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Original Research

First Report of Leaf Spot of *Conocarpus lancifolius* Caused by *Alternaria burnsii*

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Abstract

Conocarpus lancifolius leaves were observed to have typical symptoms of fungal leaf spots in botanical gardens and various nurseries, mainly during the winter and rainy seasons of 2022 and 2023. Leaves of infected plants showed round to irregular grayish-white and dark brownish to black spots with a yellow halo and central whitish color appearance that were variable in size, ranging from 0.5 to 1.5 mm in radius. After processing the samples and purification of the fungus, morphological characteristics such as its color, mycelium pattern, and spore shape were used to identify it as belonging to the genus *Alternaria*. Following morphological identification pathogenicity test was performed according to Koch's postulates, proving that the pathogenic fungus *Alternaria burnsii* was associated with *C. lancifolius* causing leaf spots. The CTAB method was used to extract DNA to perform molecular characterization and further species-level identification. The fungal DNA was amplified using the ITS and GAPDH genes. Sequencing was done by Macrogen, Korea. A phylogenetic tree was constructed against both genes (ITS and GADPH) by using the sequence of 20 isolates; 19 belong to the Alternaria family and 1 is an outgroup. The suspected isolate with accession no OQ689862, and OQ910482 showed 99.48% and 100% similarity with OP985911 and MN718663, respectively. After submitting sequences to NCBI

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BanKit, the following accession numbers were assigned against the ITS and GAPDH gene sequences: OQ689862 and OQ910482, respectively. After molecular and morphological characterization, it was determined that *Alternaria burnsii* was isolated and associated with the leaf spots of the *C. lancifolius* plant by satisfying Koch's postulates. According to our research, this is the first report of the leaf spot of *C. lancifolius* caused by *A. burnsii*. It is suspected that in the future, *C. lancifolius* will be infected by other pathogenic microbes. Therefore, to control the disease spread, highly effective management strategies should be adopted.

Keywords: *Conocarpus lancifolius*, leaf spot, molecular characterization, ITS, GAPDH, Koch's postulates

Introduction

Conocarpus lancifolius is an evergreen ornamental tree belonging to the Combretaceae family [1–3]. These plants are found in the world's subtropical and tropical regions, including Western Africa, Saudi Arabia, The United States, The United Arab Emirates, Kuwait, Iraq, and Pakistan [4]. There are around 500 species in this family [5]. This low-branching, multi-trunked, shrubby, evergreen tree bears glaucous medium-green leaves with little greenish flowers. These have thick cone-shaped heads that develop in terminal panicles in the spring and are followed by half-inch-long red to brown fruits with a cone-like shape. Usually, *C. lancifolius* is 1 to 10 m tall [6]. The appealing dark brown bark is ridged and scaly [7]. It is indigenous to Florida's woodland habitat in North America [8]. Around the world, this species can be found along shorelines in tropical and subtropical climates [9]. It was brought to Pakistan because it is tolerant to heat and drought, and can absorb brackish water [10].

Conocarpus trees are among the significant ornamental trees produced primarily for aesthetic, climatic, and recreational purposes [11]. Due to their resistance to climatic conditions like high temperatures and salinity, these can even be grown in desert areas [12]. These trees are valued for their ability to purify the air as well as their ornamental value in public gardens, sidewalks, and streets [13]. Anemia, catarrh, conjunctivitis, diabetes, fever, headache, bleeding, tumors, orchitis, prickly heat, swellings, and syphilis are among the conditions for which the plant is used as a folk treatment [14]. For fever, the leaves can be consumed or their decoction consumed. For fenceposts, crossties, turnery, boats, firewood, and landscaping, wood is employed. In parks, boulevards, and other urban green places like yards, this shrub is grown purely for decorative purposes [15].

Conocarpus trees are susceptible to many plant diseases brought on by various fungi, which can result in significant losses across the globe [16]. Fungi belonging to the genus *Alternaria* have been identified as causing disease in Conocarpus [9]. *Alternaria spp.* has been found to affect *C. lancifolius,* as well as different hosts and species, such as pumpkin seeds and cumin plants [17]. The goal of this work was to isolate and identify a fungal pathogen linked with leaf spot disease. As a result, the current study was designed to characterize the pathogens affecting *C. lancifolius* plants.

Experimental Procedures

Survey and Sample Collection

In 2023, a thorough survey was carried out, examining 300 trees near the Islamia University of Bahawalpur shown in Fig. 1. The diseased foliage was gathered and preserved in sterile polyethylene bags. Samples were kept cold at 4°C until analyzed further [18].

Isolation and Purification of Pathogens

From trees, leaves exhibiting symptoms were collected. After being cut into tiny (5 mm) pieces and surface sterilized for one minute with 1% sodium hypochlorite, the diseased samples were thrice washed with distilled water. After air-drying on sterile filter paper, the leaf tissues were put on potato dextrose agar (PDA) plates and incubated for three days at 28±2°C. A 5 mm disk cut from the edge of the fungal culture was used to collect and purify the mycelia that had formed from the tissues and put them on newly prepared PDA plates. Throughout the research, these plates were periodically multiplied and sub-cultured [19].

Morphological identification

Microscopic examination was performed to identify the isolated fungi depending on their morphological features, including culture colony color, spore size, and shape [19, 20].

Pathogenicity Tests on Detached Leaves

Healthy *C. lancifolius* leaves were surface sterilized and washed with sterile water three times for a pathogenicity test. A sterile inoculation needle was used to create a cross on the leaf. With the mycelia pointing downward, a mycelial plug (5 mm in diameter) was punched out from the margin of a colony that had been growing for three days and placed into the cross's center. Wet, sterile

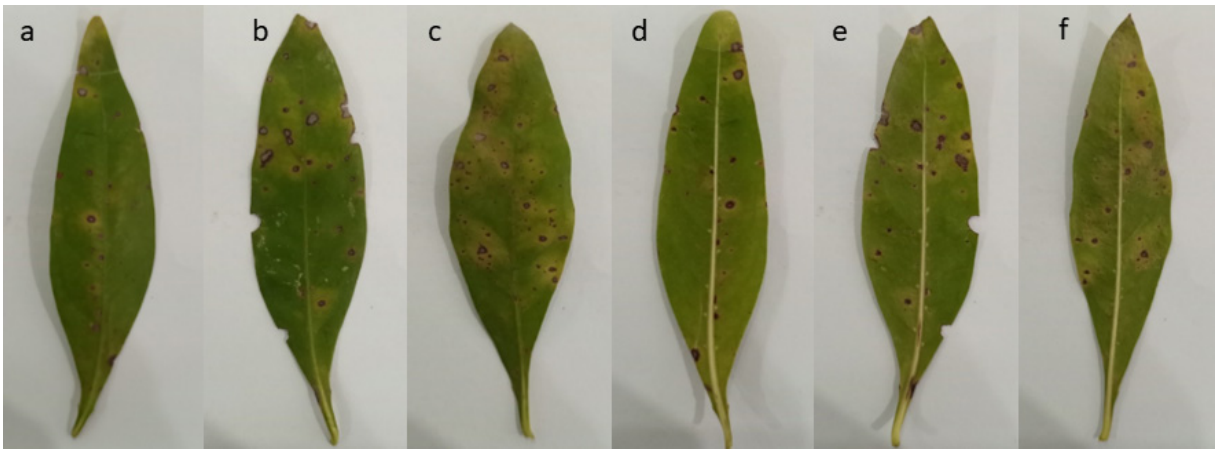

Fig. 1. (a, b, c) shows the front side of the leaf having leaf spots that range in size that were discussed above, and (d, e, f) shows the back side of the leaves having spots on them.

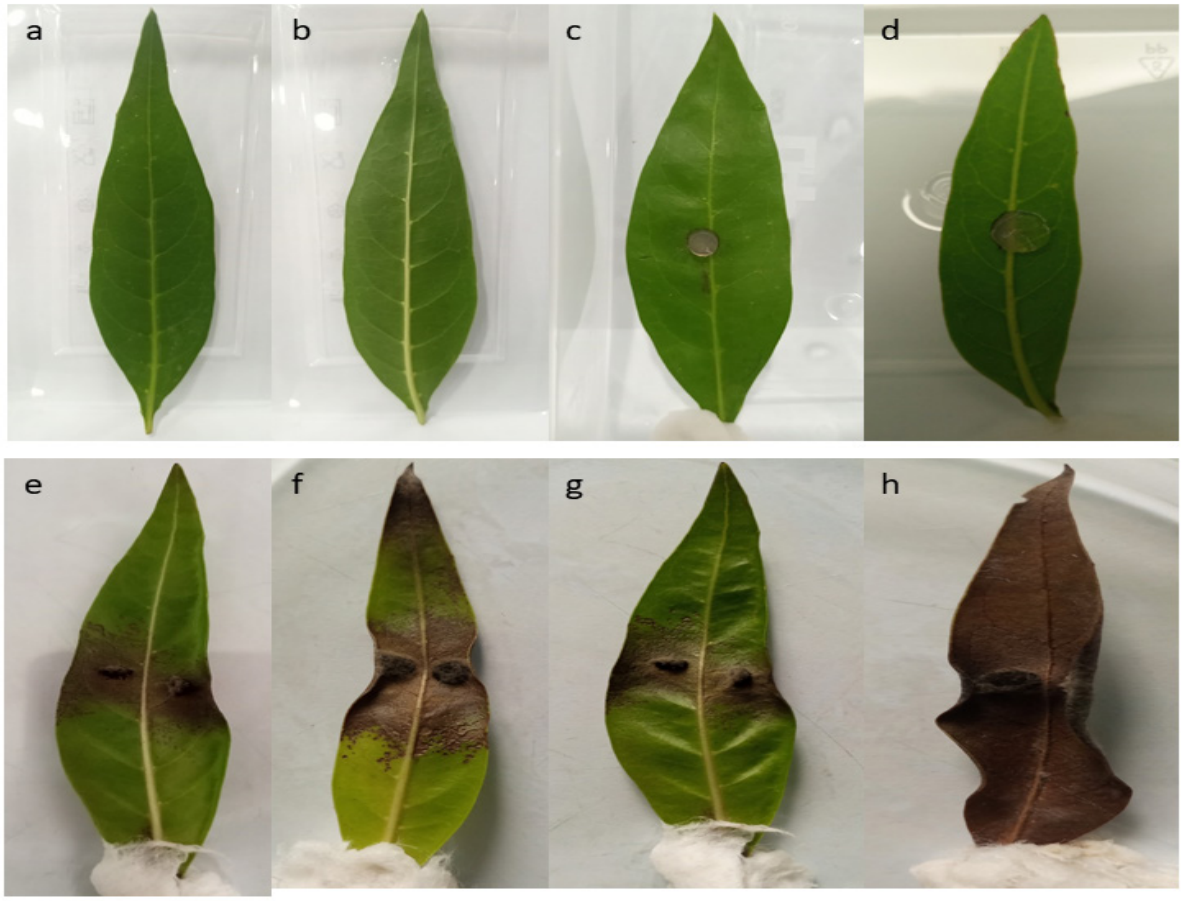

Fig. 2. (a, b) shows the front and back side of the healthy plant, (c, d) shows the control leaf front and back side, (e, f, g, h) shows the presence of fungus on the leaf that was infecting the leaf.

cotton is placed over the petioles of leaves. The treated leaves were then arranged in a plastic box. As controls, PDA plugs devoid of mycelia were utilized. Every treated leaf was maintained at 28±2°C in an incubator, as shown in (Fig. 2). The experiment was conducted on triplets, and after five days, the leaves were visually inspected, along with the sizes of symptomatic areas on the leaves [21]. To satisfy Koch's postulates [22], the fungi were

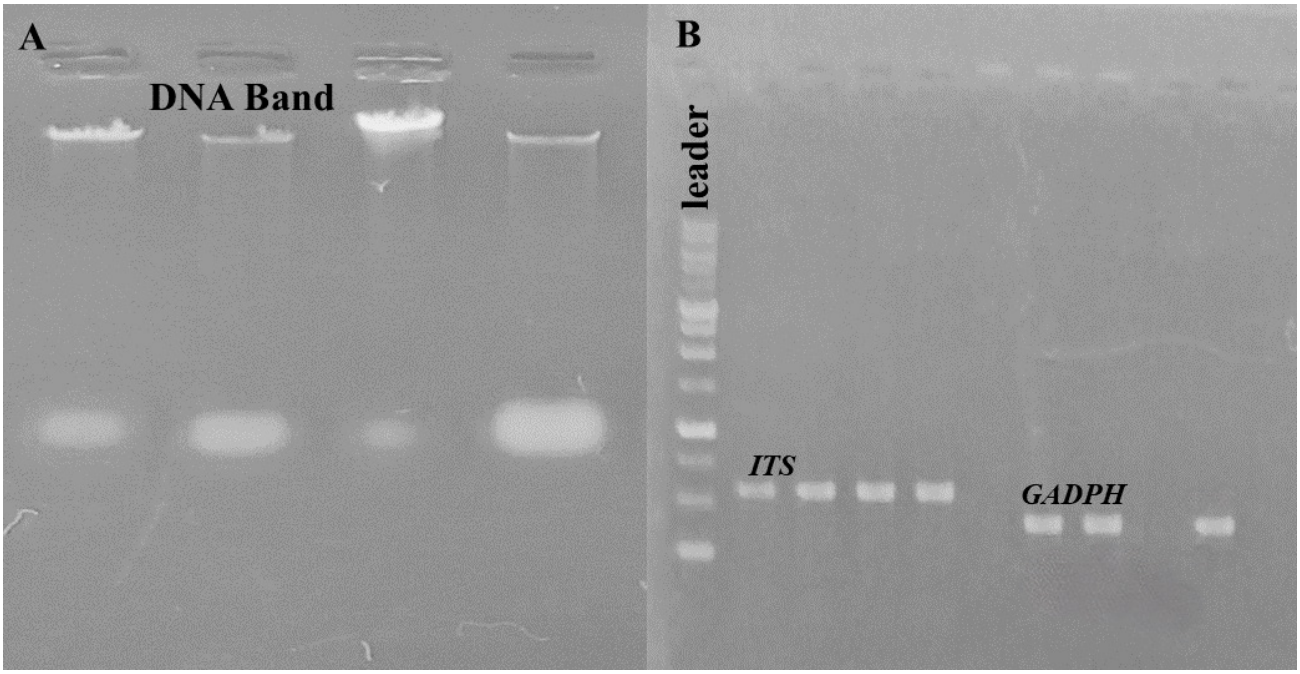

Fig. 3. (A) shows the DNA band of the isolated fungus, and (B) shows the band of ITS and GAPDH amplified product.

reisolated from the infected leaves and identified based on colony and conidial morphology accompanied by ITS and GAPDH sequencing.

Fungal DNA Extraction

Fungal DNA extraction was done by using the CTAB method for molecular characterization [23]. A fungal broth was prepared by putting 3 plugs of mycelium in 100 ml of potato dextrose broth (PDB). Four-day-old mycelium culture was harvested from PDB by using a centrifuge machine. 3 g of mycelium was shifted in 1.5 mL of Eppendorf tube. 1 mL of CTAB was added to the Eppendorf tube and mycelium was crushed with the help of a micro pestle. After crushing tubes were centrifuged at 1000 rpm for 10 min. 900 µL CTAB was shifted to a new Eppendorf tube of 2 mL. Add an equal amount of PCI in the tube and centrifuge the tubes at 1500 rpm for 15 min. Separate the uppermost layer to a new Eppendorf of 1.5 mL and add an equal amount of chilled isoamyl alcohol. Store the tubes at 40°C for 24 h. After that, the tubes were centrifuged at 1000 rpm for 10 min in a refrigerated centrifuge machine. Discard the upper layer and keep the pallet in the tube. Allow the tubes to air dry for 15 to 20 min. Add 50 µL of TE buffer to dissolve the DNA pallet for the usage of gel electrophoresis. After the confirmation of DNA extraction, the internal transcribed spacer (ITS) and glyceraldehyde-3-phosphate dehydrogenase GAPDH gene were amplified and sequenced. Sterile distilled water is used as a negative control [23].

PCR Amplification

The isolate was characterized by amplification and sequencing of the ITS and GAPDH region, using ITS1 and GAPDH primers. PCRs were performed in a volume of 25 µL in a thermal cycler [24]. The reaction mixture contained 12.5 µL Thermo Scientific Green Taq PCR Master Mix 2×, 1 µL of each ITS1 and GAPDH primer, 2 µL of DNA template, and 8.5 µl DNase/RNase-Free Distilled Water. Cycling conditions were as follows: initial denaturation of 5 min at 94°C; 35 cycles of 40 s denaturation at 94°C, 40 s annealing at 55°C, 1 min extension at 72°C; and a final extension for 10 min at 72°C.

Sequencing and Phylogenetic Aanalysis

A genomic DNA ladder of 1Kb was used to measure the size of the amplification and confirm the amplified PCR products on a 1% w/v agarose gel [25]. After getting the positive bands on the gel shown in (Fig. 3), samples were sent to Korea (Macrogen) for sequencing. The sequences that were acquired were entered into the NCBI public database to receive GenBank accession numbers. ITS and GAPGH sequences of *A. burnsii* were retrieved from the NCBI and compared with the isolate used in this investigation. Using Basic Local Alignment Standard Tools, the genetic similarity between the local and prior isolates was further verified (BLAST).

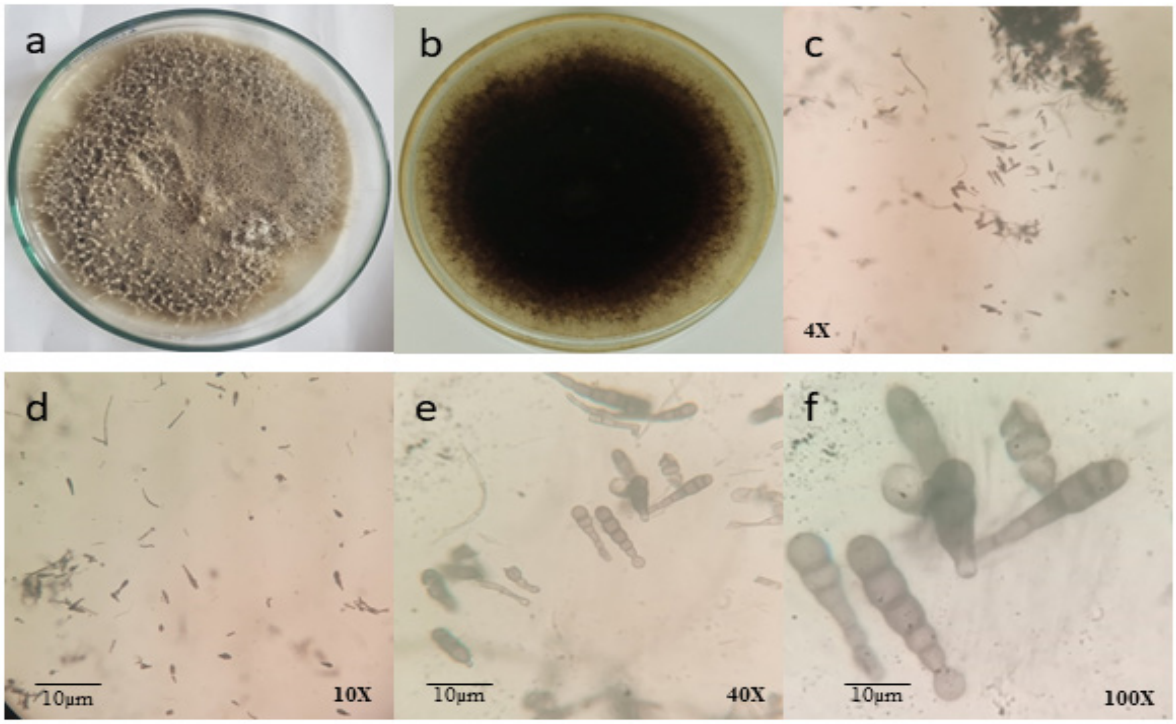

Fig. 4. (a) front view of pure colonies, (b) back view of pure colonies, (c) mycelium at 4X in the light microscope, (d) spores at the resolution power of 10X, (e, f) shows the fungal spore at 40X and 100X respectively of the causal agent A. burnsii.

Results

The survey depicted that no nursery was devoid of leaf spot infections. Every eight out of ten plants were associated with leaf spot. Infected plants showed spots that were round to irregular in shape and light to dark black with chlorotic borders with a central whitish color appearance, ranging in diameter from 0.5 to 1.5 mm. The percentage of the natural infection was ranging from 80 to 83%. Following the pathogenic fungus's purification using the single spore technique, the fungal isolate was examined under a light microscope to determine the sporulation and spore morphologies of fungal cultures that were five to seven days old [20]. Pure colonies feature an uneven, fast-growing obverse with a grayish-white color and a dark brownish reverse with a black inside and a brown outside shown in Fig. 4. As the mycelium was grown on PDA and Malt Dextrose Agar, it displayed aerial hyphae and superficial branches. Conidia were usually found in short to somewhat long chains of two to eight, sometimes more. Their typical length was 15~60 (~90)×6.5~14 (~16) µm, were ellipsoid, long ellipsoid, obclavate, or ovoid, with 1~3 (~4) longitudinal septa and 3~12 transverse septa. These were also occasionally (rarely) branched. *Alternaria burnsii* was identified as a fungus based on its morphological features (Paul NC et all 2015). To verify the legitimacy of this disease, a pathogenicity test was conducted. The pathogenicity on the initial host was confirmed by applying Koch's postulates. A detached leaf assay was carried out by implanting agar plugs containing fungal cultures on excised *C. lancifolius* leaves to evaluate the pathogenicity of the fungal isolates on *C. lancifolius* plants. Disease symptoms induced by the fungus *A. burnsii*, which developed full symptoms after 7 days of inoculation, revealed minor changes. A disease of leaf spot afflicted a large number of plants, resulting in considerable losses. Two days later, the lesions began to overlap each other as these quickly spread from the inoculation sites.

Molecular identification was done by the extraction of DNA by following the protocol with minor modifications [26]. Polymerase chain reaction was performed for the amplification of DNA fragments and PCR products were sent to Macrogen (Korea) for sequencing. Both regions of the internal transcribed spacer and glyceraldehyde-3-phosphate dehydrogenase (ITS1 and GAPDH) of the rDNA of the isolate were amplified. The sequence homology was checked and submitted to NCBI as *A. burnsii*. After critical analysis and verification by NCBI, accession numbers were assigned against the sequence submitted. These are OQ689862 and OQ910482 respectively showing the similarity with OP985911, and MN718663 as shown in (Fig. 5 and Fig. 6).

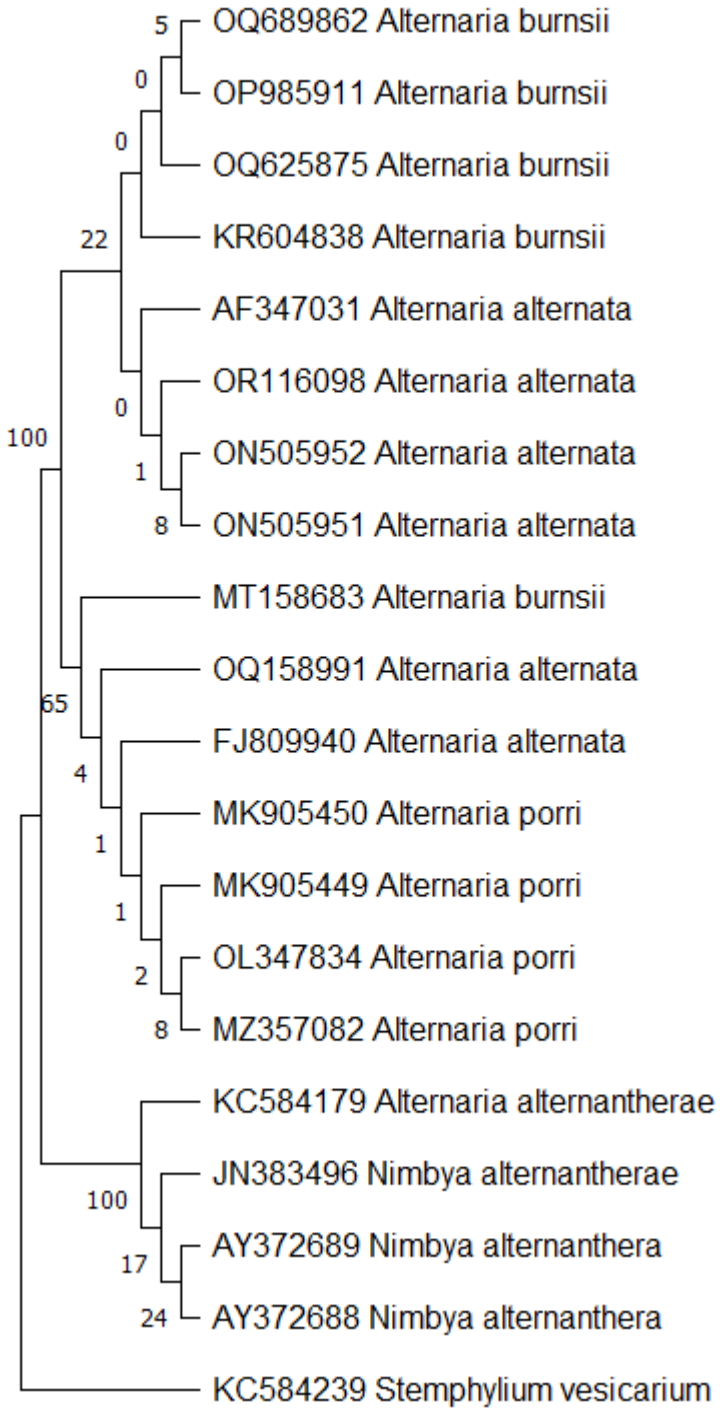

Fig. 5. Maximum likelihood (ML) phylogenetic tree obtained from consensus sequences of *Alternaria* isolates using ITS rDNA primers. Numbers above the branches are bootstrap values from 1000 replicates. The scale indicates the genetic distance between the species. *Stemphylium vesicarium* was selected as an outgroup. The isolate OQ689862 was found highly similar to OP985911.

Discussion

High humidity and temperature fluctuations are the two most frequent issues in Punjab that plants deal with [27]. *C. lancifolius* was brought to the area as a species that could withstand dryness and salinity [28]. *C. lancifolius* can withstand summer temperatures of 47°C and needs very little nutrients in the soil [29]. Plant pathogenic bacteria can thrive in saline environments, but before the year 2000, nothing was known about phytopathogenic fungi

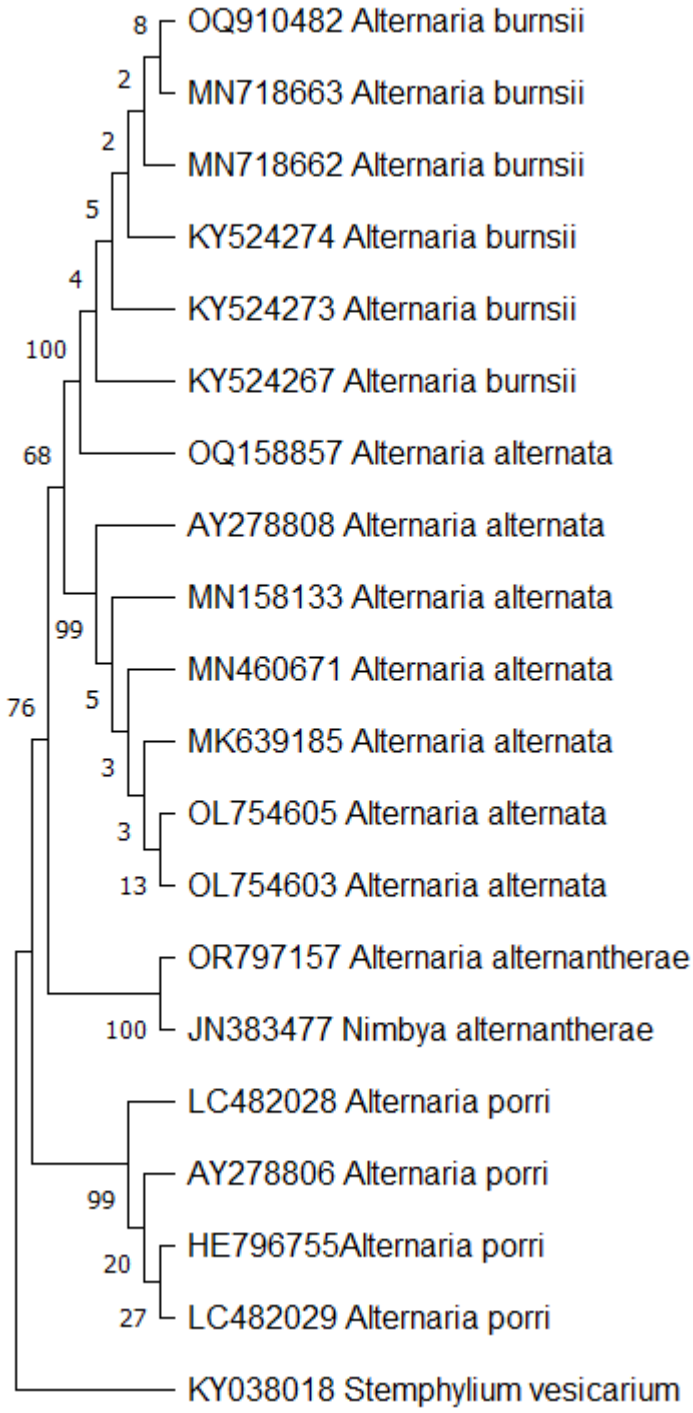

Fig. 6. Maximum likelihood (ML) phylogenetic tree obtained from consensus sequences of *Alternaria* isolates using GAPDH rDNA primers. Numbers above the branches are bootstrap values from 1000 replicates. The scale indicates the genetic distance between the species. *Stemphylium vesicarium* was selected as an outgroup. The isolate OQ910482 was found highly similar to MN718663.

that were prevalent in highly salinized environments [30]. The current study accomplished a novel disease grading scale for *A. burnsii* leaf spot infecting *C. lancifolius* for the first time. Samples were collected following symptoms during the first stage of diagnosis. The symptoms of *A. burnsii* leaf spot can overlap with many biotic and abiotic causes, making the symptomology an unreliable tool for accurately confirming the condition. *A. burnsii* causes cumin blight disease on cumin plants [31], leaf spots on New Guinea impatiens plant [32, 33], and leaf

blight on maize plants [32]. To resolve this ambiguity, specific morphological and molecular characteristics were employed for identification, including colony color, the size of the main and secondary conidiophores, the size, shape, and quantity of conidia, as well as the total number of longitudinal and transverse septations [34]. *A. burnsii* was partially confirmed as the causative agent of leaf spot on *C. lancifolius* by the observed morphological characterization. At the species level, accurate identification of fungal diseases is now feasible because of molecular techniques including PCR amplification, sequencing, and sequence analysis. To solve this issue, the ITS and GAPDH regions of the representative isolate were amplified using PCR assays using ITS and GAPDH primers. The resulting nucleotide sequence evidence was then submitted to the NCBI in order to get GenBank accession numbers. Due to the significant degree of variation in closely related species, the comparison of the ITS and GAPDH regions is a useful tool for the taxonomy and molecular phylogeny of phytopathogenic fungi [35]. It is regarded as the phytopathogenic fungus universal barcode area and offers details on the genetic variability among various isolates of the same species. The ITS and GAPDH regions of every isolate that has previously been published were examined to see how similar the tested isolate's genetic makeup was [36]. The genetic similarity between the tested isolate and the previously described *A. burnsii* from Bahawalpur, Punjab, Pakistan, ranged from 99.964% to 99.982%. To the best of our knowledge, this is the first instance of a buttonwood leaf spot caused by *A. burnsii* in Bahawalpur, Punjab, Pakistan.

Conclusions

For the first time, leaf spot disease caused by *A. burnsii* on *C. lancifolius* was identified in this investigation. The precise confirmation of *A. burnsii* on *C. lancifolius* was achieved through the utilization of observed morphological characterizations and derived nucleotide evidence. According to this study, *A. burnsii* leaf spot is rapidly spreading throughout the province and is turning into a deadly infection of *C. lancifolius*. According to our findings, this is the first record of leaf spot on *C. lancifolius* in Bahawalpur, Punjab, Pakistan, brought on by *A. burnsii*. By keeping in view *A. burnsii's* rapid spreading, it would be a threat to other ornamental plants, fruit trees, and vegetables. An effective and quick treatment is required to control the spread of *A. burnsii*. Moreover, detailed research is required to check the host range of *A. burnsii*, which facilitates the farmer to optimize their control measures against fungal pathogens.

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Conflict of Interest

The authors declare no conflict of interest.

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