Journal of Agriculture and Veterinary Science
ISSN: 2959-1198 (Print), 2959-1201 (Online)

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Research Article

First report of *Alternaria* leaf blight of Radish (*Brassica napus*): A Growing Concern in Pakistan's Agriculture

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ABSTRACT

Alternaria species are necrotic plant pathogens that cause dark spot disease to nearly one hundred host plants, affecting them from vegetative to reproductive stages. Infection typically appeared on leaves and stems as dark brownish to black spots that form concentric ring patterns as they enlarge, typically showing blight symptoms. The objective of the study was to characterize *Alternaria* species associated with radish leaf blight in Pakistan. In this study, a total of six isolates causing typical symptoms of leaf blight were collected from radish leaves (cv. Sufaid Pari) during a survey in February 2023 at the Vegetable Research Station Sahiwal (VRSS) Punjab, Pakistan. Being the first identification, all recovered isolates from symptomatic radish leaves were characterized based on morphological traits, pathogenicity and molecular analysis of internal transcribed spacer regions (ITS) of the 5.8S rDNA gene. Sequence analysis showed that radish isolates grouped to three *Alternaria* species: mainly *A. brassicae, A. brassicicola* and *A. alternata* respectively. Pathogenicity tests demonstrated that all isolates could produce similar necrotic lesions on detached radish leaves as observed during the field survey. Morphological characteristics act as a primary method for identifying *Alternaria* spp. although it is not feasible due to some *Alternaria* genera are morphologically related, consequently, molecular method of identification was sufficient for accurate species identification. This study represents ~~is~~ the first comprehensive investigation of *Alternaria* spp. causing leaf blight of radish in Pakistan.

Keywords: *Alternaria*, Pathogenicity, Characterization, Molecular Analysis.

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Article History
Received: May 25, 2024
Accepted: June 27, 2024
Published: August 30, 2024

INTRODUCTION

Leaf blight caused by *Alternaria* spp. is an emerging disease of radish crop, with a wide host range across the Brassicaceae family including Broccoli (*B. oleracea* var. *italica*) cabbage *(B. oleracea* var. *capitate*) and cauliflower (*B. oleracea* var. *botrytis*) (Saharan et al., 2016). Worldwide, more than 70% yield reduction has been reported on Brassica spp. which could present a threat to Pakistan (Kumar et al., 2014). *Alternaria* genus has a broad host range causing leaf spots, blight, and rotting on over 100 host plants (Thomma, 2003).

Under favorable conditions, the pathogen can spread through wind and rain splashes leading to symptoms including leaf and stem spots, silique blight, and seed infection in humid weather conditions with optimum temperature ranges of 15-21 ºC. The disease initially appears as tiny dark circular to elliptical spots with pale brown to black lesions. Later, these spots increase gradually in diameter forming concentric rings pattern on the leaves (Neeraj and Verma, 2010). In severe conditions, the disease causes defoliation of plants and breakdown of plant tissues, resulting in the death of the above ground tissues (Al-Lami et al., 2019). *Alternaria* genus contains 299 species which have been classified morphologically based on conidial production mainly large, multicellular, and dark-colored longitudinal conidia with extensive bases which gradually narrow to linear beaks forming club-like appearance (Kirk et al., 2008). Although the *Alternaria* genus has been primarily classified based on morphological and cultural characteristics, but these taxonomic identifications are difficult to distinguish among different species of the *Alternaria* genus (Pryor and Gilbertson, 2000). Meanwhile, all these conventional methods are time-consuming, difficult, and not reliable for studying the diversification among the *Alternaria* genus (Mohammadi and Bahramikia, 2019). Therefore, along with the conventional methods for fungal identification, recent molecular approaches have been adopted by researchers due to accurate identification of different *Alternaria* species (Ma et al., 2021). In addition, these molecular diagnostic approaches are effective, faster and easier than conventional methods in detecting fungal pathogens causing symptomatic plant diseases. Moreover, Polymerase chain reaction (PCR) amplification through internal transcribed spacer regions (ITS) and gene sequencing are useful tools in the establishment of species-level relationship within the *Alternaria* genus (Chitrakani et al., 2019). With this background in mind, the aim of this research work was to identify different *Alternaria* species isolated from radish leaves causing leaf blight disease through morphological descriptions and ITS gene sequencing.

MATERIALS AND METHODS

Sample collection, Cultural and morphological Characterization

In February 2023, Infected leaf samples of radish (cv. Sufaid Pari) were collected at the Vegetable Research Station Sahiwal (VRS), Pakistan (30°63′11.6″N, 73°1′93.8″E) showing typical symptoms such as dark brownish to black spots forming concentric rings and eventually enlarged as appearance of blight (Figure 01 and Figure 2). All Infected samples were brought to Plant Pathology Research Institute, Faisalabad (PPRI) for cultural and morphological studies, based on the fungal taxonomic key proposed (Simmons, 1995).

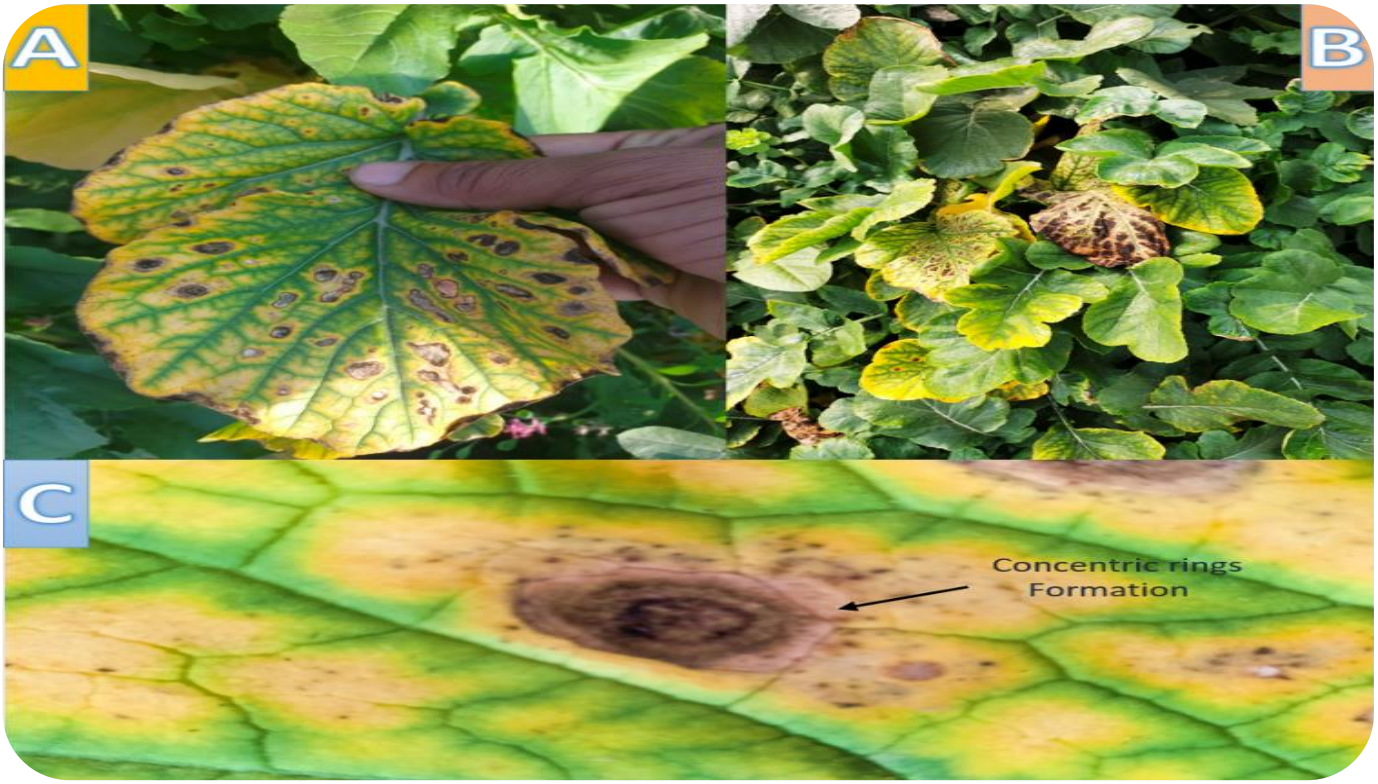

Figure 1. Symptoms of *Alternaria* blight caused by *Alternaria* spp. on radish leaves sampled from VRS, Sahiwal. (A) Radish leave showing typical symptoms of well-defined black spots (B) Enlargement of black spots showing blight on radish leaves (C) circular black spots with concentric rings formation.

Detached leaf assay technique

For confirmation of pathogenicity, five detached asymptomatic mature radish leaves were surface sterilized with 1% sodium hypochlorite, inoculated by immersion into spore suspension for 30 seconds and placed in Petri dishes (150x15mm) with cotton moistened with 3 mL of tap water. The Petri dishes were kept at 24°C in incubator ~~at~~ with 80-100% relative humidity (RH). The humidity of the Petri dishes was monitored and maintained by watering every third day throughout the incubation period. Dew formation in Petri dishes facilitated the development of the pathogen. Furthermore, leaves that were sprayed with sterile distilled water served as negative controls. The experiment was repeated three times (Rezene et al., 2018).

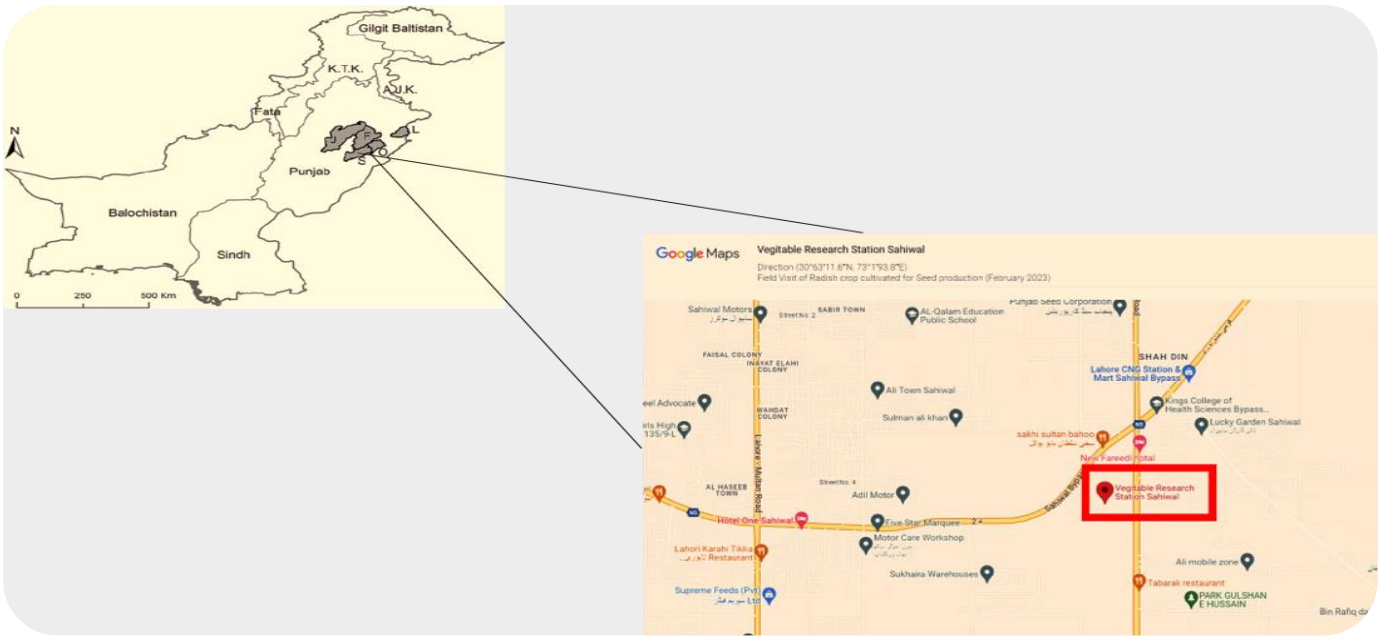

Figure 2. Location of Disease sampling.

Molecular diagnosis:

Following protocols are used for molecular identification such as DNA Extraction (Ghuffar et al., 2021), PCR amplification through ITS gene (White et al., 1990), gel documentations and PCR product purification (Gurjar et al., 2009), sequencing and editing of purified samples (Hall, 1999) for phylogenetic tree construction (Kumar et al., 2016).

RESULTS

Cultural and morphological studies:

Six isolates of *Alternaria* genus causing leaf blight of radish (cv. Sufaid Pari) were collected from Vegetable Research Station (VRS) Sahiwal, Punjab, Pakistan. During cultural studies, among six isolates different variations were observed in terms of colony color (CC), colony type (CT), and colony margins (CM) respectively. Isolates: VrsRaa01 and VrsRaa02 were found to be olivaceous green in colony color. In contrast, colony type was observed to be velvety and cottony at the center, whereas colony margins appeared slightly irregular olivaceous green to light green in color. Meanwhile isolates: Vrsab01 and Vrsab02, colony color was recorded as olivaceous to dark green on Potato Dextrose Agar (PDA) media, colony type was found velvety, appressed at center whereas, appearance of colony margins observed whitish rim with brown margins. The colony color of isolates VrsRabr01 and VrsRabr02 recorded as greenish with grayish surface, growth pattern was observed as cottony and appressed at the center in case of colony margins found dark olivaceous, appressed with light green rim respectively. Conidia of isolated *Alternaria* genus from radish crop has significant variations in terms of size and septations shown in (Table. 1 & Fig 3 A, B, C & D). Pathogenicity test revealed that all isolates found virulent and further used for molecular studies.

Phylogenetic analysis:

Morphologically and pathogenically characterized six isolates (VrsRaa01, VrsRaa02, Vrsab01, Vrsab02, VrsRabr01 and VrsRabr02) were further subjected to molecular studies using universal primers. Gel electrophoresis showed a single band length of 650 bp with amplified ITS product (Figure 2). After amplification, final sequences were

submitted in the NCBI public data base under the accession numbers of ITS gene **(**OQ633119, OQ633076, OQ633074, OQ633056, OQ648025 & OQ648026) exhibiting more than 99% genetic similarity with isolates of *Alternaria alternata, Alternaria brassicae* and *Alternaria brassicicola* available at public nucleotide database system. Phylogenetic analysis of Internal transcribed spacer region of *A. alternata, A. brassicae* and *A. brassicicola* revealed varying levels of genetic diversity. In phylogenetic tree the reference sequences were used as *A. dauci, A. tenuissima* and *A. solani,* respectively. Furthermore, *Penicillium crustosum* was used as an outgroup shown in (Figure 4).

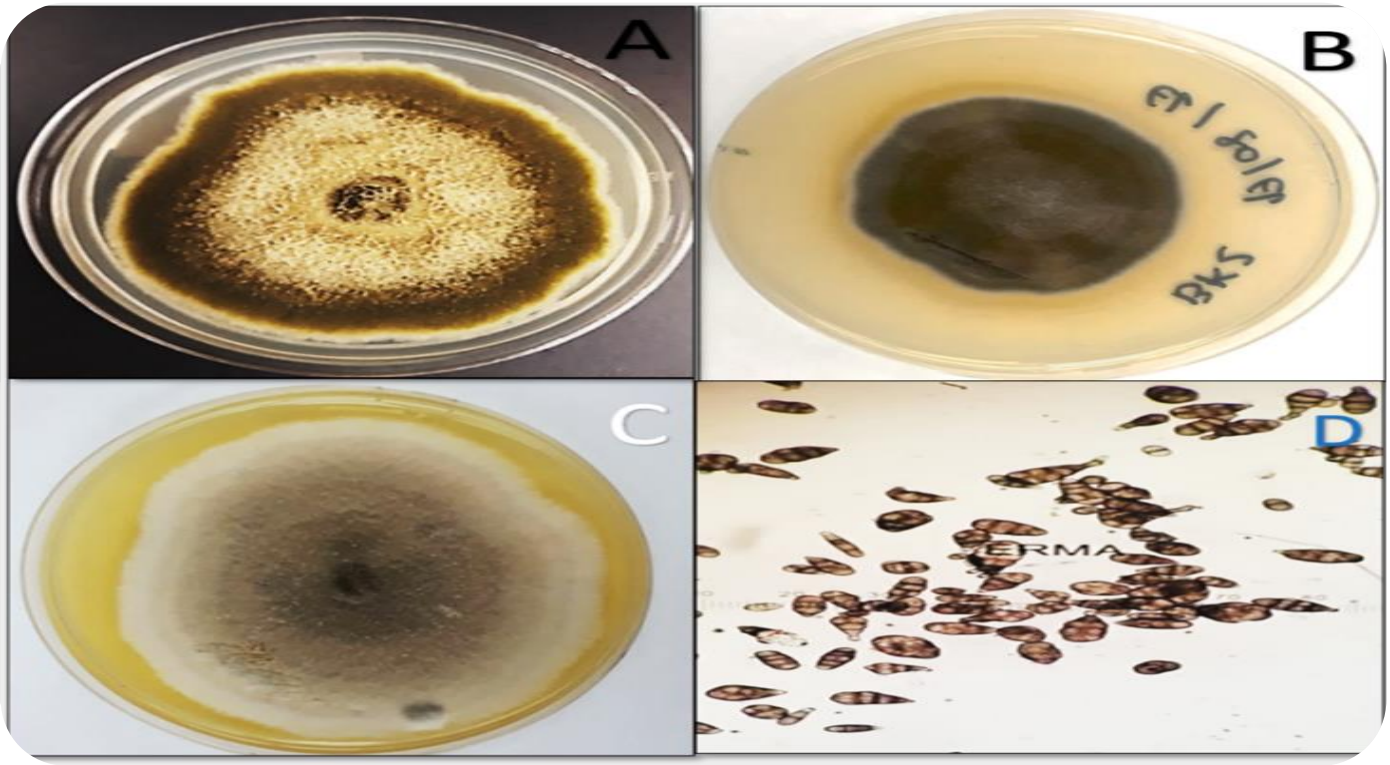

Figure 3 (A-D): Cultural and microscopic features of *Alternaria* genus.

DISCUSSION

To our knowledge, this research marks the first study about *Alternaria* spp. causing *Alternaria* leaf spot of radish in Pakistan. Our findings revealed symptoms characterized by dark brown to black spots that developed concentric rings and eventually expanded, as the appearance of blight. Similar disease patterns have been studied previously in horseradish plant (*Armoracia rusticana*) belonging to the Brassicaceae family (Blagojević et al., 2020). During morphological studies, two isolates, VrsRaa01 and VrsRaa02, exhibited olivaceous green colony color (CC) with velvety and cottony colony types (CT) at the center. The colony margins appeared slightly irregular, ranging from olivaceous green to light green. For isolates Vrsab01 and Vrsab02, the colony color ranged from olivaceous to dark green, with a velvety, appressed center and margins displaying a whitish rim with brown edges. Isolates VrsRabr01 and VrsRabr02 were observed to have a greenish color with a grayish surface. Their growth pattern was cottony and appressed at the center, while the colony margins were dark olivaceous, appressed, with a light green rim. All the results consistent with prior research on the cultural characteristics of Alternaria spp. in PDA medium (Ma et al., 2021) (Pryor and Michailides, 2002). Microscopic observations of conidia were conducted, in line with studies by (DeShields and Kc, 2021). Pathogenicity tests demonstrated that all fungal isolates exhibited virulence. Similar studies involving Alternaria isolates on strawberry leaves showed comparable symptoms after inoculation (Mehmood et al., 2018). Following the phenotypic and pathological analyses of Alternaria genus, molecular analysis was performed to achieve accurate species-level identification. Using the PCR technique with universal primers, the internal transcribed spacer (ITS) regions of the 5.8S rDNA gene were amplified, producing approximately 650 base pair amplicons, consistent with previous studies. This gene region proved useful in illustrating the phylogenetic relationship among Alternaria species causing leaf blight in various crops belonging to the Brassicaceae family, aligning with prior research (Blagojević et al., 2020). Our findings are supported by (Aoki et al., 2004), who emphasized the utility of molecular studies for species-level identification, especially when morphological

characterization is challenging. Similarly, (Waalwijk et al., 2004) advocated for the use of PCR amplification of the ITS gene in fungal pathogens for more accurate identification compared to conventional techniques. (Mule et al., 2005) further highlighted the efficacy of DNA sequencing in the precise identification of biotic diseases at the species level. To our knowledge, this research marks the first report about Alternaria spp. causing Alternaria leaf spot of radish in Pakistan. Our findings revealed symptoms characterized by dark brown to black spots that developed concentric rings and eventually expanded, as the appearance of blight. Similar disease patterns have been studied previously in horseradish plant (Armoracia rusticana) belonging to the Brassicaceae family (Blagojević et al., 2020).

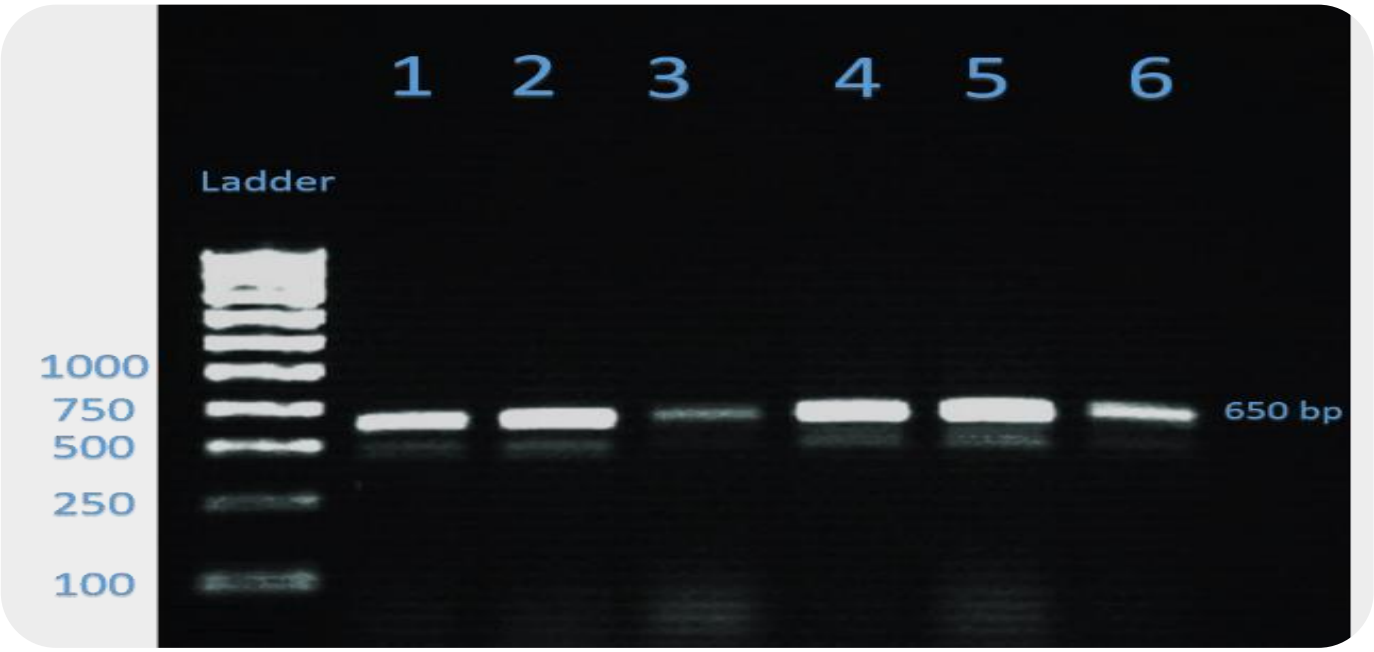

Figure 4: Gel band documentation of PCR product

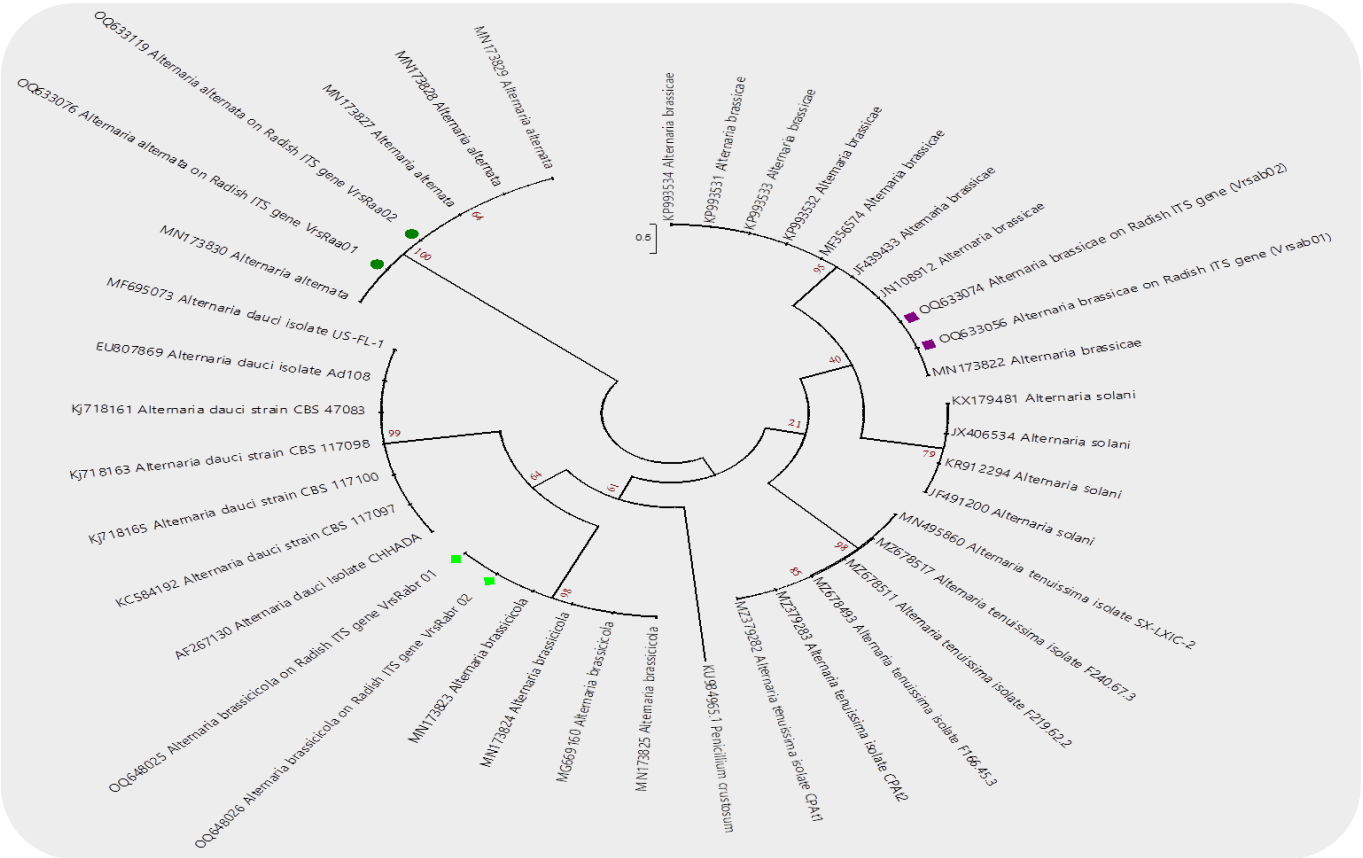

Figure 5. Phylogenetic analysis

During morphological studies, two isolates, VrsRaa01 and VrsRaa02, exhibited olivaceous green colony color (CC) with velvety and cottony colony types (CT) at the center. The colony margins appeared slightly irregular, ranging from olivaceous green to light green. For isolates Vrsab01 and Vrsab02, the colony color ranged from olivaceous to dark green, with a velvety, appressed center and margins displaying a whitish rim with brown edges. Isolates VrsRabr01 and VrsRabr02 were observed to have a greenish color with a grayish surface. Their growth pattern was cottony and appressed at the center, while the colony margins were dark olivaceous, appressed, with a light green rim. All the results were consistent with prior previous research on the cultural characteristics of Alternaria spp. on PDA medium (Ma et al., 2021) (Pryor and Michailides, 2002). Microscopic observations of conidia were in line with studies by (DeShields and Kc, 2021). Pathogenicity tests demonstrated that all fungal isolates exhibited virulence. Similar studies involving Alternaria isolates on strawberry leaves showed comparable symptoms after inoculation (Mehmood et al., 2018). Following the phenotypic and pathological analyses of Alternaria genus, molecular analysis was performed to achieve accurate species-level identification. Using the PCR technique with universal primers, the internal transcribed spacer (ITS) regions of the 5.8S rDNA gene were amplified, producing approximately 650 base pair amplicons, consistent with previous studies. This gene region proved useful in illustrating the phylogenetic relationship among Alternaria species causing leaf blight in various crops belonging to the Brassicaceae family, aligning with previous research (Blagojević et al., 2020). Our findings were supported by (Aoki et al., 2004), who emphasized the significance of molecular studies for species-level identification, especially when morphological characterization is challenging. Similarly, (Waalwijk et al., 2004) advocated for the use of PCR amplification of the ITS gene in fungal pathogens for more accurate identification compared to conventional techniques. (Mule et al., 2005) further highlighted the efficacy of DNA sequencing in the precise identification of biotic diseases at the species level.

Table 1: Cultural and morphological characterization of *Alternaria* spp.

Sr.	Isolation ID	Colony			Conidia			
		Colony Color	Colony Type	Colony Margins	Length (um)	Breadth (um)	Transverse Septation	Longitudinal Septation
1	VrsRaa 01	Olivaceous green	velvety, cottony at center	Slightly irregular olivaceous green to irregular light green with white rim	25.2 ±0.3	12.26 ±0.1	4	3
2	VrsRaa 02	-	-	-	21.8 ±0.15	11.75 ±0.6	3	2
3	Vrsab01	Olivaceous to dark green	velvety, appressed at center	Appressed with white rim to brownish margins	28.02 ±0.5	15.7 ±0.3	5	4
4	Vrsab02	-	-	-	24.21 ±0.32	14.21 ±0.1	5	4
5	VrsRabr 01	Greenish with grayish surface	Cottony, slightly furrow with appressed center	Dark olivaceous, regular, appressed, brownish with white and green rim	30.7 ±1.5	18.19 ±0.4	6	4
6	VrsRabr 02	-	-	-	29.3 ±0.4	17.34 ±0.5	5	4

CONCLUSION

Based on our findings, we report for the first time the occurrence of *Alternaria* leaf blight on radish in Pakistan, emphasizing the importance of molecular studies in accurately identifying pathogens at the species level.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors declare no competing interests.

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