The Pharma Innovation Journal 2022; 11(12): 1777-1782

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(12): 1777-1782

www.thepharmajournal.com
Received: 27-10-2022
Accepted: 29-11-2022

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Characterization of pathogen associated with the early blight of potato in Gujarat, India

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Abstract
Potato (*Solanum tuberosum* Linn.) is an important vegetable crop cultivated throughout the world. It is a perennial herb belonging to the family *Solanaceae*, a native of the Andean Plateau of South America. However, the early blight of potatoes caused by *Alternaria solani* is a prominently observed disease in most potato-growing areas. Early blight disease is one of the most common and widespread diseases of potatoes. Early blight disease appeared as brown, round to oval necrotic spots with concentric rings on infected leaves. The isolation from diseased plant leaves revealed the association of *Alternaria* sp., which further satisfied Koch's postulates. Based on cultural and morphological characters of pathogen and confirmation by sequencing of ITS rDNA region of a pathogen (NCBI accession number: MZ557830), proved that early blight of potato caused by *Alternaria solani* (Ellis and Martin) Jones and Grout in Gujarat conditions.

Keywords: Potato, *Alternaria solani*, ITS1, ITS4

Introduction
Potato (*Solanum tuberosum* Linn.) is an important vegetable crop cultivated throughout the world. The power of potato is known for sustaining millions of lives by providing nutritious food in times of war and hunger and is also used as a staple food in several countries of the world. In Gujarat, it is mainly cultivated in Banaskantha, Aravalli, Sabarkantha, Gandhinagar, Mehsana, Kheda, Anand, Patan, Vadodara, Mahisagar, Panchmahal and Ahmedabad districts. Among all diseases of potato, early blight disease caused by *Alternaria solani* (Ellis and Martin) Jones and Grout is widely distributed and highly destructive depending upon the variety grown, weather conditions and inoculum load in the soil. This disease can cause an average annual yield loss of approximately 79 percent of the total production of potato. To overcome such issues, eco-friendly inputs are one of the best, low-cost and ecologically sustainable methods for managing plant diseases. Considering the importance of the disease, the present study on various aspects of the disease was carried out at the Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand from 2021 to 2022 for developing effective and eco-safe management strategies for the disease. The isolation was made from potato leaves showing characteristic symptoms, revealed the association of *A. solani*. A total of 36 isolates were collected from major potato growing areas of different agro-climatic zones of Gujarat. The isolated fungus satisfied Koch's postulates on potato plants by employing the spore suspension spray inoculation method. The identification of the pathogen was confirmed based on cultural and morphology characters as well as through DNA sequencing. The ITS rDNA region-based sequencing of *A. solani* proved accurate for species-level identification of the pathogen. The phylogenetic tree was also constructed and compared with other similar worldwide fungal isolates available in the NCBI database. It is evident from the ITS rDNA sequence that pathogenic *Alternaria solani* (GenBank Accession No. MZ557830) is responsible for causing early blight in potato growing regions of Gujarat

Materials and Methods
Information about work place
Location
Anand Agricultural University is located in Anand, Gujarat, at 22^0-35' North latitude and 72^0-55' East longitude with a 45-meter elevation above mean sea level. It falls under Central Gujarat Agro-climatic Zone (III).

Climate

Anand Agricultural University, Anand has an average rainfall of the tract is about 1333 mm. It starts in the middle of June and ends in the middle of September. Winter is fairly cool and dry, while summer is quite hot, where the temperature ranges from 30 to 45 °C.

Sterilization of soil and earthen Pots

Sandy loam soil was mixed with Farm Yard Manure (FYM) in a 3:1 ratio and autoclaved at 1.2 kg/cm^2 pressure for two hours and two days before being placed in surface-sterilized earthen pots for the experiment. Before usage, the earthen pots were carefully cleaned with tap water and disinfected for two minutes with a 4 percent formaldehyde (Formalin 40 %) solution.

Isolation, purification, identification and pathogenicity of the causal agent

Collection of Diseased Samples/Isolates

A total 36 isolates of *Alternaria* sp. were collected during *Rabi* 2020-21 from different potato growing districts of Gujarat *viz*., Anand, Kheda, Gandhinagar, Aravalli, Sabarkantha and Banaskantha (Table 1). The diseased leaves of potato showing the typical symptoms of early blight having target spots were collected, placed in brown paper bags and brought to the laboratory for microscopic examination and tissue isolation of the causal agent for further studies.

Symptomatology

The visual and microscopic examination of typical early blight samples was carried out to confirm the presence of the pathogen. The typical symptoms of early blight on leaves of potato under field conditions were visually observed and recorded (Plate 1).

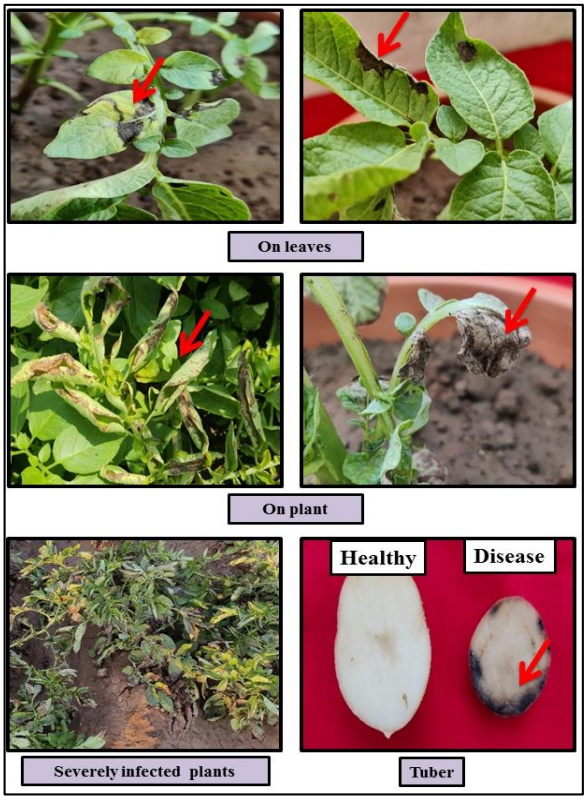

Plate 1: Typical symptoms of early blight of potato caused by *A. solani* on plant parts

Plate 2: Photomicrographs of cultural and morphological characters of *A. solani* (As1 isolate) causing early blight potato

Isolation and Purification of the Pathogen

The fungus was isolated from leaf tissues of potato showing characteristic early blight symptoms. Standard tissue isolation procedure was followed for isolation of the pathogen (Tuite, 1969) [9] as given below:

Purification

1. The culture obtained was purified by the hyphal tip method (Rangaswami, 1972) [7].
2. The obtained culture was maintained on PDA slants for further investigations.

Storage and Maintenance of Isolates of *A. solani*

The pure culture, thus obtained was maintained on PDA slants and kept at 28±1 °C for further investigation. Subsequent, sub-culturing was done once in 15 days on PDA slants and preserved in a refrigerator at 4 °C for further experimental use.

Morphological Identification of the Pathogen

Identification of pathogen causing early blight of potato grown on PDA medium was examined visually as well as microscopically for cultural and morphological characters. The cultural characteristics were recorded right from the initiation of growth up to 15 days including colony colour, the topography of colony, colony margin, colony diameter, sporulation and hyphal measurement. The morphological characters *viz*., length and width of conidia, conidial septation were measured under a microscope with high power magnification from 10 days old culture of *A. solani* and compared with those of the standard measurements given by Ellis (1971) [2] to identify the pathogen. The

photomicrographs of the same were also taken. Alternatively, genetic identification was also carried out to identify the fungal pathogen at the species level.

Identification of Pathogen through Internal Transcribed Spacer (ITS) Region

In the era of "Molecular Plant Pathology", DNA markers have become a powerful tool to study taxonomy and molecular genetics of a variety of organisms. Among the different DNA marker techniques, the ITS region of fungal DNA (rDNA) has been recognized as the official barcode for fungi (Schoch *et al.*, 2012) [8]. The ITS region is the widely sequenced DNA region in fungi useful for molecular systematic and has often been used in fungal diversity studies (Nilsson *et al*., 2009). The total genomic DNA was isolated from fungal mycelia using CTAB (Cetyl trimethyl ammonium bromide) DNA extraction protocol with some modification.

Quantitative analysis of extracted genomic DNA

The DNA concentration was determined by measuring the absorbance at 230, 260 and 280 nm, using NanoDrop Spectrophotometer (NanoDrop 1000, USA). The amount of DNA in each sample was quantified by taking the readings at A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}.

Table 1: List of primers

Sr. No.	Primers	Sequence	Reference
1	Forward primer ITS 1	5´-TCCGTAGGTGAACCTGCGG-3´	White *et al.* (1990) [11]
2	Reverse primer ITS 4	5´-TCCTCCGCTTATTGATATGC-3´	

Table 2: Components for PCR reaction

Sr. No.	Reagents	Quantity (µl/reaction)
1	Dream *Taq* green PCR Master mix (MBI, Fermentas AG)	12.50
2	Forward primer (10 pM/µl)	1.00
3	Reverse primer (10 pM/µl)	1.00
4	Nuclease free water	8.00
5	Template DNA (50ng/µl)	2.50
	Total	25.00

PCR programming

Initial denaturation 96 °C- 4 min
Denaturation 96 °C- 1 min
Annealing 53 °C- 50 Sec
Extension – 72 °C- 1 min
Final Extension- 72 °C- 5 min
No. of cycles- 35

Homology analysis of the ITS sequences and phylogenetic analysis

Sequences were searched using the BLAST (Basic Local Alignment Search Tool) programme from the Genbank database of NCBI (National Centre for Biotechnology Information), USA (Altschul *et al*., 1997) [1].

Pathogenicity Test

The pathogenicity test of *A. solani* (As1 isolate) was proved through artificial inoculation of the pathogen by following the standard method of inoculation (Koch's postulates).

The pathogenicity test on the leaves of the potato (variety: Kufri Lauvkar) was carried out in a pot under glasshouse conditions. Ten earthen pots (30 cm diameter) were filled with sterilized soil and FYM at the ratio of 3:1. Potato tubers were grown in earthen pots, covered with plastic to avoid any airborne infection. The pots were labelled, watered gently and arranged in the glasshouse. The pots were watered up to saturation in the morning. The leaves were surface sterilized with 1 percent sodium hypochlorite solution and washed thoroughly with sterile distilled water to remove the traces. Thirty days old plants were used for inoculation purposes. Spore suspensions (2×10^6 spores/ml) spray inoculation was done in the evening hours with the help of an atomizer. Ten repetitions were kept by maintaining one control pot. The control pot without inoculation was sprayed with sterilized distilled water. The inoculated and uninoculated pots were covered with polyethene bags for 48 hrs. to provide high humidity. The observations on the disease development on leaves were recorded periodically from the initiation of the disease. Re-isolation was done from the artificially inoculated plants showing typical early blight symptoms by tissue isolation method and the identification of the fungus was confirmed as per the original description. The culture obtained by re-isolation was transferred on PDA slants for comparison with the original culture and further investigations.

Results and Discussion

Pathological Investigations

Collection of Diseased Samples/ Isolates

The diseased samples showing typical early blight symptoms were collected from major potato-growing regions of Gujarat. The presence of the pathogen was confirmed by a critical examination of the hand section of diseased tissue under the microscope. After proper drying, the identified diseased samples were labelled and preserved.

Symptomatology

To confirm the presence of the pathogen, a typical early blight sample was examined visually and microscopically. The typical symptoms of early blight on plant parts of potato under natural field conditions were visually observed and recorded. The symptoms of early blight of potato were mainly observed on the leaves. The disease appeared as characteristic dark brown to black lesions with concentric rings which produce a target spot effect. Symptoms were initially observed on older, senescing leaves. Enlarging lesions were often surrounded by a narrow chlorotic halo. Later, these spots increased in size and coalesced covering a larger leaf area and leading to a blighted appearance, under severe conditions brownish lesions were observed on the tuber. Similar types of symptoms were also observed by Mayee and Datar (1986) [5], Kucharek (2000) [4] and Waals *et al.* (2001) [10]. They reported that the disease appears on a leaf as pin-point size brown to black spots, usually on the older leaves. These lesions expand in size up to one and a half inches across, remaining brown with or without yellowing surrounding the spot. The concentric rings were usually seen within the enlarged spots.

Isolation and Purification of Pathogen

The diseased sample/fungal isolates were collected from major potato growing districts of Gujarat *viz.*, Anand, Kheda, Gandhinagar, Aravalli, Sabarkantha and Banaskantha and confirm the presence of a pathogen under the microscope. After confirming the presence of the pathogen by microscopy, the diseased tissues from infected leaf parts were subjected to tissue isolation. The isolated fungal culture isolates were further purified by a single hyphal tip method. The pure culture of the pathogen isolates was maintained by periodically transferring on PDA slants throughout the investigations (Plate 2).

Zheng *et al*. (2015) [12] and Kaur *et al*. (2020a) [3] isolated the *A. solani* from early blight infected tissues of potato on PDA media and purified them by a single hyphal tip method.

A total of 36 isolates were collected from major potato growing areas of different agro-ecological zones of Gujarat. For convenience, the isolates of *Alternaria solani* were designated as As1 to As36 (Table 1).

Taxonomy and Identification of the Pathogen

The fungus isolated from diseased leaf samples was purified by a single hyphal tip method. The cultural and morphological characteristics of fungus grown on PDA were studied and the molecular tool, Internal Transcribed Spacer (ITS) region was used to validate the identification of the fungal pathogen.

Cultural characters

Fungal colonies grew very fast on PDA and attained a diameter of 70-80 mm in five days at 28±1 °C Mycelial growth was linear, fluffy, initially greenish when young, but later turned dark black to white. The colony margin was rough and regular. Conidia were generated singly or in a chain after seven days of fungal development.

Morphological characters

Microscopic examination revealed that mycelium was septate and hyaline Conidia were muriform, septate, light to dark brown and measured 23.2-58.7 x 11.5- 25.9 µm on average. Conidia were found in a single or a mass form, broadly rounded base with 4-6 transverse septa and 1-2 longitudinal septa. The size of the beak was 11.9-24.1×1.6-6.1 µm (Plate 2B). Based on cultural and morphological characteristics, the pathogen is primarily identified as *Alternaria solani* (Ellis and Martin) Jones and Grout. The above cultural and morphological characteristics of *A. solani* isolated from the early blight infected potato plant were closely identical to the description of *A. solani* which was given by Ellis (1971) [2].

DNA extraction and quantification

As mentioned in the materials and methods section, DNA was extracted using the CTAB (Cetyl trimethyl ammonium bromide) DNA extraction procedure. The amount of DNA in a sample was quantified using NanoDrop Spectrophotometer by taking the readings at A260nm/A280nm (1.86) and A260nm/A230nm (1.91). For further investigation, samples with a high quantity of DNA (4941.0 ng/l).

Polymerase Chain Reaction (PCR)

The ITS rDNA region was amplified by using universal primer pairs ITS1 and ITS4. The primer pair ITS1 and ITS4 resulted in a 540 bp amplicon for *A. solani*. A 1.5 percent agarose gel was used to examine the amplified products (Plate 3).

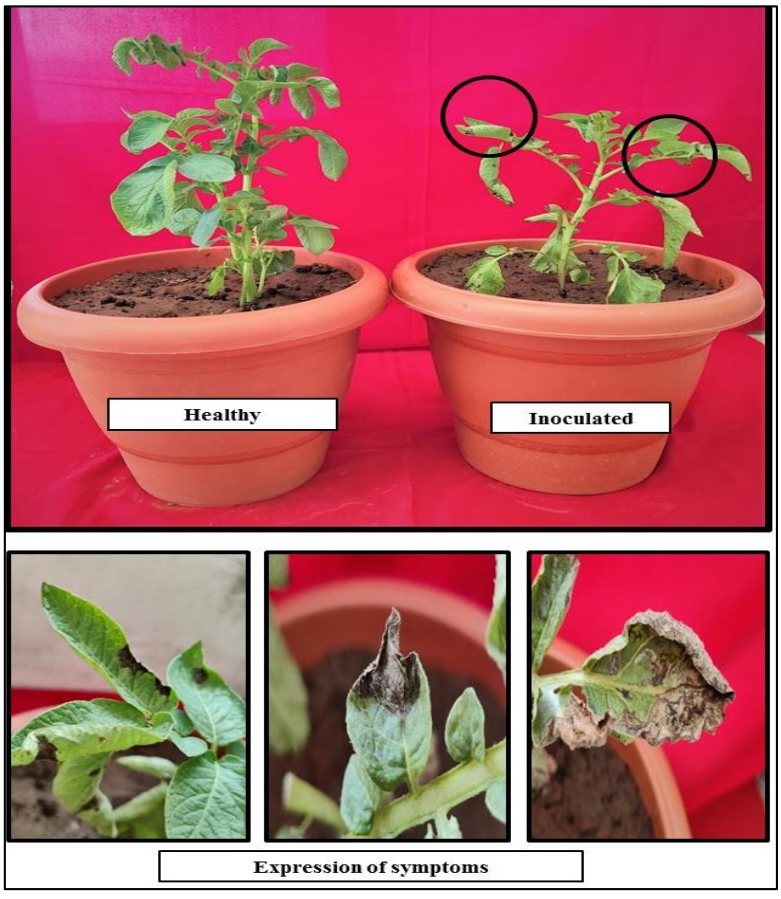

Plate 3: Pathogenicity test

Pathogenicity Test

The pathogenicity test of one representative isolate of *A. solani* (As1) was carried out on the K. Lauvkar variety of potato by using the standard inoculation method as described in materials and methods. Fifteen days after inoculation, the symptoms appeared on inoculated leaves as brown, round to oval necrotic spots with concentric rings (Plate 3). The plants which were not inoculated with the fungal spore suspension did not show any symptoms of the disease. Leaves were collected from artificially inoculated and infected potato plants and re-isolated separately on PDA. The re-isolation yielded the pure culture of *A. solani* identical to the original in all the respect thus proving Koch's postulates. By this pathogenicity test, it was confirmed that the causal organism of early blight of potato is *A. solani* (Ellis and Martin) Jones and Grout (Plate 4).

Similar types of results were obtained by and Kaur *et al.* (2020a) [3] who successfully proved the pathogenicity of early blight of potato by using the spore suspension spray inoculation method.

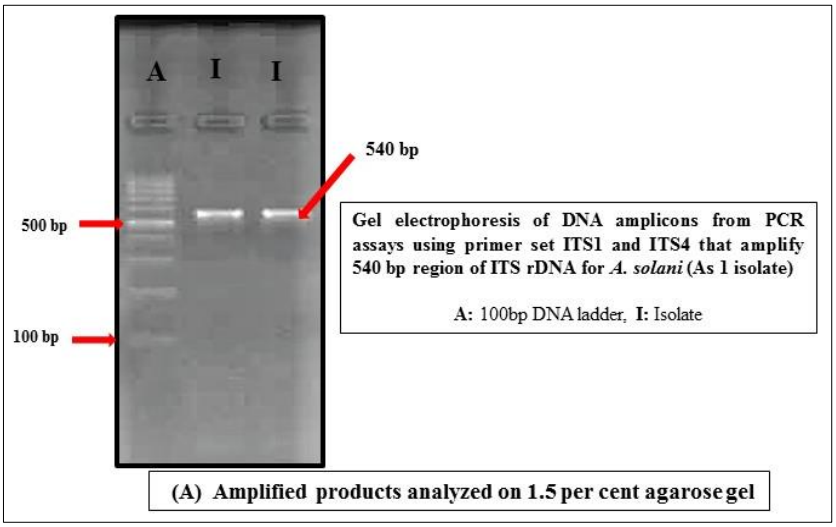

Plate 4: DNA of isolates of *A. solani* obtained on agarose gel electrophoresis

Table 3: Sources of isolates of *A. solani* collected from the major potato growing regions of Gujarat

Sr. No.	Isolate No.	District	Taluka	Place	GPS Location
1	As1	Anand	Anand	Anand-1	22.536471°N 72.980321°W
2	As2			Anand-2	22.536636°N 72.982272°W
3	As3			Bakrol	22.573503°N 72.910713°W
4	As4			Lambhvel	22.591490°N 72.937305°W
5	As5			Boriyavi	22.616162°N 72.932162°W
6	As6			Khambhodaj	22.575454°N 73.087227°W
7	As7	Kheda	Nadiad	Narsanda	22.627235°N 72.887928°W
8	As8			Chaklasi	22.657834°N 72.944251°W
9	As9			Kanjari	22.622818°N 72.913822°W
10	As10			Vadtal	22.5980023°N 72.872672°W
11	As11		Kathalal	Shahpur	22.902035°N 73.017003°W
12	As12		Kapadvanj	Unda ni muvadi	22.980844°N 73.010760°W
13	As13			Kevadiya	23.083597°N 72.992592°W
14	As14	Gandhinagar	Dehgam	Khanpur	23.233534°N 72.896837°W
15	As15			Rakhiyal	23.255550°N 72.903576°W
16	As16			Babalpura	23.214071°N 72.864214°W
17	As17	Aravalli	Bayad	Borol	23.238635°N

					73.219916°W
18	As18			Madhav kampo	23.192463°N 73.228671°W
19	As19			Demai	23.175435°N 73.206352°W
20	As20			Bhukhel	23.245282°N 73.183691°W
21	As21			Bibipura	23.267255°N 73.218824°W
22	As22	Sabarkantha	Prantij	Dhanpura kampa	23.238320°N 73.081441°W
23	As23			Vadrad	23.432129°N 72.893863°W
24	As24			Dalpur	23.515901°N 72.928882°W
25	As25		Bhiloda	Bhavanath	23.787943°N 73.244761°W
26	As26		Idar	Kanpur	23.832948°N 73.100565°W
27	As27			Gorol	23.875795°N 73.129281°W
28	As28			Badoli	23.846663°N 73.068925°W
29	As29			Netramani	23.852467°N 72.967876°W
30	As30		Vadali	Chorivad	23.929413°N 73.125230°W
31	As31	Banaskantha	Deesa	Deesa-1	24.286991°N 72.156044°W
32	As32			Aseda	24.211109°N 72.209240°W
33	As33			Deesa-2	24.267374°N 72.178397°W
34	As34		Dantiwada	Dantiwada	24.325896°N 72.332206°W
35	As35			Nani bhakhar	24.280839°N 72.299247°W
36	As36			Vagharol	24.085406°N 72.244315°W

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