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Morphological and molecular characterization of *Alternaria alternata* causing leaf spot of Ashwagandha

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Abstract
A key ingredient in Ayurveda is an herb called Ashwagandha. It is a small shrub in the *Solanaceae* family. Ashwagandha is invaded by many plant pathogens. Alternaria leaf spot, caused by *Alternaria alternata*, emerges as a prominent disease. The pathological investigations of the Alternaria leaf spot were undertaken by focusing on dark brown to black lesions symptoms on leaves that exhibit concentric rings. Based on the examination of cultural and morphological characteristics, the pathogen's identification was confirmed. The microscopic observation revealed that conidia were dark brown, rounded at the base and had a beak that was short and thick. Molecular identification of the fungal isolate was accomplished by conducting PCR amplification of the ITS region, followed by sequencing. The ITS rDNA sequence indicates that the pathogenic *Alternaria alternata* is responsible for the development of Alternaria leaf spot in Ashwagandha.

Keywords: Ashwagandha, *Alternaria alternata*, leaf spot, pathogenicity, ITS1, ITS4

Introduction
Ashwagandha (*Withania somnifera* (L.) Dunal) is an ancient herb that has gained widespread recognition for its potential health benefits. It is belonging to the *Solanaceae* family and holds immense significance in traditional medicinal systems like Ayurveda, Siddha, and Unani due to its therapeutic properties. There is now more demand for Ashwagandha-based products due to greater awareness of the benefits of natural healthcare products. However, *W. somnifera* was invaded by many phytopathogens especially fungal disease infections were observed frequently. Among the various diseases, the most commonly found is the leaf spot disease, which is caused by *Alternaria alternata*, significantly impairing the production of secondary metabolites. Given the significance of the disease, a comprehensive study focusing on various aspects of the disease was undertaken at the Department of Plant Pathology, B.A. College of Agriculture, Anand Agricultural University, Anand. The isolation was carried out from Ashwagandha leaves exhibiting characteristic symptoms, identifying the presence of *Alternaria* spp. By utilizing the spore suspension spray inoculation method, the isolated fungus fulfilled Koch's postulates on Ashwagandha plants. The identification was confirmed by evaluating its cultural and morphological characteristics, along with the utilization of DNA sequencing. The sequencing of the ITS rDNA region in *A. alternata* provided precise species-level identification. A phylogenetic tree was formed and subsequently compared to other homogenous fungal isolates worldwide, which were accessible in the NCBI database. The ITS rDNA sequence (GenBank Accession No. ON778577) provides clear evidence that the pathogenic *Alternaria alternata* is responsible for causing Alternaria leaf spot in Ashwagandha.

Materials and Methods
Location
The Anand Agricultural University is situated in the city of Anand, Gujarat, at 22°35' North latitude and 72°55' East longitude, situated at a height of 45 meters above mean sea level. It belongs to the Central Agroclimatic Zone of Gujarat (III).

Climate
The average annual rainfall in the region around Anand Agricultural University is 1333 mm. It begins in the middle of June and lasts until the middle of September. Summers are quite hot, with temperatures ranging from 30 to 45 °C, while winters are relatively mild and dry.

Sterilization of soil and earthen pots
A mixture of sandy loam soil and FYM in a ratio of 3:1 was prepared and autoclaved at a pressure of 1.2 kg/cm^2 for two hours and two days before being placed in surface-sterilized earthen pots for the experiment. Prior to use, the earthen pots were thoroughly washed with tap water and sterilized for two minutes using a 4 percent formaldehyde solution.

Collection of diseased Samples
The infected leaves of Ashwagandha exhibiting the characteristics symptoms of Alternaria leaf spot having concentric rings were collected, put into brown paper bags from Medicinal and Aromatic Plants Research Station, AAU, Anand during *rabi* 2021- 22 and taken to the laboratory to facilitate microscopic investigation and isolation for further investigations.

Symptomatology
The presence of the pathogen was confirmed through examination of Alternaria leaf spot samples, utilizing both visual and microscopic inspections. The characteristic symptoms of Alternaria leaf spot on leaves of Ashwagandha under field conditions were carefully examined and critically recorded.

Isolation and purification of the pathogen
The pathogen was obtained from the leaf tissues of Ashwagandha through isolation, which exhibited typical symptoms of Alternaria leaf spot. The isolation of the pathogen was carried out by the standard tissue isolation method (Tuite, 1969) [9]. The obtained culture was purified using the hyphal tip method (Rangaswami, 1972) [8].

Storage and maintenance of *Alternaria* sp.
After isolation, the pure culture was preserved on PDA slants and incubated at 28±1 °C for subsequent research. Following that, sub-culturing was carried out at intervals of 15 days on PDA slants, and the resulting cultures were kept in a refrigerator at 4 °C for future experimental utilization.

Morphological characterization
For the identification of the pathogen responsible for causing the Alternaria leaf spot in Ashwagandha, visual and microscopic examinations were conducted to assess the cultural and morphological characters of the pathogen on PDA medium. The cultural characteristics, including colony color, colony topography, colony margin, colony diameter, and sporulation were carefully recorded throughout a 15-day period, starting from the initiation of growth. Under high-power magnification, the morphological characteristics of *Alternaria* sp. were measured from a 10-day-old culture, and set in comparison with those provided in the literature. The microphotographs of the same were also recorded.

Pathogenicity test
The pathogenicity of *A. alternata* was established by artificially inoculating the pathogen, following the standard method of inoculation. The pathogenicity test was conducted in a pot within a controlled glasshouse environment. Ten earthen pots were filled with sterilized soil and FYM mixture in a 3:1 ratio. Ashwagandha seeds were sown in earthen pots and protected with plastic to prevent aerial contamination during the growth period. The pots were appropriately labeled, given a gentle watering and placed within the glasshouse. In the morning, the pots were watered until saturated. The leaves were then disinfected with a 1% sodium hypochlorite solution and rinsed with sterile distilled water to remove any residues. For inoculation, thirty-day-old plants were used. In the evening, a spore suspension (2×10^6 spores/ml) was sprayed on plants using an atomizer. Ten repetitions were maintained with one pot serving as control. Sterilized distilled water was applied by spraying to the control pot, which remain uninoculated. To maintain high humidity levels, polyethylene bags were utilized to cover both the inoculated and uninoculated pots for a duration of 48 hrs. Periodic observations on the progression of the disease were recorded starting from the onset of the disease. Using the tissue isolation method, the pathogen was re-isolated from the artificially infected plants exhibiting characteristic symptoms and the identification was verified based on the initial description. The obtained culture was transferred to PDA slants for comparing it with the original culture and conducting additional research.

Molecular characterization
DNA extraction
Fungal mycelium (300 mg) from a seven-day-old culture of *A. alternata* grown on PDA was crushed to powder form in liquid nitrogen (LN_2). Pre-warmed CTAB extraction buffer (1 ml) was added in mortar and pestle and crushed up to sample turned into liquid form. The crushed liquid solution was taken into a 2 ml centrifuge tube and a short spin was given for 30 to 60 seconds. The tubes were put in a laboratory water bath and incubated at a temperature of 65 °C for a period of 1 hour, with continuous agitation. After incubation, an equivalent amount of chilled chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min at 4 °C to separate the clear phases. A centrifuge tube (1.5 ml) was used to properly transfer the supernatant. The aforementioned procedures, commencing with the introduction of chloroform: isoamyl alcohol (24:1) and resulting in the removal of the supernatant, were carried out two more times in a consecutive manner. To precipitate desired components, supernatant was thoroughly mixed by double the volume of absolute ethanol. The tubes were left to incubate overnight at -20 °C to allow for DNA precipitation. Following the overnight incubation, the sample underwent centrifugation at 12,000 rpm for 15 minutes at 4 °C to separate its components. The supernatant was discarded and 200 µl of 70 percent ethanol was added to wash the pellet. The sample was then centrifuged at 6,000 rpm at 4 °C for 8 minutes. This process was repeated. The ethanol was removed completely and pellet was left to air dry on tissue paper for 5 to 10 minutes. The pellet was dissolved into 50 µl of TE buffer. To the dissolved DNA stock, 0.8 µl of RNase was added and incubated in a water bath at 37 °C for 45 min, followed by 65 °C for 10 minutes to deactivate the enzyme. The samples were preserved at a temperature of -20 °C in a deep freeze for future long-term use.

Quantitative and qualitative analysis of extracted genomic DNA
Using a Nano Drop Spectrophotometer, the absorbance at 230 nm, 260 nm, and 280 nm was quantified to determine the DNA concentration. The DNA content of each sample was decided by quantifying the absorbance at A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratio. The quality of extracted total DNA was

decided through 0.8% agars gel electrophoresis. The PCR conditions required for ITS region showed in Table 1.

Table 1: PCR conditions for ITS region

Steps	Temp. (°C)	Duration (min)
Initial denaturation	96	4
Denaturation	96	1
Annealing	53	50 second
Extension	72	1
Final extension	72	5
Hold	4	
No. of cycles	35	

Amplification of nucleotide sequences

Sequences were amplified using primer pairs ITS1 and ITS4 as described by White *et al*. 1990 [10]. The sequencing of the amplified product was carried out. These sequences were analyzed using the BLAST program to identify homologous matches in the Gen Bank database of NCBI, USA [1].

Results and Discussion

Symptomatology

The symptoms of the Alternaria leaf spot appeared as typical lesions of dark brown colour with clearly visible concentric rings (Plate 1A). Symptoms initially observed on mature leaves, were characterized by enlarging lesions and exhibited a narrow halo of chlorate discoloration. As the disease progressed, these spots increased in size and coalesced resulting in the coverage of a larger leaf area. Maiti *et al*. (2007) [4] and Meena *et al*. (2019) [5] also recorded corresponding symptoms. They observed the development of small, brown spots on the upper surface of older leaves, which gradually transformed into irregular, dark lesions with a surrounding water-soaked yellow halo.

Isolation and purification of pathogen

The samples exhibiting symptoms of leaf spot were collected from the Ashwagandha field and carefully examined under a microscope to verify the existence of the pathogen. The diseased tissues were isolated from infected parts and the resulting fungal culture was purified using a single hyphal tip method. To sustain the pure culture throughout the study, regular transfers were performed onto PDA slants at specific intervals. Pati *et al*. (2008) [6] and Meena *et al*. (2019) [5] isolated the *A. alternata* from Alternaria leaf spot-infected leaves of Ashwagandha on PDA media.

Cultural characters

The fungus (*A. alternata*) produced dense and fluffy mycelial growth, ranging in colour from black to dark black with a circular margin. On PDA, fungal colonies grew fast, reaching a diameter of 70-80 mm within ten days at 28±1 °C temperature. After seven days of fungal growth, the conidia were produced singly or in a chain.

Morphological characters

The microscopic observations revealed that the conidiophores of *A. alternata* were simple, septate, branched, flexuous and dark brown. Conidia were dark brown, cylindrical, ovoid, rounded base, echinulate or verrucose and usually had a beak that was short, thick, elongated and tapering or fusiform (Plate 1B). Conidia were measured 14.80-32.8 × 3.12-7.56 µm on average. The conidia occurred either singly or in clusters, with 3-4 transverse septa and 1-2 longitudinal septa. The size of the beak was 5.68-11.45 × 0.46-2.85 µm. The cultural and morphological characteristics of *A. alternata* closely resembled the original description given by Keissler (1912) [3].

(A) (B)

Plate 1: (A) Symptoms of Alternaria leaf spot and (B) Morphological characters of *A. alternata*

Pathogenicity test

The pathogenicity test was carried out as described in material and methods. At fifteen days post-inoculation, the presence of symptoms was evident on the inoculated leaves, characterized by light brown necrotic spots exhibiting concentric rings. (Plate 2). No disease symptoms were observed in non-inoculated plants. Leaves from artificially inoculated Ashwagandha plants were collected and subsequently re-isolated on PDA media. The re-isolated pure culture of *A. alternata* remained indistinguishable from the original culture, confirmed the fulfillment of Koch's postulates. This pathogenicity test proved that *A. alternata* (Fr.) Keissler is the causal agent of Alternaria leaf spot in Ashwagandha. Kalieswari *et al*. (2016) [2], Meena *et al*. (2019) [5], and Rahman *et al*. (2020) [7] obtained analogous results, providing comprehensive evidence of Ashwagandha leaf spot pathogenicity through the spore suspension spray inoculation method.

DNA extraction and quantification

DNA isolation was conducted employing the CTAB (Cetyl trim ethyl ammonium bromide) DNA extraction technique, as detailed in the materials and methods section. Nano Drop Spectrophotometer, the amount of DNA in the sample was measured at A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}. For subsequent analysis, samples containing a substantial DNA concentration (2786.34 ng/µl) and free from RNA/protein contamination were selected.

Polymerase chain reaction

The primer pairs ITS1 and ITS4 were successful in amplifying the ITS rDNA region, resulting in a 528 bp amplicon specifically for *A. alternata*. The products of amplification were assessed using a 1.5 percent agarose gel. On an agarose gel, representative images of the PCR results are depicted in Plate 3.

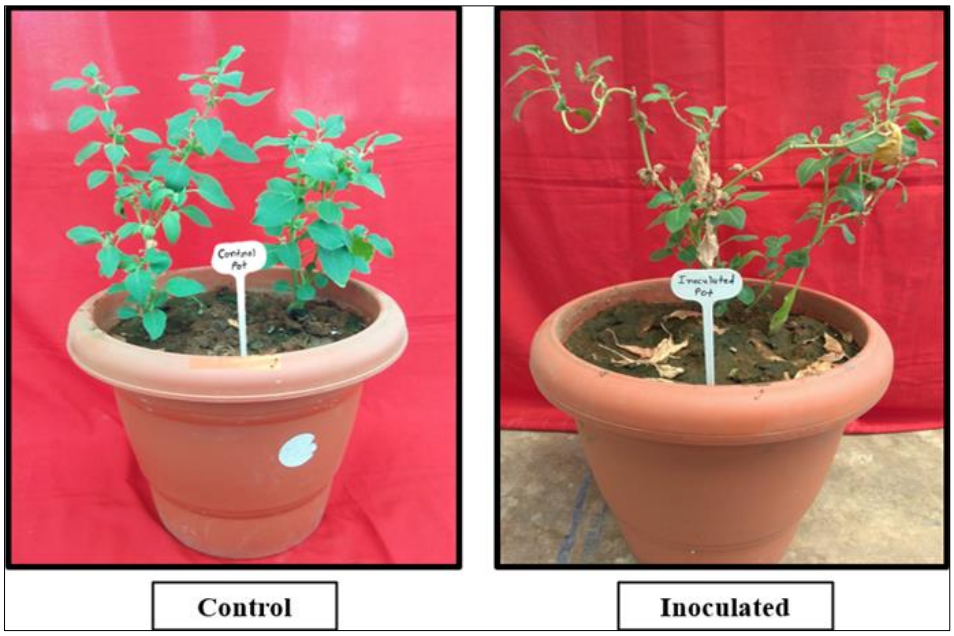

Plate 2: Pathogenicity test of *A. alternata* on Ashwagandha plants

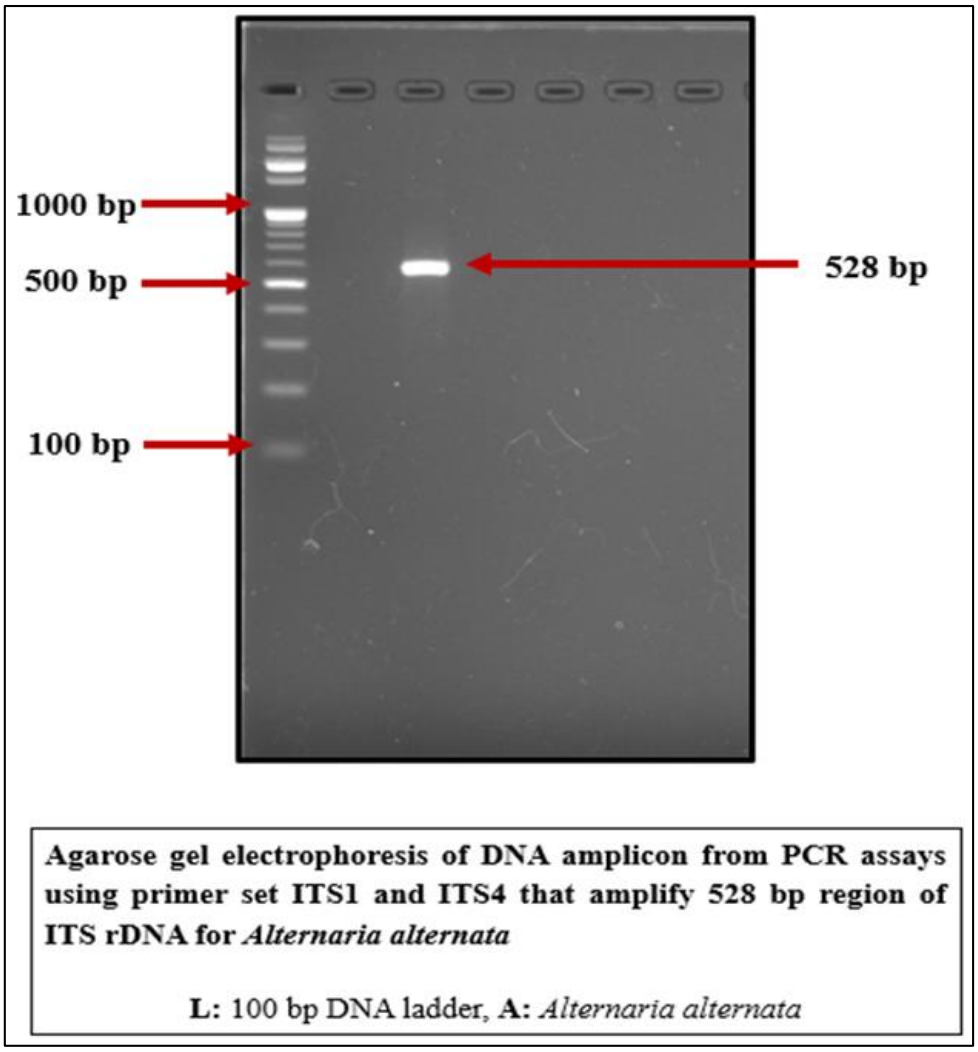

Plate 3: Agarose gel electrophoresis of PCR product of *A. alternata*

Conclusions

Ashwagandha has been used for centuries due to its powerful adapt genic properties. Alternaria leaf spot is a substantial disease affecting Ashwagandha plants. Considering the gravity of this disease and the economic significance of the crop, the current investigation was conducted to perform DNA sequencing using the ITS region and carried out cultural and morphological studies of *A. alternata*. Alternaria leaf spot

symptoms showed a narrow chlorotic halo around affected areas. Gradually, spots enlarged, coalesce and covered a larger leaf area. The morphological characteristics of the pathogen were identical to *A. alternata.* Sequencing of the ITS rDNA region in *A. alternata* provided precise species-level identification of the pathogen.

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