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Research Article

First Report of *Alternaria alstroemeriae* Causing Leaf Spot of Soybean in Uzbekistan Based on Morphological Characterization and ITS Phylogenetic Analysis

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ABSTRACT

Soybean (*Glycine max* L.) is an important legume crop valued for its high protein and oil content and its contribution to sustainable agriculture. In Uzbekistan, soybean cultivation has expanded in recent years, increasing the need for accurate identification of fungal pathogens affecting crop production. This study aimed to identify *Alternaria* species associated with soybean leaf spot using morphological characteristics and molecular analysis. Symptomatic soybean leaves were collected from fields in the Syrdarya region, and three fungal isolates were obtained. Colony morphology and microscopic examination revealed characteristics typical of *Alternaria*, including dark olivaceous colonies, septate conidiophores, and muriform conidia. A representative isolate (R-21) was subjected to ITS rDNA sequencing, and phylogenetic analysis confirmed its close relationship with reference isolates of *Alternaria alstroemeriae* available in GenBank. The combined morphological and molecular evidence identified the isolate as *A. alstroemeriae* associated with soybean leaf spot in Uzbekistan. Although pathogenicity tests and multilocus phylogenetic analyses were beyond the scope of the present study, these findings provide the first molecular evidence of the occurrence of *A. alstroemeriae* associated with soybean leaf spot in Uzbekistan. The results establish a baseline for future studies on pathogenicity, population diversity, epidemiology, and integrated disease management of this pathogen.

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Introduction

Soybean (*Glycine max* L.) is among the most economically important legume crops worldwide due to its high protein and oil content and its extensive utilization in food, feed, and industrial applications (Hartman et al., 2011; Akhmadaliev et al., 2024; Sherimbetov et al., 2024a). As a rich source of plant-based protein and essential fatty acids, soybean plays a critical role in global nutrition and sustainable

agriculture. In addition to its nutritional value, soybean contributes to agroecosystem sustainability through biological nitrogen fixation, which improves soil fertility and reduces dependence on synthetic fertilizers.

In recent years, soybean cultivation has expanded in Central Asia, including Uzbekistan, driven by national agricultural strategies aimed at crop diversification, soil fertility improvement, and food security enhancement (FAO, 2020; Abduvaliev et al., 2024; Sherimbetov et al.,

2025). The increasing demand for plant-based protein, along with the need to optimize crop rotations in irrigated and rainfed systems, has further stimulated soybean production in the region. However, the successful expansion of soybean cultivation is closely linked to effective management of biotic and abiotic stresses that threaten crop productivity.

Among biotic constraints, fungal diseases represent one of the most significant limiting factors for soybean yield and seed quality worldwide (Wrather and Koenning, 2006). Fungal pathogens not only reduce yield but also impair seed quality, market value, and storability. Under favorable environmental conditions, disease outbreaks can lead to substantial economic losses, particularly in regions where soybean is a relatively new crop and disease monitoring systems are still developing.

Leaf spot diseases caused by fungi of the genus *Alternaria* are widely distributed and associated with considerable economic losses in many agricultural crops (Thomma, 2003). Species of *Alternaria* are necrotrophic pathogens capable of infecting a broad range of hosts and surviving under diverse environmental conditions (Rotem, 1994). Their ability to produce abundant spores, survive on plant residues, and rapidly colonize host tissues makes them successful pathogens in various agroecological zones.

In soybean, *Alternaria* leaf spot typically appears as circular to irregular necrotic lesions with concentric rings, often surrounded by chlorotic halos. Severe infections may cause premature leaf senescence, defoliation, and reduced photosynthetic capacity, ultimately leading to yield reduction and weakened plant vigor (Bisht et al., 2014). Moreover, *Alternaria* infections may predispose plants to secondary pathogens, compounding overall disease impact.

The genus *Alternaria* constitutes a taxonomically complex group of species with overlapping morphological characteristics, which complicates reliable species-level identification based solely on classical morphological criteria (Simmons, 2007). Traditional identification relying on colony morphology and conidial features is often insufficient due to phenotypic plasticity and environmental influences on fungal growth. As a result, misidentification is common, particularly among small-spored species complexes.

Alternaria alstroemeriae, originally described as a pathogen of ornamental plants, has recently been reported on various hosts, suggesting a broader

ecological amplitude than previously recognized (Woudenberg et al., 2013). Its morphological resemblance to other small-spored *Alternaria* species frequently leads to diagnostic uncertainty (Lawrence et al., 2013). Therefore, molecular diagnostic approaches have become indispensable in modern fungal taxonomy. Sequence-based identification methods, particularly DNA barcoding using the internal transcribed spacer (ITS) region and other gene markers, have emerged as reliable tools for species delimitation and phylogenetic analysis in *Alternaria* (Woudenberg et al., 2015; Sherimbetov et al., 2024b). Molecular techniques allow precise discrimination among closely related species and contribute to a better understanding of pathogen diversity, evolution, and geographic distribution. The integration of morphological and molecular data provides a robust framework for resolving taxonomic ambiguities and ensuring accurate pathogen identification.

Despite the increasing importance of soybean cultivation in Uzbekistan, studies addressing fungal pathogens affecting this crop remain limited. Information on the occurrence, diversity, and identification of *Alternaria* species associated with soybean in the region is particularly scarce. This knowledge gap hampers the development of effective disease monitoring and management strategies.

Therefore, the present study aimed to conduct a comprehensive morphological and molecular characterization of *A. alstroemeriae* associated with soybean leaf spot in Uzbekistan. By combining classical mycological observations with molecular phylogenetic analysis, this research seeks to clarify the taxonomic status of the pathogen and contribute to a better understanding of its occurrence and potential threat to soybean production in the region.

Materials and methods

Sample collection and fungal isolation

Soybean (*Glycine max* L.) plants exhibiting symptoms typical of *Alternaria* infection, including leaf blight, necrotic lesions with concentric rings, stem lesions, and premature leaf senescence, were collected during the growing season from soybean fields. To ensure representative sampling, plant material was randomly collected from three locations within each field. Both symptomatic and asymptomatic leaves and stems were sampled, placed in sterile polyethylene bags, labeled, and transported to the laboratory.

Plant tissues were washed under running tap water to remove debris and surface contaminants. Small tissue segments (5-7 mm) were excised from lesion margins and surface-sterilized by immersion in 70% ethanol for 30 sec followed by 1% sodium hypochlorite for 4 min, then rinsed three times in sterile distilled water and dried on sterile filter paper under aseptic conditions. This sterilization protocol follows standard phytopathological isolation procedures (Sambrook and Russell, 2001).

Sterilized tissues were plated on potato dextrose agar (PDA) amended with streptomycin (50 mg L⁻¹) to suppress bacterial growth. Plates were incubated at 25 ± 2°C under a 12 h light/12 h dark photoperiod to stimulate sporulation. Fungal colonies resembling *Alternaria* were subcultured to obtain pure isolates using hyphal-tip or single-spore isolation techniques. Preliminary identification was based on colony morphology and microscopic observation of conidiophores and conidia, following standard taxonomic criteria for *Alternaria* (Woudenberg et al., 2013). Pure cultures were maintained on PDA at 4°C for short-term storage and preserved in 20% glycerol at -80°C for long-term storage.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from a representative isolate (strain R-21) grown on PDA at 25 ± 1°C for 7-10 days. DNA extraction was performed using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. DNA quality and concentration were assessed spectrophotometrically and by agarose gel electrophoresis (Sambrook and Russell, 2001).

The internal transcribed spacer (ITS) region of rDNA was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), widely accepted as fungal DNA barcoding markers (White et al., 1990). PCR reactions (25 µL) contained PCR buffer, MgCl₂, dNTPs, primers, Taq polymerase, and genomic DNA template. Thermal cycling included initial denaturation at 95°C for 5 min; 35 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec), and extension (72°C, 1 min); followed by final extension at 72°C for 10 min.

PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Amplicons were purified and subjected to bidirectional Sanger sequencing.

Phylogenetic analysis

Sequences were edited and assembled using SnapGene software. Preliminary identification was performed using BLAST searches against the NCBI database (Altschul et al., 1990). Closely related sequences were retrieved from GenBank for comparative analysis.

Multiple sequence alignment was conducted using the MUSCLE algorithm, known for high accuracy in fungal phylogenetics (Edgar, 2004). Phylogenetic trees were constructed in MEGA version 12 using the Maximum Likelihood method with the Kimura 2-parameter model. Node support was evaluated using 1,000 bootstrap replicates (Kumar et al., 2018). The final tree was visualized and edited in MEGA.

Results

Morphological characterization

During the mycological investigation of symptomatic soybean plants collected from the Syrdarya district (Syrdarya region), three fungal isolates with similar cultural and microscopic characteristics were consistently recovered from infected tissues. Fungal growth became visible within 3-4 days after incubation on PDA, indicating relatively fast colony development typical of *Alternaria* species.

Initially, colonies appeared white to light gray with sparse aerial mycelium. As incubation progressed, colony pigmentation gradually intensified, turning dark gray to olivaceous. After 7-10 days, mature colonies displayed a dense, cottony to velvety mycelial texture with irregular and slightly undulate margins. Zonation was occasionally visible in older cultures, reflecting periodic growth patterns. The colony surface became progressively darker due to conidial production. The reverse side of colonies exhibited a characteristic dark olive-green to black pigmentation, a feature commonly associated with melanized *Alternaria* spp.

Microscopic examination revealed well-developed, septate, and branched hyphae that were brown to olivaceous in color, indicating melanin deposition in the cell walls. Hyphal cells were smooth-walled and varied slightly in width, forming a compact mycelial network. Conidiophores were observed arising singly or in small fascicles from the aerial and substrate mycelium. They were straight to slightly flexuous, septate, and pale to dark brown in coloration. Conidiophores measured moderate lengths and frequently showed geniculate growth at points of conidium formation. Terminal and

intercalary conidiogenous loci were visible, indicating active conidiogenesis.

Conidia were abundantly produced in acropetal chains, a diagnostic feature of the genus. Individual conidia were obclavate to ellipsoid, occasionally ovoid, and exhibited both transverse and longitudinal septa (muriform structure). The conidial walls were smooth to finely roughened and moderately thickened. Conidial dimensions ranged from $17.1\text{--}22.8 \times 9.7\text{--}11.4 \mu\text{m}$ (mean = $19.95 \times 10.55 \mu\text{m}$). Many conidia possessed a short to moderately elongated apical beak, which was tapered and sometimes slightly curved. The number of transverse septa typically ranged from 3-6, with 1-2 longitudinal septa, consistent with small-spored *Alternaria* morphology.

No significant morphological variability was observed among the three isolates, suggesting they belonged to the same species. The combination of colony

pigmentation, muriform conidia, conidial size range, and beak formation strongly corresponded to diagnostic features described for *A. alstroemeriae*.

Overall, the morphological characteristics observed in the present isolates were in close agreement with published descriptions of *A. alstroemeriae* (Simmons, 2007; Woudenberg et al., 2013). These phenotypic traits provided preliminary taxonomic evidence and were later corroborated by molecular identification, thereby strengthening the reliability of species determination. Representative morphological characteristics of *A. alstroemeriae* strain R-21 are presented in Figure 1.

Molecular Analysis

PCR amplification of ITS region of R-21 strain yielded band of expected size. Amplicon was successfully sequenced and deposited in GenBank under accession number OQ073449. The Maximum Likelihood phylogenetic tree based on ITS rDNA sequences is shown in Figure 2.

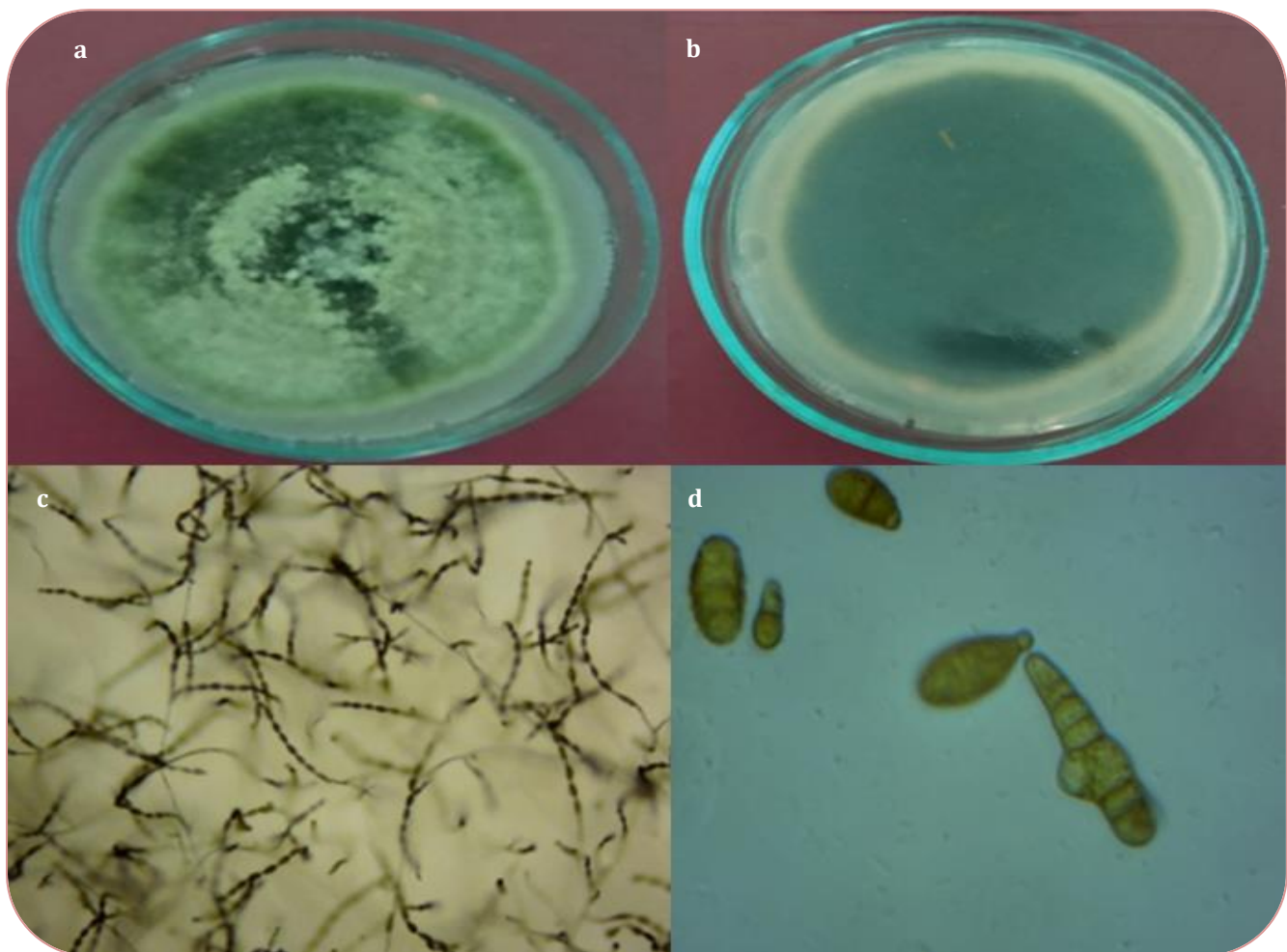


Figure 1. Morphological characteristics of *A. alstroemeriae* strain R-21 grown on PDA medium for 7 days: (a) colony obverse view, (b) colony reverse view, (c) conidiophores, and (d) conidia.

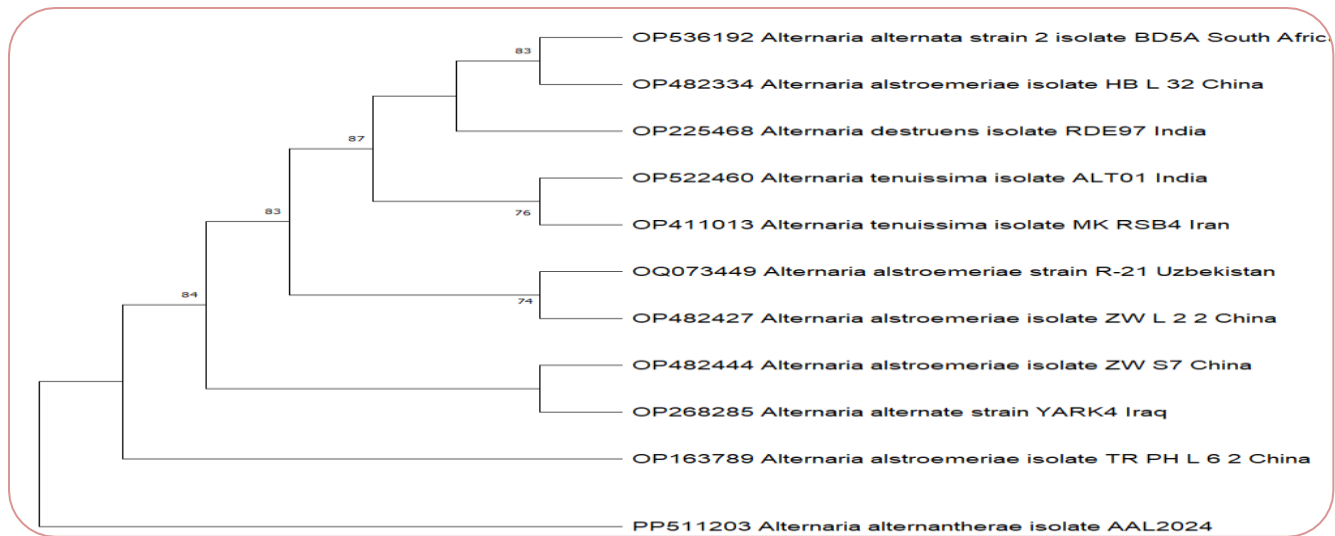


Figure 2. Maximum Likelihood phylogenetic tree based on ITS rDNA sequences showing the relationship of *A. alstroemeriae* strain R-21 with closely related *Alternaria* species. Bootstrap support values ($\geq 50\%$) from 1,000 replicates are indicated at the nodes. *A. alternantherae* (PP511203) was used as the outgroup.

The phylogenetic analysis placed *A. alstroemeriae* strain R-21 (OQ073449), isolated from Uzbekistan, within the *A. alstroemeriae* lineage. The isolate clustered with reference *A. alstroemeriae* sequences from GenBank and was supported by a bootstrap value of 74%, indicating moderate support for its phylogenetic placement. The bootstrap value of 74% provides moderate statistical support for the placement of isolate R-21 within the *A. alstroemeriae* clade. The combined morphological characteristics and ITS sequence similarity further support its identification as *A. alstroemeriae*. The *A. alstroemeriae* clade is clearly separated from closely related species within the genus *Alternaria*, including *A. tenuissima*, *A. destruens*, and *A. alternata*. These species form independent lineages with comparatively high bootstrap values (76-87%), reflecting stable species-level divergence. The clear separation between *A. alstroemeriae* and members of the *A. alternata* species complex demonstrates that strain R-21 does not belong to a cryptic lineage but represents a well-defined taxonomic entity within the genus. Within the broader phylogenetic framework, the clustering pattern suggests that *A. alstroemeriae* exhibits low intraspecific genetic variation across wide geographic regions, including Central and East Asia. The close relationship between the Uzbekistan isolate (R-21) and Chinese isolates implies either recent divergence or ongoing gene flow, possibly facilitated by plant host movement or shared ecological niches. This pattern is consistent with the biology of *Alternaria*

species, which are known for their wide distribution and capacity for dispersal through airborne conidia. The phylogenetic tree is rooted using *A. alternantherae* isolate AAL2024 (PP511203) as an outgroup, providing a clear evolutionary reference point and confirming the directionality of divergence among the examined taxa. The basal position of the outgroup supports the stability of the internal nodes and reinforces the evolutionary distinction between *A. alstroemeriae* and other *Alternaria* species included in the analysis. Overall, the phylogenetic placement of *A. alstroemeriae* strain R-21 confirms its taxonomic identity and highlights its close evolutionary relationship with other *A. alstroemeriae* isolates. Although the bootstrap support for the *A. alstroemeriae* clade was moderate (74%), the phylogenetic placement was consistent with morphological observations and ITS sequence similarity. Future studies using multilocus sequence data and Maximum Likelihood or Bayesian phylogenetic analyses are expected to provide improved phylogenetic resolution and stronger statistical support. These findings contribute to a better understanding of the evolutionary relationships within the genus *Alternaria* and provide molecular evidence supporting the identification and global distribution of *A. alstroemeriae*.

Discussion

The present study provides the first integrative morphological and molecular evidence confirming *A. alstroemeriae* as the causal agent of soybean leaf spot in

Uzbekistan and demonstrates the importance of combining phenotypic and phylogenetic approaches for accurate species delimitation within the genus *Alternaria*, where misidentification is common. The isolates obtained from soybean tissues showed colony pigmentation, growth patterns, and conidial morphology consistent with small-spored *Alternaria* species, including dark olivaceous colonies, muriform conidia with both transverse and longitudinal septa, and short to moderately elongated beaks, all of which correspond closely to published descriptions of *A. alstroemeriae* (Simmons, 2007; Woudenberg et al., 2013). The relatively stable conidial dimensions and septation patterns among isolates further suggested taxonomic uniformity, although it is well recognized that morphological traits in *Alternaria* can vary depending on culture conditions and environmental factors, making morphology alone insufficient for definitive identification. In this context, molecular analysis provided decisive confirmation, as ITS rDNA sequences of strain R-21 showed high similarity to authenticated *A. alstroemeriae* sequences and clustered within a well-supported *A. alstroemeriae* clade, while being clearly separated from closely related taxa such as *A. alternata*, *A. tenuissima*, and *A. destruens*, demonstrating the resolving power of the molecular marker used. The moderate to high bootstrap support values obtained indicate stable and reliable phylogenetic relationships (Hillis and Bull, 1993) and the congruence between morphological and molecular data strengthens the reliability of species identification in accordance with modern polyphasic taxonomy. In the present study, only one representative isolate (R-21) was selected for molecular analysis because all recovered isolates displayed identical morphological characteristics. ITS sequencing provided reliable preliminary identification and confirmed the placement of the isolate within the *A. alstroemeriae* clade. However, the ITS region alone may not provide sufficient resolution to distinguish closely related species within the genus *Alternaria*. Additional loci, such as *gapdh*, *tef1*, and *rpb2*, have been shown to improve phylogenetic resolution and species delimitation, particularly among small-spored *Alternaria* species. Furthermore, the clustering of isolate R-21 with reference *A. alstroemeriae* sequences from GenBank suggests a close phylogenetic relationship, although broader sampling and multilocus analyses are required to evaluate intraspecific genetic diversity and population

structure. Therefore, future studies should include multilocus sequencing of isolates collected from different soybean-growing regions of Uzbekistan together with more robust phylogenetic analyses to further validate species identification and improve taxonomic resolution. The expanding soybean cultivation in Uzbekistan and increasing movement of planting material could further facilitate pathogen introduction and establishment. From a phytopathological perspective, the detection of *A. alstroemeriae* on soybean is particularly significant because this species was historically associated mainly with ornamental hosts, and its occurrence on soybean suggests either a broader host range than previously recognized or a host shift driven by ecological and climatic conditions, since warm temperatures and periods of high humidity typical of soybean-growing regions in Uzbekistan are known to favor *Alternaria* infection and sporulation (Rotem, 1994). Epidemiologically, infected soybean fields may function as reservoirs of inoculum, increasing the likelihood of disease recurrence and regional spread, while the genetic similarity between Uzbek and foreign isolates also points to possible transboundary movement and highlights the need for strengthened phytosanitary monitoring and disease surveillance. The combined morphological and molecular evidence presented here confirms that *A. alstroemeriae* is a true pathogen associated with soybean leaf spot rather than an incidental contaminant, thereby expanding both the known host range and geographic distribution of the species and underscoring the necessity of integrating phylogenetic tools into routine diagnostic workflows, particularly in regions where fungal biodiversity remains underexplored (Lawrence et al., 2013; Woudenberg et al., 2015). The present study has several limitations that should be acknowledged. The bootstrap support obtained should be interpreted with caution because phylogenetic reconstruction was based on a single ITS locus and the Neighbor-Joining algorithm. Although the ITS region is widely accepted as the primary fungal barcode, additional loci such as *gapdh*, *tef1*, and *rpb2* provide higher phylogenetic resolution for closely related *Alternaria* species. Furthermore, only one representative isolate was subjected to molecular analysis, and pathogenicity tests were not conducted. Future studies incorporating multilocus datasets and more robust phylogenetic approaches, such as Maximum

Likelihood or Bayesian inference, together with pathogenicity assays, will provide stronger evidence for species delimitation and the biological significance of this pathogen. Overall, the integrative approach applied in this study contributes to a better understanding of *Alternaria* diversity in Central Asia and indicates that *A. alstroemeriae* may represent an emerging disease risk for soybean production systems, emphasizing the importance of future studies focused on pathogenicity testing, yield loss assessment, population genetics, and development of effective management strategies to mitigate potential impacts on soybean cultivation.

Conclusion

This study provides the first molecular evidence of *Alternaria alstroemeriae* associated with soybean leaf spot in Uzbekistan. The integration of morphological characteristics with ITS rDNA sequence analysis enabled reliable identification of the representative isolate (R-21) and confirmed its close phylogenetic relationship with reference *A. alstroemeriae* isolates available in GenBank. These findings expand the known geographical occurrence of *A. alstroemeriae* and contribute baseline information on *Alternaria* diversity associated with soybean in Uzbekistan. However, because pathogenicity assays and multilocus phylogenetic analyses were not included in the present study, further research is required to confirm the pathogenic role of this fungus and improve species resolution. Future investigations should incorporate Koch's postulates, additional molecular markers (e.g., *gapdh*, *tef1*, and *rpb2*), and a broader collection of isolates to better understand the genetic diversity, epidemiology, and potential impact of *A. alstroemeriae* on soybean production. The results presented here provide a useful foundation for future disease monitoring and integrated management strategies in the expanding soybean production systems of Uzbekistan.

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Author Contributions

DR, AS, and JS conceptualized and designed the study.

DR carried out the experimental work and field investigations. BA provided overall supervision and ensured the scientific rigor, methodological accuracy, and consistency of the research throughout the study. AS, BA, and TM contributed to the experimental design, data interpretation, and provided valuable scientific guidance at various stages of the research. DR, AS, BA, TM, JR, and JS participated in data analysis, manuscript writing, and revision. All authors critically reviewed the manuscript, contributed to its intellectual content, verified the data and analyses, and approved the final version of the manuscript for publication.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger

SDG 12: Responsible Consumption and Production

SDG 15: Life on Land

Policy Addressed

The first molecular identification of *Alternaria alstroemeriae* associated with soybean leaf spot in Uzbekistan supports plant health surveillance, agricultural biosecurity, and sustainable crop protection. The findings provide baseline data for disease monitoring, quarantine risk assessment, IPM development, and efforts to enhance crop diversification, agricultural resilience, and food security.

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