aspergillus niger* was very simple looking for its structure. For clearer visualization of the structure, then change the magnification of the light microscope to 100X, then turned the focus dial to refocus the lens for photograph clarification (Sebti *et al.*, 2005). Take noted of the various structures inside the *Aspergillus niger* by microscopic magnification 40X and 100.

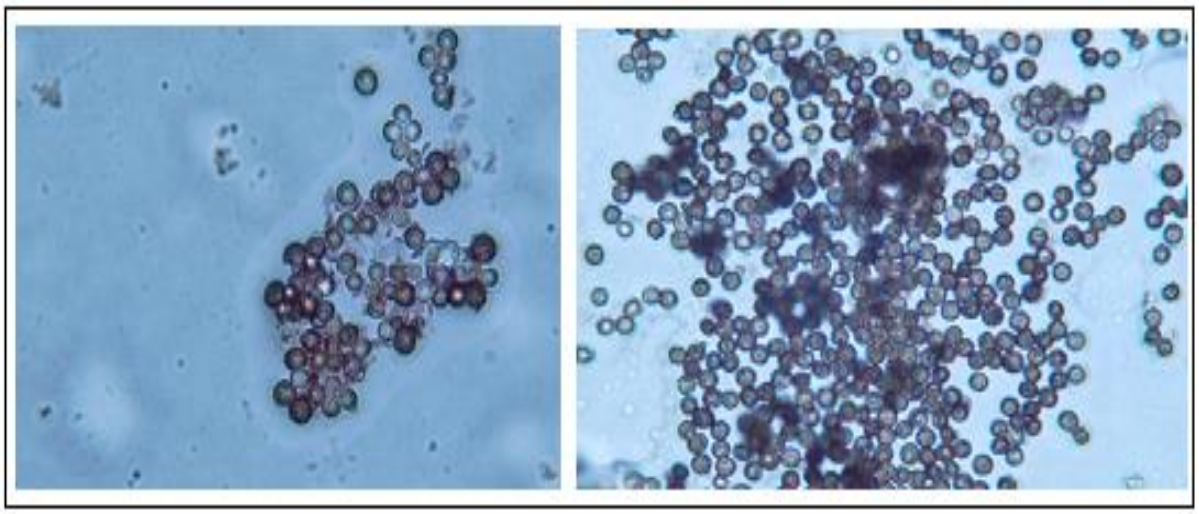

Fig. 8. Light microscopic analysis of *Aspergillus niger* mycelium and spores under 100X magnification.

A number of images of *Aspergillus niger* were digitally produced with higher magnification. It was ideally suited for observation of intact sporing structure over a wide range of magnification, duplicating and supplementing information. SEM provides results of different ranges of mycelium in 4 mm, 200 µm and 100 µm with the magnification of 40X, 250X and 500X at 9 KV.

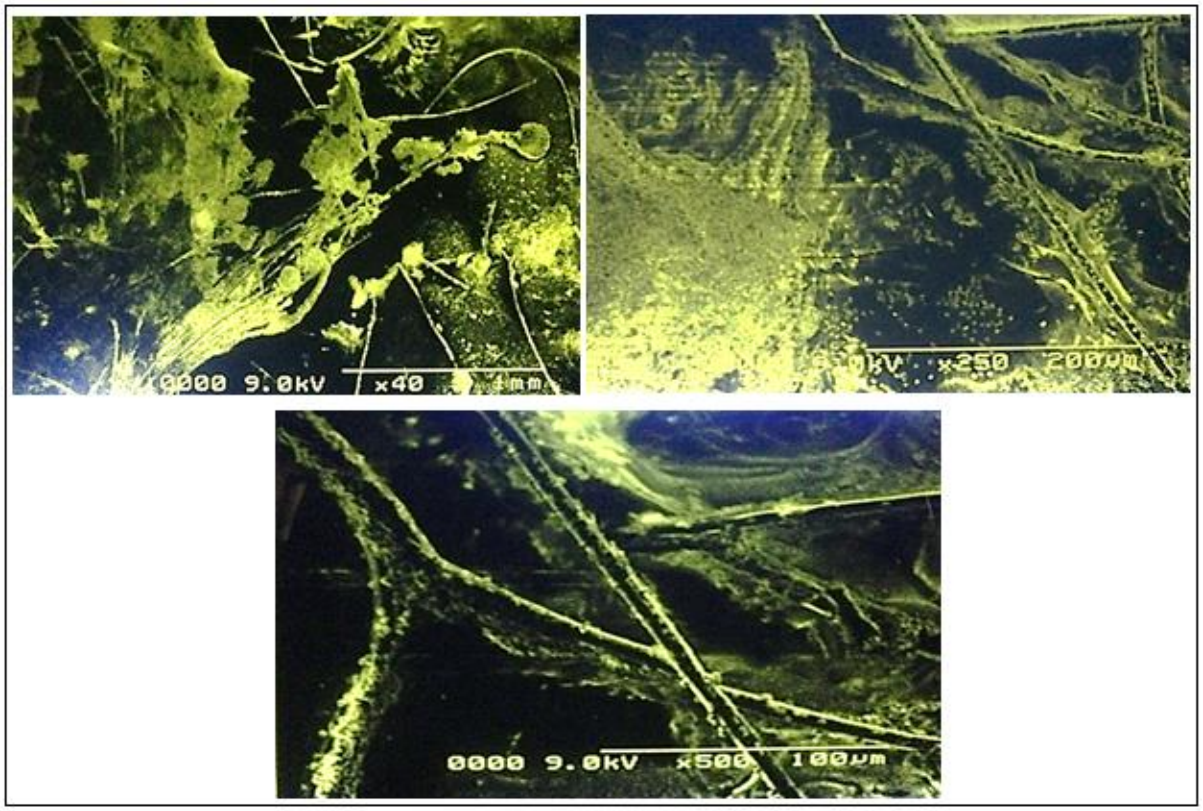

Fig. 9. *Aspergillus niger* under a scanning electron microscope at 9 kV.

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