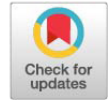

Research Article

Analysis of Fungal rDNA for the Identification of the Pathogen Associated Black Rot of Pumpkin

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Abstract | Pumpkin (*Cucurbita pepo*: *Cucurbitaceae*) is a heat-labile plant, having nutritional value containing large number of active compounds like carotenoids, alkaloids and flavonoids and necessary for food security against disease causing agents like bacteria, fungi etc. Black rot is a serious disease in pumpkin caused by fungus (*Didymella bryoniae*) and damaged fruit with the symptoms of dark brown lesions but causal organism identification through DNA is still unknown. We collected infected leaf from *C. pepp* from the vicinity of The University of Agriculture, Faisalabad and isolated *D. bryoniae* on the respective media. Then morphologically identification was observed through fungal growth pattern, growth color, spores shape, spores color, hyphal septation. For the confirmation of Koch's postulates, the pathogenicity test was performed. CTAB method and Internal Transcribed Spacer region were applied to isolate and amplify the fungal genomic DNA respectively, by using universal primers ITS1/ITS4. The analysis of polygenetic of an amplified product revealed that black rot caused by *D. bryoniae* in pumpkin.

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Introduction

Pumpkin has been used as a conventional cure in Pakistan, Yugoslavia, Mexican areas, China, India, Argentina and Brazil, where pumpkins are commonly cultivated for fruit, oil seeds to fulfill the nutritional requirements as well as a low-calorie vegetable (Ulrich *et al*., 2022). Commonly, three different types of pumpkin naming Butternut Squash (*Cucurbita moschata*), Calabacín (*Cucurbita pepo*) and Hubbard Squash (*Cucurbita maxima)* are found throughout the world (Syed *et al*., 2019). Pumpkin is the widely grown in warm-temperate to temperate areas and having fruit weighs 100 g to a maximum of 20 kg with different shaped like flate and round (Lust and Paris, 2016). Butternut Squash have a good taste mostly used in making stews, custards, jams, pies, and curries even used in medicine (Muchirah *et al*., 2018).

Butternut Squash pumpkin grown for seed oil extraction, toco-pherols content and fatty acid composition with the oil value in that pumpkin variety ranged from 13 to 29 %, with 70-31% of unsaturated fatty acid content. Further, linoleic, oleic, stearic and palmitic acid was observed in Pumpkins with a-toco-pherols ranges from 25 to 64 mg/g (Ryan *et al*., 2007).

Pumpkin has wonderful power to combat against different diseases and tablets or suspension can be prepared by extracting and concentrating active compounds of pumpkin for therapeutic purposes and delayed the development of hypertension, lowering gastric level and therapy of breast, lung, and colorectal cancers (Asif *et al*., 2017) The antioxidants used against cancer. From 2007 to 2011, the sauces and seasoning categories were recognized as one of the top ten ethnic items, and it is predicted to maintain its strong development, as sauce consumption grew owing to typical home cooking all over the world. Consumers are interested to buy new products that promote health, which are ready to eat and have high nutritional value and exert positive health effect (Bochnak and Świeca, 2020).

The pumpkin seed and extracted oil in Europe cover around a multimillion-dollar industry. Austria, Hungary, Serbia and Slovenia are pumpkin oil producers. Its success is emerging in other parts of the world, which is gaining popularity slowly but steadily (Patel, 2013). Pumpkin seeds are outstanding nutrient sources consist of minerals primarily copper, magnesium, iron, sodium, zinc, potassium, manganese including potassium recognized act as a reliable source of fighting infections as well as a weapon against diseases such as prostate cancer, arthritis and inflammation and considered as discarded portions but now nutritional elements, which are present in discarded materials, play a significant role in the meat. They can be consumed daily without even any bad impact on people's health.

Black colored rot is fungal disease which produced black colored on the rind and fruit may fall in during or after harvest that got affected by this disease. *D. bryoniae* infects the vegetative parts of pumpkins and other cucurbit plants, the disease is known as gummy stem blight. Having circular, tan to dark brown spots emerge on the leaves, frequently at or near the edges, and gradually grow until the whole leaf is blighted (Syed *et al*., 2019).

D. bryoniae pathogen was basically identified in cucumber (*C. sativus*) plants in greenhouses in several areas of Turkey's Elazg province, with a prevalence rate and disease severity (DS) of 10.79% and 20.02%, respectively (Mutlu *et al*., 2015). *D. bryoniae* can colonize and proliferate rapidly inside the host tissue that's why it is suitable for the plants of Cucurbitaceae family. *D. bryoniae* pathogen was explored and disclosed for the very first time on a muskmelon host in India. The first report of *D. bryoniae* linked with cultivated cucurbit in Italy was isolated from C. *melo* in an unknown year and termed *Diaporthe melonis. Giovanni passerini* enacted it in 1885 (Corlett, 1981).

The disease is also caused by three genetically different species but having same morphology which includes Stagonosporopsis species, *S. cucurbitacearum* (*D. bryoniae*), *S. citrulli* and *S. caricae*. These species have comparable plant symptoms and mycelial communities (Aveskamp *et al*., 2010).

D. bryoniae, an incitant of blight in ridge gourd. Pathogens survived in the form of spores on seeds and plant debris housed inside under ambient laboratory settings, since they generated viable spores for the whole twelve-month observation period (Bhat and Bhat, 2013).

D. bryoniae's survives in crop leftovers for 14 days at temperatures as low as -9°C. It is a facultative necrotrophic fungus that may infiltrate the plant

through natural wounds, especially in old leaves. High humidity and the presence of moisture on leaf surface, highly aided the establishment of pathogens (Bhat and Bhat, 2013).

Ascomycetous infects certain stages of the host plant, causing symptoms on the crop and the fungus to develop (seed-borne, airborne, or soil-borne). Black rot, a widespread disease of Crucifers, infects Brassicas, Radishes, and various Cruciferous weeds (Keinath, 2011, 2013).

Moisture and temperature are significant factors in sporulation, germination, system development and conidia colonization into plant tissue. Temperatures ranging from 61°F to 75°F and a moisture level of 85% RH induced by a persistent leaf wetness of 1-10 hours are ideal for the infection process (Olson and Santos, 2010). Several approaches, including physiological, cultural, biochemical, morphological, and molecular investigation, may be used to identify *D. bryoniae* by using a polymerase chain reaction (PCR)-based analytical approach (Keinath, 2011) and random amplified polymorphic DNA (RAPD) typing. PCR primers and RAPD markers were utilized to differentiate two *D. bryoniae* genotypes as well as virulent or nonpathogenic *Phoma* species (Somai *et al*., 2002).

A very little data is available about black rot of pumpkin in Pakistan. Therefore, in current studies pathogenicity and molecular identification of pathogen is achieved. In Pakistan, no molecular characterization was done on this pathogen for identification that is much needed for disease management. So, this study was designed for molecular characterization of the pathogen associated with black rot of pumpkin.

Materials and Methods

Collection of samples

Black rot infected pumpkin leave samples were collected in polyethylene bags from the University of Agriculture, Faisalabad. Samples were covered to avoid humidity and stored at 40°C in refrigerator for further use.

Isolation, purification, identification of pathogens and cultural characterization

The fungus associated with black rot was isolated using potato dextrose agar (PDA). Afterward the infected portion of the leaves was cut into small pieces and disinfected with 70% ethanol and distilled water 2 to 3 times. Then it was transferred aseptically to the incubated (at 26±5°C for 2-3 days) Petri plates that contained PDA medium. Isolated material was sub-cultured to obtain the pure culture. The isolated fungus was examined under light microscope and different morphological fungal characteristics were observed, for example fungal growth pattern, growth color, spores shape, spores color, hyphal septation and color. Fungi was identified and divided into various groups on morphological basis.

Initially, the hyphae from the culture colony of associated fungus were deposited on a glass slide and dropped lacto-phenol cotton blue dye on the slide and covered with cover slip. Placed the slide under the compound microscope and viewed on different magnifications such as 10X, 40X and 100X respectively, for the description of microscopic analysis of associated pathogen. The conidia are light to dark brown colored, obclavate to obpyriform or ellipsoid, transversally as well as longitudinally septate. Spores are narrow, branched and conidia emerged from the terminals of conidiophore (Figure 1). After a brief examination, the pathogen was identified on the basis of their morphological characters by using taxonomic keys and various literature of associated fungus.

Figure 1:

DNA extraction

CTAB method was used to extract the fungal genomic DNA. Almost 50 mg of mycelia of 10 days old pure fungal PDA culture was taken and the procedure was carried out as explained by Doyle and Doyle (1987).

Pathogenicity test

All isolates were undergone by the pathogenicity test, as specified by (Keinath, 2013). Pumpkin seeds were grown in earthen pots filled with sterilized soil. The spore suspension (10^6 conidia/mL) was injected/

sprayed on healthy plant leaves. Re-isolation was done from infected leaves after the appearance of symptoms. The obtained cultures were compared to the original culture to validate the pathogen's identification and pathogenicity.

Agarose gel electrophoresis

Electrophoresis examined samples of DNA in 1-percent Agarose gel which were detected after staining with EB (Ethidium bromide). Further, the samples were observed under UV light and snapped via Gel DocTM EZ imager.

Quantification of DNA

A nano drop spectrophotometer was used to measure the DNA concentration in each sample (Thermo Scientific Nano Drop 8000 UV-Vis Spectrophotometer).

PCR amplification and purification

Pathogen specific universal primers were used to establish PCR-based amplification of DNA. The complete ITS region of rDNA was amplified with universal primer ITS 1 as forward primer (5'–TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5' TCCTCCGCTTATTGATATGC-3') as reverse primer. PCR amplification conditions were optimized and final amplifications was made in the overall reaction of 25 µl. The PCR Master Mix included the enzyme Taq DNA polymerase, 2X Dream Taq buffer, 0.4 mM dGTP, dATP, dTTP, dCTP, and 4 mM MgCl2. Forward primer 0.1-1 M and reverse primer 0.1-1 M were used, as well as template DNA or mycelia ranging from 10.0 g to 1.0 g and nuclease free water to form a total volume of 25µl. Amplifications were performed in thermos-cycler programmed for 95ºC for 3 minutes. Followed by 34 cycles at 95ºC for 40s, 55º C annealing for 30s, and 72ºC for 60 seconds & a final extension for 10 minutes at 72ºC. Electrophoresis was used to separate the PCR sample in 1.0% Agarose gel which were prepared in 0.5X TBE buffer. Besides, amplified products were purified through PCR purification kit (Favor Prep PCR Clean-up Mini Kit).

Sequencing and phylogenetic analysis

Sanger sequencing was used to analyze the entire nucleotide sequences of purified PCR products with specific inner primers. Sequencing samples was resolved commercially. Besides, phylogenetic analysis of ITS region was performed using isolates of species associated with black rot disease (NCBI, DNA data bank). MEGA-7, accessible online software was used to produce Sequence alignments and phylogenetic analysis.

Sample collection

Infected leaves of pumpkin (*C. pepo*) with leaf spot disease were collected from the vicinity of University of Agriculture. The leaves showing characteristic symptoms on the round, tan to dark brown dots that occur on the leaves, usually at the edges, were collected (Figure 2). During the field demonstration, it was noticed that the disease mostly appeared at the start of June and mid of July in the rainy environment.

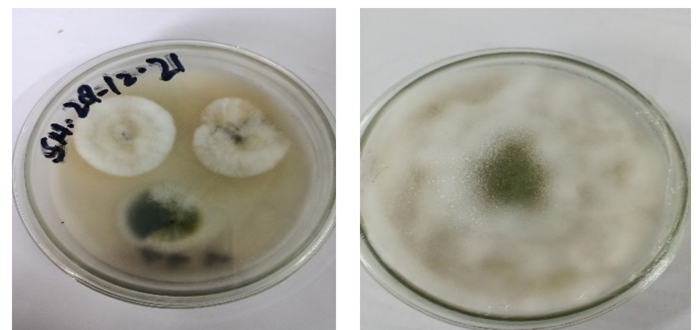

Figure 2: *The Cultural Colonies of associated fungus D. bryoniae white aerial and olivacious mycelium observed that turned to green color in the center with white fluffy hyphal margins.*

Isolation and purification of pathogen

The isolation of an associated fungus *D. bryoniae* was done on PDA (Potato Dextrose Agar) media by using the standard tissue isolation technique from infected leaf samples of the pumpkin. Isolated samples were repeated for several times to obtain the pure culture colonies of the associated fungus (Figure 1). Initially, the culture colonies of the fungus exhibit white aerial and *Olivacious mycelium* observed that turned to green color in the center and ultimately become dark black in color. After 7-8 days, white fluffy mycelium grows at the margins of the culture colonies.

Pathogen detection based on morphology

Figure 1 isolation and purification of fungal pathogen *D. bryoniae*. The infected portion of leaves was cut into small pieces and disinfected with 70% ethanol and distilled water twice or thrice. Then infected portion was transferred aseptically to the petri plates that contained Potato Dextrose Agar (PDA) medium. Besides this, the PDA plates were incubated at 26±5˚C for approximately 2-3 days. Then sub-cultured the isolated material to obtain pure culture colony.

Identification of an isolated fungal pathogen, the pathogen was done under a compound microscope and morphological traits were compared for identification. After subculturing, the related fungal spore was extracted from the hyphal border of the colony culture plate and examined under a compound microscope at 40x magnification.

Culture colonies of *D. bryoniae* with white, aerial and olivacious mycelium that became green in the middle and white, fluffy hyphal borders were seen (Figure 2). Conidiophores generate single or small groups of conidia that are branching or simple, straight or two-celled in form, and emerge from the conidiophores' terminals.

An in-depth investigation of the fungi's morphological and conidial properties, both on PDA and by microscopy, revealed that *D. bryoniae* was the causal agent of the suspected illness. In the early phases of fungal development, white aerial and *olivaceous mycelium* was identified, whereas in the later stages, olive to dark, green or black substrate mycelium was found. There was no perithecium found. The surface of the colony on the Petri dish was rough and undulated. Microscopic examination of the conidia revealed that they were round-ended, cylindrical, monoseptate, and hyaline. Conidia varied in length and diameter from 6.4 to 13.6 and 3.69 to 4.68 m, respectively.

Preservation of pathogen

D. bryoniae pathogens may be preserved as dried cultures on a sterile filter paper. A 75mm diameter sterile filter paper circle is centered on a 100mm diameter Petri dish with one-quarter-strength potato dextrose agar using conventional sterile methods. The filter paper should not entirely cover the agar. There should be a thin agar border around the dish's edges. Cut two pieces from an established culture and put them on agar, not on the filter paper, but along the border of the filter paper. As previously stated, culture is incubated for 7 to 14 days. When the colony has completely filled the filter paper circle, it is removed using sterile forceps, put in a sterile Petri dish, and left in an operational laminar flow hood for 3 to 5 days. The dried filter paper is cut into 0.5- to 1-cm square pieces using sterile forceps and sterile scissors and kept in sterile glass vials at 5°C. Cultures on the filter paper may live for up to ten years. To begin a new culture, lay one or two filter paper squares on agar and incubate as previously indicated.

Pathogenicity test

The validation of pathogen *D. bryoniae* was done by conducting the Pathogenicity test while using Koch's postulates. The pumpkin plants were grown in pots in the greenhouse (Figure 3). The spore suspension ($10^{\circ 6}$conidia/ml) was prepared from 7 days old culture of the associated fungus (*D. bryoniae* and was sprayed on the injured leaves of 1-2 weeks old pumpkin plants grown in the pots (Keinath, 2013). Symptoms appeared on the leaves after 7-14 days of inoculation. The plants apparently showed the symptoms small circular spots with tan to dark brown spots. The plants that were grown as control, were sprayed with distill water and did not show any symptoms. The pathogen was re-isolated from the infected leaves and then identified on morphological as well as microscopic characters.

Figure 3: *(A) Pathogens (Didymella bryoniae) were validated by conducting the pathogenicity test through (Didymella bryoniae) Koch's postulates. The pumpkin plants were grown in the pots in the greenhouse. The spore suspension (10°6conidia/ml) was prepared from 7 days old culture of the associated fungus (Didymella bryoniae) and was sprayed on the injured (pin prick method) leaves of 1-2 weeks old spinach plants grown in the pots. Symptoms appeared on the leaves after 7-14 days of inoculation. (B) Plants apparently showed the symptoms of light brown to dark brown spots with concentric rings and leaf margin are curled. These symptoms were compared with the original and pathogen was re-isolated to check the identity and pathogenicity.*

Results and Discussion

Current study was designed to characterize the associated pathogen at the molecular level in Pakistan. Pathogen was isolated, purified and identified on the basis of morphology such as color and shape of culture colonies, color and shape of conidia and conidiophores; septation, etc. Koch's postulates validated pathogen by conducting pathogenicity test. The plants were inoculated artificially and control

plants were sprayed with distilled water. The first symptom appeared was a small circular spot with concentric rings. Later, the symptoms developed into dark brown to black in color and leaf margins become narcotic and blighted in sever conditions. The pathogen was re-isolated from the infected plant parts and identified on the morphological basis. A standard tissue isolation technique was used for the isolation of *D. bryoniae* from the infected leaves of the plant. The pathogen was identified on morphological characters. The culture colonies of the associated fungus *D. bryoniae* were olive green in color initially then turned to dark green color with white fluffy hyphal margins. Conidia emerged singly or in small groups, branched or simple, straight and flexuous.

Following the pathogenicity test, re-isolated and purified cultures from infected plants resembled the original. The plants doused with distilled water showed no signs of illness. Fungal genomic DNA was extracted for molecular characterization of the pathogen. ITSI/ITS4 primers were used to amplify the rDNA's Internal Transcribed Spacer region.

DNA isolation and gel electrophoresis for DNA samples

We developed a protocol used to extract fungal genomic DNA. Then, DNA was resolved electrophoretically in 1% agarose gel with 6X DNA loading dye at 120 volts for 40 minutes. 6X DNA loading dye =0.5 µl. The results of gel electrophoresis for DNA samples are given below in Figure 4.

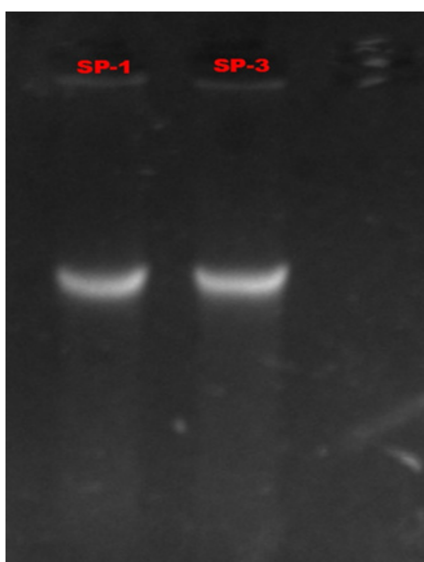

Figure 4: *Total genomic DNA of associated pathogen (D. bryoniae) from two isolated SP1 and SP3.*

Polymerase chain reaction (PCR)

After DNA extraction, PCR was conducted for the amplification of ITS region of rDNA. ITS1/ITS4 primers were used for the amplification of Internal Transcribed Spacer (ITS) region of the rDNA. The observed amplicon size was approximately 500-600 base pairs. PCR profile was set as shown in the Figure 5.

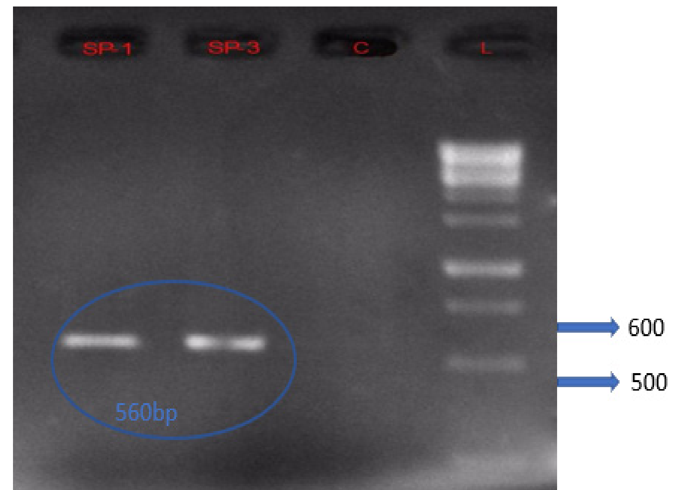

Figure 5: *The results of PCR of internal transcribed spacer (ITS) region of rDNA and mitochondrial small-subunit of rDNA.*

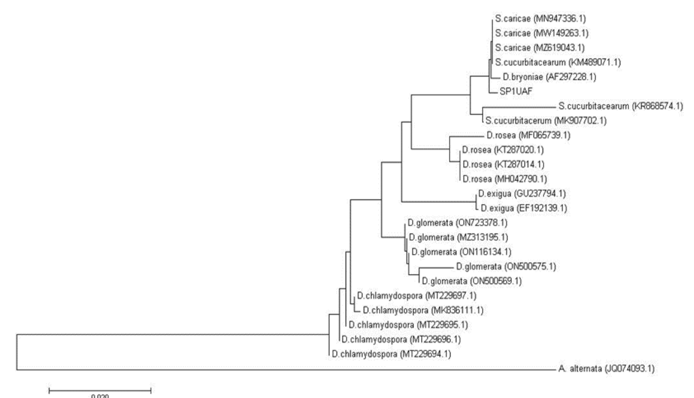

Figure 6: *Neighbor joining phylogenetic dendrograms based on an alignment of D. bryoniae and selected additional species' projected nucleotide sequences (ITS region). The numbers at the nodes represent the percentage bootstrap confidence values (1000 replicates). Horizontal lines are proportional to predicted mutation distances, whereas vertical lines are arbitrary. The tree is randomly rooted to the nucleotide sequence of the distantly related Alternaria alternate (JQ074093.1) fungus.*

Analysis of internal transcribed spacer (ITS) region of rDNA and mt SSU of rDNA

Sequencing: After trimming the obtained sequence was submitted to GenBank. Sequencing was done commercially. Phylogenetic analysis shows that the associated fungus is closely related to *D. bryoniae* in the phylogenetic tree. We downloaded the BLAST sequences from the NCBI site that showed 100% identity and 100% query cover and further proceeded by the use of Mega 7/CLC main work bench 8 software.

There has been a tremendous expansion in the evolution and use of various molecular techniques in phylogenetic study of various fungal species and populations. Molecular methodologies and techniques are more stable, faster, sensitive, and less dependent on environmental conditions than the morphological ones (Faggi *et al*., 2001). To detect and discriminate fungus, fungal genomic DNA characterization has been used (Gil-Lamaignere *et al*., 2003). Various DNA-based approaches can reveal a fungal variety in ecosystems, with the great benefits of extremely sensitive and fast detection (Gonzalez and Saiz-Jimenez, 2005). For molecular identification and other species level tracking in many fungus groups, ITS (Internal Transcribed Spacer) region of rDNA has become the definitive genetic marker. Lama Fungal species identification has mostly relied on varied rDNA (ribosomal DNA) ITS (Internal Transcribed Spacer) sections. Because of its large copy number in the fungal genome as a portion of repeated nrDNA (nuclear ribosomal DNA), the non-coding ITS region comprising of ITSI, ITS2 and 5.8S DNA should be used to construct an extraordinarily sensitive probe for amplification of the target gene sequence. Internal Transcribed Spacer region sequences from several fruiting bodies of a well-known pathogen are accessible for the expected number of 1% fungal species. The sequencing of ITS region is mostly done (Nilsson *et al*., 2005). When some conserved coding regions of ribosomal RNA (rRNA) genes are compared with ITS region, the rapid rate of evolution benefits these ITS regions, which results in maximum distinction in sequence between those species that are closely related to each other. To summarize, DNA sequences in the internal transcribed spacer (TTS) region yield more taxonomic resolution than other coding regions (Anderson *et al*., 2003; Lord *et al*., 2002). Furthermore, exceedingly varied DNA sequences in the Internal Transcribed Spacer (ITS) region may serve as the marker for groups that are taxonomically more obscure from one another. Most significant open archives for sequenced material included The European Molecular Biology Laboratory (EMBL), the International Nucleotide Sequence Database (INSD: GenBank), and the DNA Database of Japan (DDBJ) (Anderson *et al*., 2003). Internal Transcribed Spacer region ITS1/ITS2 Intergenic spacer IGS are the most often sequenced and compared in the rRNA (rRNA) gene cluster for phylogenetic analysis. This is due to the fact that these multicopy genes are highly conserved within the species, but might be completely different between species).

Phylogenetic analysis

A phylogenetic tree tells us the evolutionary relationships or evolutionary history among a set of objects. The example of objects is categories of species, biological species, proteins and nucleic acids. We used 'Mega7' software to create the Polygenetic tree. This software is used to draw the conclusion related to the mutation distance of the test strain with pathogenic, nonpathogenic beneficial fungal species based on the DNA sequence of ITS (Internal Transcribed Spacer) region of rDNA. We produced a phylogenetic tree to indicate the estimated period, and one of the fungal species evolved from the nearest neighbour, revealing the pathogenic fungus's evolutionary link (*D. bryoniae*). In order to understand the evolutionary relationship of isolates, we sequenced ITS region and constructed the phylogenetic tree from various species of *D. bryoniae* such as *S. cucurbitacearum, S. caricae, D. glomerata, D.rosea, D. exigua, D. chlamydospora*, *D. hellebori*, available in GenBank. *Alternaria alternata* (JQ074093.1) was used as the out group. For phylogenetic analysis the data was recovered from NCBI, the sequence of genomic DNA from the ITS region was analyzed for molecular characterization of the pathogen. 25 sequences were documented in the form of 'FASTA file' and the FASTA file was added to software (MEGA 7/CLC main work bench 8). Use of MUSCLE alignment made any of these sequences aligned, and selection of delete just the chosen block. Deleted all sites with gaps and missing data in this phase. The whole aligned sequence was retained for later use in creating phylogenetic dendrograms. We used the evolutionary history to build the phylogenetic tree. Then we made the use of Neighbor Joining method' in software to build the phylogenetic tree. We discovered that the proportion of duplicate trees created is followed by associating all of the species grouped together in a test Bootstrap utilizing 1000 repetitions for tree construction. This is done to eliminate mistakes and improve accuracy in the tree. *D. bryoniae* is widespread on all six inhabited continents, while the number of confirmed occurrences from South America and Africa is minimal (Keinath, 2011).

Conclusions and Recommendations

The current study was conducted at the University of Agriculture in the department of plant pathology

from 2021 to 2022 to identify the causal organism of red rot of pumpkin in Pakistan. After a comprehensive study based on symptomatic data, morphological data, pathogenicity, molecular and phylogenetic analysis, it was concluded that the responsible pathogen for Black rot of pumpkin disease in Pakistan is *D. bryoniae.*

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Author's Contribution

Sidra Hafeez conducted the study. All other authors helped her in writing, data analysis and submission of the research article.

Conflicts of interest

The authors declare no conflict of interest.

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