GIS-BASED SPATIAL ASSESSMENT AND MOLECULAR CHARACTERIZATION OF EARLY BLIGHT (*ALTERNARIA SOLANI*) IN POTATO-GROWING REGIONS OF PUNJAB, PAKISTAN

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ABSTRACT

Potato early blight, caused by the necrotrophic fungus *Alternaria solani*, is one of the most economically significant foliar diseases of potato worldwide, causing yield losses of up to 50% under favorable epidemic conditions. In Pakistan, especially in Punjab province, systematic data on the diversity of *A. solani* and spatial disease distribution are scarce. This study was designed to quantify disease incidence, prevalence, and plant mortality across 40 sites in four major potato-producing districts of Punjab, and to characterize *A. solani* isolates through morphological, microscopic, and ITS-based molecular analysis. Spatial analysis identified Okara and Khanewal as high-risk hotspots, with site-level disease incidence ranging from 21% to 68%, disease prevalence up to 62%, and plant mortality up to 64%. District-level means showed Okara with the highest disease incidence (56.7%), prevalence (54.8%), and mortality (51.8%), while Sahiwal recorded the lowest values across all three parameters. Morphological and microscopic examinations revealed variation among isolates in colony and conidial characteristics. According to the sequence analysis by the information technology service (ITS), the similarity between the sequence and reference sequences in the NCBI database was 99.1-99.8%. The results obtained along with the morphological and microscopic features helped in the identification of the studied isolates as *A. solani.* This is the first GIS-based epidemiological and molecular study of potato early blight in key potato-growing districts of Punjab, providing a spatially explicit framework for disease surveillance and management.

Keywords: GIS-based Mapping; Early Blight of Potato; *Alternaria solani*; Disease Incidence; Disease Prevalence; Spatial Epidemiology; ITS Phylogenetics; Punjab Pakistan

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INTRODUCTION

Potato (*Solanum tuberosum* L.) ranks third globally in food crop production after sugarcane and tomato (FAO, 2024). It forms the foundation of horticulture in Pakistan, where Punjab province produces more than 90 percent of the national potato output (Pakistan Bureau of Statistics, 2022). Despite its agricultural importance, potato production faces a persistent biotic challenge from early blight caused by the necrotrophic fungus *Alternaria solani*, which significantly threatens both production and tuber quality on a global scale (Chaerani and Voorrips, 2006; Cristina *et al*., 2021).

Early blight manifests as concentric, target-shaped lesions on leaves, stems, and tubers, leading to premature defoliation, reduced photosynthetic capacity, and yield losses that can exceed 50% under favorable environmental conditions (Rotem, 1994; van der Waals *et al*., 2001). The disease is especially widespread in warm and humid climates prevalent in most parts of the Indo-Gangetic plains, including Punjab, Pakistan. Survival of *A. solani* in crop residues and soil, combined with high genetic variability, makes long-term disease management and resistance breeding challenging (Leiminger and Hausladen, 2012).

The main methods of plant disease assessment are visual field observations and manual sampling. They offer useful information on disease occurrence and severity, but can be labour intensive, time consuming and observer dependent (Bock et al., 2010; Buja et al., 2021). Geographic Information Systems (GIS) can be used to make maps and analyse the spatial distribution of plant diseases and determine regions with relatively high disease levels. GIS-based analysis can also incorporate disease observations with environmental and geographical factors, if available, to aid in the surveillance and management of the disease, thereby helping in decision making (Tsouros et al., 2019; Mahanta et al., 2024).

Accurate pathogen identification is critical for effective disease management. Although morphological characterization provides preliminary diagnostic information, molecular techniques such as Internal Transcribed Spacer (ITS) sequencing have been widely employed for identification of *Alternaria solani* and closely related species with high

specificity (Woudenberg *et al*., 2014; El-Ganainy *et al*., 2021; Ayad *et al*., 2019). GIS applications in agriculture, reviewed by Mathenge *et al*. (2022), further support spatial analysis of disease distribution.

In Punjab, despite frequent reports of early blight outbreaks in potato-growing fields, there is a general lack of systematic research on the spatial distribution of the disease, molecular identity of local isolates, and science-based disease management. No GIS-based epidemiological and molecular study of early blight has been conducted in this region, representing a critical knowledge gap. Accordingly, there is an urgent need for spatially explicit, science-based approaches integrating field surveillance, GIS-based mapping, and molecular diagnostics.

The objectives of this study were to: (1) quantify the disease incidence, prevalence, and plant mortality of potato early blight across 40 sites in four major potato-producing districts of Punjab, Pakistan; (2) generate GIS-based spatial distribution maps to identify disease hotspots and spatial patterns of disease intensity; (3) characterize *Alternaria solani* isolates through colony morphology and conidial microscopy; and (4) confirm pathogen identity through ITS-based PCR amplification, BLAST analysis, and Maximum Likelihood phylogenetic inference.

MATERIALS AND METHODS

Survey and Sample Collection: A field survey was conducted at 40 potato-growing sites across four districts of Punjab, Pakistan: Multan, Khanewal, Sahiwal, and Okara. Ten sites were surveyed in each district. The survey was conducted across both urban and rural potato-growing regions to assess disease incidence, prevalence, and severity during the potato growing season.

Each location was subdivided into quadrants systematically in an X formation, and samples were picked on the lines that cut across. Specifically, leaves with the typical symptoms of the disease turn into rounded, irregular spots about 2-5 mm in diameter, and their color is dark brown to black; thus, were selected in the field specifically. Infected potato leaves and roots, as well as soil in rhizosphere, were sampled with a sharp knife and put in polythene sampling bags. All bags were well labeled with the date and locality where the sample was taken. The obtained samples were further taken to the Plant Pathology Laboratory, BZU Multan, where they were cultured and their pathotypes of *A. solani* determined.

Assessment of Early Blight Disease: During surveys, 50–150 plots with 100–500 plants per plot using an X pattern were observed at each site. The level of disease was determined by visual estimation of the percentage of plant tissue showing early blight symptoms. For consistency and standardization, a disease rating scale of 0–5 was used (James, 1971), with modifications as described by Chaudhary *et al*. (2021) (Table 1).

Table 1. Rating Scale for The Assessment of Early Blight of Potato

Categories	Ratings	Infected Area (%)	Description of the symptoms
I	0	0	No infection on leaves
II	1	1 - 10	Small irregular spots on leaves
III	2	11 - 25	Small irregular brown spots with concentric rings
IV	3	26 - 50	Small brown spots develop brown lesions with concentric rings
V	4	51 - 75	Lesions coalesce to form irregular and appears as a typical blight symptom
VI	5	76 - 100	Severe typical blight symptoms and sometimes dead leaves

Disease prevalence, disease incidence, and crop mortality were calculated using the standard epidemiological formulas as defined by Campbell and Madden (1990) and Nutter *et al*., (1991):

$$\text{Disease Prevalence (\%)} = \frac{\text{No. of locations showing symptoms}}{\text{Total No. of locations studied}} \text{ X } 100$$

$$\text{Disease Incidence (\%)} = \frac{\text{No. of infected plants}}{\text{Total No. of plants studied}} \text{ X } 100$$

$$\text{Mortality of crop plants (\%)} = \frac{\text{No. of dead plants}}{\text{Total No. of plants studied}} \text{ X } 100$$

Statistical Analysis: This study was observational in design. Disease incidence, prevalence, and plant mortality data collected from 40 survey sites across four districts were analyzed using one-way ANOVA in Statistix 8.1 to test for significant differences among districts ($\alpha = 0.05$). The assumptions of ANOVA (normality and homogeneity of variance) were verified using residual plots and Levene's test, respectively. Proportional data (disease incidence and prevalence)

were arcsine square-root transformed prior to analysis. Pairwise mean comparisons were performed using Fisher's Least Significant Difference (LSD) test (Steel and Torrie, 1997). Pearson's correlation coefficient (r) was calculated to assess the strength and direction of linear relationships among disease incidence, disease prevalence, and plant mortality using Statistix 8.1. Correlation strength was interpreted as: $|r| \geq 0.70$ = strong; $0.40 \leq |r| < 0.70$ = moderate; $|r| < 0.40$ = weak. Direction was expressed as positive or negative. Correlations were considered statistically significant at $p \leq 0.05$.

GIS-based Spatial Analysis: The spatial distribution of disease intensity and the examination of potential associations between environmental factors and severity of the disease were examined with the help of GIS techniques, including the buffer analysis and interpolation. The data about the disease and GPS coordinates on each location were measured, and the data set was saved and processed through ArcGIS after weeding out unreliable data points. The table data were transferred into a point feature layer by using the Display XY Data tool and using the appropriate latitude and longitude options. The severity of the disease was demonstrated on the map using equal intervals with the help of a color range that the points received due to the disease rating, and a clear legend to define the levels of severity. The map that was developed finally had all the necessary features like a title, north arrow and a scale bar and a detailed legend showing which all the map layers were in detail, such that the information would be easily read. Mapping processes were conducted with the ArcGIS software version 10.8, and other GIS techniques that had been reported by Mahanta *et al.* (2024).

Pathogen Isolation and Purification: Leaf samples were surface-sterilized by rinsing with distilled water, treating with 0.5% sodium hypochlorite solution for 2 minutes (Barnett and Hunter, 1998), and rinsing again with sterile distilled water to remove surface contaminants. Leaf pieces (5 mm^2) including both diseased and healthy tissue were plated onto potato dextrose agar (PDA) supplemented with streptomycin sulfate (100 $\mu g\ mL^{-1}$) under laminar airflow conditions (Dhingra and Sinclair, 1995). Plates were incubated at 28 ± 1°C under a 12-h light/dark cycle. Emerged fungal colonies were purified by single-spore isolation and transferred to PDA slants for storage as reference cultures.

Morphological Characterization of the *A. solani*: Morphological observations were conducted after the cultures grew to three weeks, following the procedure outlined by Yu *et al.* (2003). Morphological characteristics were noted and included colony size, color, shape of the colony edges and patterns found on the colony surface (rings or zones). Spores' shape, color, size, and the count of septa were viewed using a Zeiss Primo Star microscope with an AxioCam ERC 5s camera under observation at 40x and 100x magnifications.

Molecular Characterization of the Pathogen

DNA extraction: DNA of the pathogenic fungus was isolated by employing the CTAB technique outlined by Doyle and Doyle (1987). Tris-HCl, NaCl, EDTA, CTAB and double-distilled water were combined to create an extraction buffer; b-mercaptoethanol was added after heating was performed to activate the solution and enhance the yield of DNA. Pure cultures of *A. solani* were used to obtain fungal mycelium collected and ground into fine powder using liquid nitrogen. The mycelium was scraped off in Eppendorf tubes filled with CTAB buffer and b-mercaptoethanol, scraped and incubated at 65 degrees Celsius with frequent shaking to ensure full cell lysis and release of DNA. The incubation was followed by the addition of chloroform-isoamyl alcohol to separate the DNA and the impurities. Centrifuged them and the DNA in the clear upper layer moved to new tubes. The solutions were then kept at -20 degree centigrade to allow the removal of DNA by the addition of cold isopropanol. The liquid phase was discarded after centrifugation, and the pellet containing DNA was washed with 70% ethanol in order to eliminate the traces of salts and contaminants. The pellet was subsequently dried in the air and put into the double-distilled water. The DNA solution was transferred to -80 degree Celsius and kept at that temperature to be used in the future.

Polymerase Chain Reaction (PCR): The ITS of the 5.8S rDNA was also amplified with the primers of the ITS4 and ITS5 according to an established technique with slight modifications. The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was amplified using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). A total of 26.5 ul reaction mixture was prepared in each PCR reaction (Table 2). The reactions were performed with the aid of the BK-AI Standard Thermal Cycler (Biobase), and the temperature and time parameters of each step of the amplification are provided in Table 3.

Table 2. Recipe for Reaction Mixture of PCR

Sr. No.	Chemical Components	Amount Required
1	DNA Template	4µL
2	Primer ITS4 10 Pmol	3 µL
3	Primer ITS5 10 Pmol	3 µL
4	10X PCR buffer	2 µL
5	25mM MgCl2	3 µL
6	1mM dNTPs	2 µL
7	2U/µl Taq Polymerase	1.5 µL
8	H_2O	8 µL
	Total Volume	**26.5 µL**

This reaction mixture was composed of all the vital materials necessary to carry out a successful DNA amplification in the process of PCR.

Table 3. PCR conditions for the amplification of *A. solani* isolates

Sr. No.	Stage	Temperature	Duration
1	Initial denaturation	94°C	5 minutes
2	Denaturation	94°C	1 minutes
3	Annealing	55°C	50 seconds
4	Elongation	72°C	2 minutes
5	Final Extension	72°C	7 minutes

Gel Electrophoresis: A 1.25% (w/v) agarose gel was prepared by dissolving 1.5 g of agarose in 120 mL of Tris-acetate-EDTA (TAE) buffer. The mixture was kept in the hot water until the agarose was fully dissolved, and then the mixture was poured into the gel casting tray and left until fully solidified, after which the combs were removed and the tray locks opened. The solid gel, and the tray were transferred to the electrophoresis, where TAE buffer was added until the gel was submerged by a 2-4 mm layer of buffer as per the normal procedure, as explained by Sambrook and Russell (2001). Each DNA sample was mixed with 6X loading buffer using a gentle twirl, and 20 uL of the DNA sample and the loading buffer were added to the agarose gel using individual wells. Moreover, 10 ul of primers ITS4 and ITS5 were placed in each well, as in El-Ganainy *et al.* (2021).

Amplification results were verified by loading 6 uL of the PCR product into a 1% ethidium bromide-stained agarose gel and running it at 85 volts for 1 hr. Once the dye moved to the terminal of the gel, the power supply was reverted off, and the cables were pulled out. The lid of the electrophoresis chamber was then opened, and the gel was finally examined under UV light with the use of a gel documentation system.

Genome Sequencing and Phylogenetic Analysis: Purified PCR products from all five isolates were submitted to Macrogen Inc. (Seoul, Republic of Korea) for Sanger sequencing using the ITS4 forward primer. The resulting sequences were deposited in the NCBI GenBank database (Accession Numbers: PZ544711, PZ544780, and three additional accession numbers pending GenBank processing; to be updated in the final proof). Sequence alignment and BLAST searches were conducted against the NCBI database to identify closely related reference sequences. Phylogenetic analysis was performed in MEGA 11 (Tamura *et al*., 2021) using the Maximum Likelihood method with the Tamura-Nei substitution model (Tamura and Nei, 1993) and 1,000 bootstrap replicates. *Curvularia lunata* (FG129235) was used as the outgroup.

RESULTS

Survey of 4 Potato Growing Districts in Punjab: A survey conducted on the characteristics of the disease in 40 sites in 4 districts in Punjab, Pakistan, to investigate the severity of the disease, incidence, prevalence and mortality rate reveals different findings. Table 4 contains details of various sites, such as the GPS coordinates. The disease symptoms on potato plants were assessed by documenting the disease scores based on the disease rating scale (Figure 1; Table 1).

Table 4. Details of survey and sampling sites

Districts	Locations	GPS Coordinates
Multan	Gopalpur	30.052682N, 71.475900E
	Moza Jhok Jammu	29.9599220N, 71.4611340E
	5 Faiz	29.9536560N 71.5105540E
	19 Kasi Jhandy wala	30.1073624N 71.6054287E
	Duniya pur road near Faridpur	30.0469129N, 71.5819714E
	Basti Malook	29.8469892N, 71.5278428E
	Bhudla Sant	30.1527341N, 71.7096875E
	Nandla (Head Muhammad Wala)	30.2480987N, 71.4550724E
	Umerpur	30.2651089N, 71.4286185E
	Khanpur Gaziyan near Shujabad	29.9642077 N, 71.2902815E
Khanewal	15/V Khanewal	30.343447 N,71.905454E
	132/16 L Mian Channu	30.459121 N,72.313691E
	9/V Khanewal	30.407510 N,71.940807E
	Moza Nabi pur Kabirwala	30.4334404 N,71.872468E
	Chak No. 12/AH	30.369823 N,71.946163E
	Chak No. 171/10-R	30.234002 N,71.877328E
	Chak No. 108/15L	30.340229 N, 72.229116E
	Noori Suhag	30.481958 N,72.221662E
	Chak No. 36/10.R	30.347890 N,72.126230E
	Chak No. 88/10-R	30.304051 N,71.964569E
Sahiwal	97/6R	30.658713 N, 73.010650E
	Muhammad Pur	30.715926 N, 73.015277E
	87/6R	30.706048 N,73.095991E
	49/3R	30.822603 N, 73.280498E
	6/14-L Sahiwal	30.480216 N, 72.508164E
	69/4R Yousaf walla	30.703967 N, 73.191469E
	56/5L Sahiwal	30.727399 N, 73.25423E
	118/12L Kasowal	30.481570 N, 72.540536E
	77/5R Noor Shah Sahiwal	30.823762 N, 73.203215E
	10/11. L Chicha Watni	30.452853 N, 72.716104E
Okara	41/3R Okara	30.817164 N,73.367256E
	Adda Gambir Okara	30.7463508 N,73.3150204E
	55/2L Okara	30.816770 N, 73.481210E
	24/2L Adda Kissan Okara	30.837986 N,73.535653E
	5/1A Renala Khurd	30.893704 N, 73.595718E
	40/D Depalpur	30.732562 N, 73.579146E
	6/1 Shergarh Road Depalpur	30.778805 N, 73.691171E
	Salowal Depalpur	30.716719 N, 73.746726E
	Sharifabad Depalpur	30.554157 N, 73.666279E
	Landianwala	30.589937 N, 73.708261E

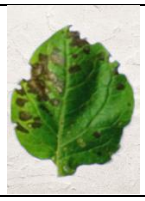
Rating scale	**0**	**1**	**2**	**3**	**4**	**5**
Level of Infection	**0**	**1-10**	**11-25**	**26-50**	**51-75**	**76-100**
Symptoms	Healthy leaves	Brown lesions started appear on the leaves	Disease in progress, lesions started enlarging	 Lesions became darker in colour and expanding towards mid rib	Severe infection: lesions turned darker, expanded to leaf margin and covered by yellow hallow.	At peak lesions became water soaked, bull's eye shaped with concentric rings and leaf chlorosis.

Figure 1: Disease rating scale explaining the different levels of infection due to Early Blight of Potato

One-way ANOVA revealed significant differences among districts for all three disease parameters ($p \leq 0.05$). Okara recorded the highest disease incidence (56.7%), disease prevalence (54.8%), and plant mortality (51.8%), followed by Khanewal (50.4%; 53.3%; 50.5%), Multan (47.2%; 45.8%; 46.0%), and Sahiwal (40.9%; 38.3%; 37.8%). For incidence, all four district means differed significantly (LSD = 2.028). For prevalence and mortality alike, all district pairs differed significantly except Okara and Khanewal (54.8% vs 53.3% and 51.8% vs 50.5%, differences below the LSD) (Table 5).

Table 5. Mean disease incidence (%), disease prevalence (%), and plant mortality (%) of potato early blight across four surveyed districts of Punjab, Pakistan, with LSD pairwise comparisons (ANOVA, $p \leq 0.05$).

Districts	**Disease Incidence (%)**	**Disease Prevalence (%)**	**Mortality (%)**
Okara	56.7	54.8	51.8
Khanewal	50.4	53.3	50.5
Multan	47.2	45.8	46.0
Sahiwal	40.9	38.3	37.8

LSD = 2.028

Pearson correlation analysis revealed a strong positive correlation between disease incidence and disease prevalence ($r = 0.7159$, $p \leq 0.05$). A moderate positive correlation was observed between disease incidence and plant mortality ($r = 0.6446$, $p \leq 0.05$). Disease prevalence and plant mortality exhibited a strong positive correlation ($r = 0.8588$, $p \leq 0.05$) (Table 6).

Table 6. Pearson correlation among disease incidence, prevalence, and mortality

	Disease Incidence (%)	**Disease Prevalence (%)**
Disease Prevalence (%)	0.7159*	
Mortality (%)	0.6446*	0.8588*

** Indicates statistically significant Pearson correlation coefficient at $p \leq 0.05$ (two-tailed). Positive r values indicate a positive linear association between variables*

GIS-based Distribution of Potato Early Blight Disease in Selected Districts of Punjab, Pakistan: GIS maps were used to show the distribution and the severity of the disease amongst the areas under study, and they were used to depict the general patterns and magnitude of the disease in the regions that were under the study.

Incidence (%) of Potato Early Blight Disease in Selected Districts of Punjab, Pakistan: Experiments revealed definite variations in the percent diseases incidence in different localities, indicating that not all the localities were impacted by the disease. In general, the lowest disease incidence was observed in Sahiwal and Khanewal through spatial

analysis. The highest incidence of disease was reported in Khanewal (Moza Nabipur Kabirwala 68%), 41/3R (67%) and Adda Gambir (62%) in Okara. Conversely, the disease was observed to be lower at Gopalpur (21%) at Multan, followed by 97/6R (22) at Sahiwal, meaning that the sites were less susceptible to the disease. There was several other sites that had moderate to high disease incidence that provided further insight into the total disease situation in the area. As an example, 6 /1 Shergharh Road, Depalpur had a disease incidence of 61%. Likewise, 55/2L Okara, 19 Kasi Jhandaywala, 40/D Depalpur, Umerpur, Chak No. 171/10-R, 5 Faiz, and 69/4R Yousafwala had values of incidence of diseases in the range of 51% 59% (Figure 2).

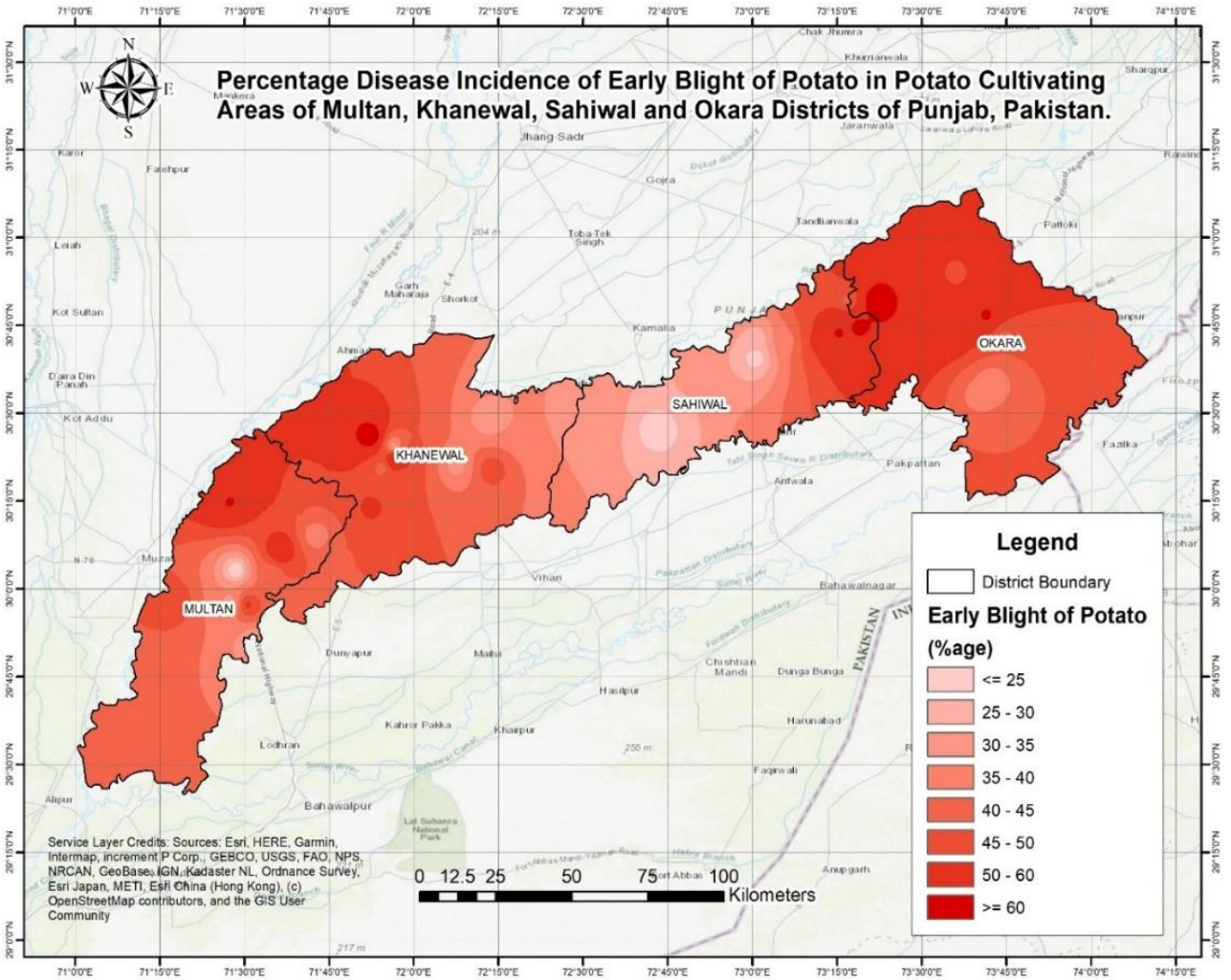


Figure 2. GIS map of Disease Incidence (%) of Early Blight of Potato in selected potato-cultivating areas of Multan, Khanewal, Sahiwal, and Okara districts of Punjab, Pakistan.

Prevalence (%) of Potato Early Blight Disease in Selected Districts of Punjab, Pakistan: The highest site-level disease prevalence was recorded at 41/3R in Okara (62%), followed by Moza Nabipur Kabirwala in Khanewal (61%). Adda Gambir in Okara and 56/5L in Sahiwal each recorded 59% disease prevalence. On the contrary, Gopalpur (17) in Multan, then 10/11L Chichawatni (18), and 97/6R (19) in Sahiwal had the lowest disease prevalence. There were a few other sites that had moderate and high percentages of Early blight, thus giving more reasons for the general occurrence of the disease. Chak No. 12/AH and 6/1 Shergarh Road Depalpur, both had a prevalence of 58%. On the same note, 55/2L Okara had a 57 percent prevalence, whereas 40/D Depalpur had a 55 percent prevalence. The prevalence of the disease was 54 percent in the sites 24/2L Adda Kissan Okara and Umerpur, and 52 percent in 19 Kasi Jhandywala (Figure 3).

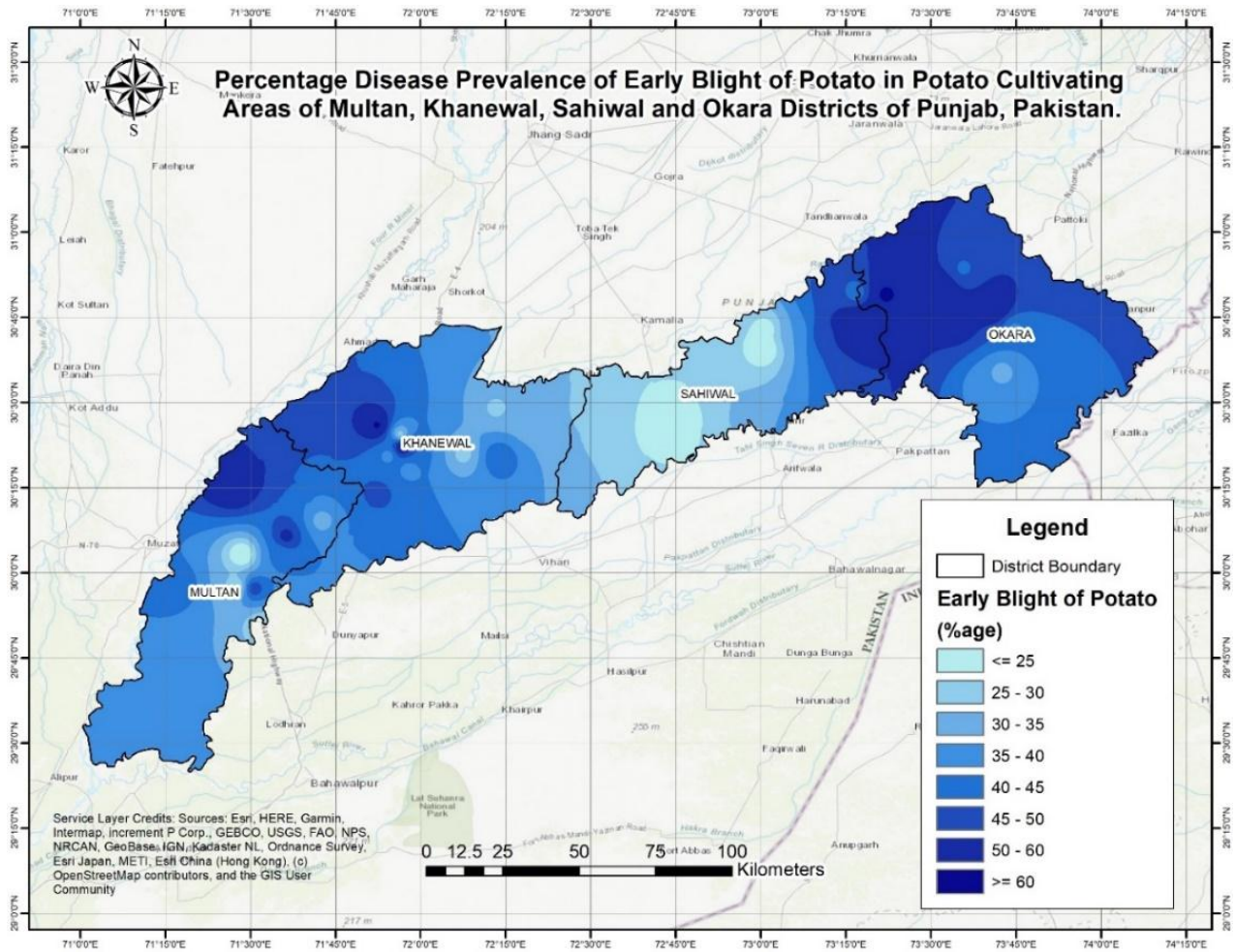


Figure 3. GIS map of Disease Prevalence (%) of Early Blight of Potato in selected potato-cultivating areas of Multan, Khanewal, Sahiwal, and Okara districts of Punjab, Pakistan.

Mortality (%) of Potato Early Blight Disease in Selected Districts of Punjab, Pakistan: The death of the potato plants due to Early blight during the field conditions was evaluated, but the results showed an obvious difference between the sampled sites. The highest mortality of plants was observed at the Moza Nabipur Kabirwala (64%) at Khanewal, and 41/3R (61) and Adda Gambir (60) in Okara. However, there were also lower levels of mortality of plants in some sites, such as Gopalpur (19%) in Multan and 10/11 Chichawatni (19%) in Sahiwal, with 97/6R (22) and Muhammadpur (24) in Sahiwal, respectively. There were also other sites that had moderate plant mortality. Both Chak No. 12/AH and 6/1 Shergarh road Depalpur registered mortality rates of 59%. Similarly, mortality rates at 56/5L Sahiwal were found to be 56, 55/2L Okara was 55 and 53 at 19 Kasi Jhandywala (Figure 4).

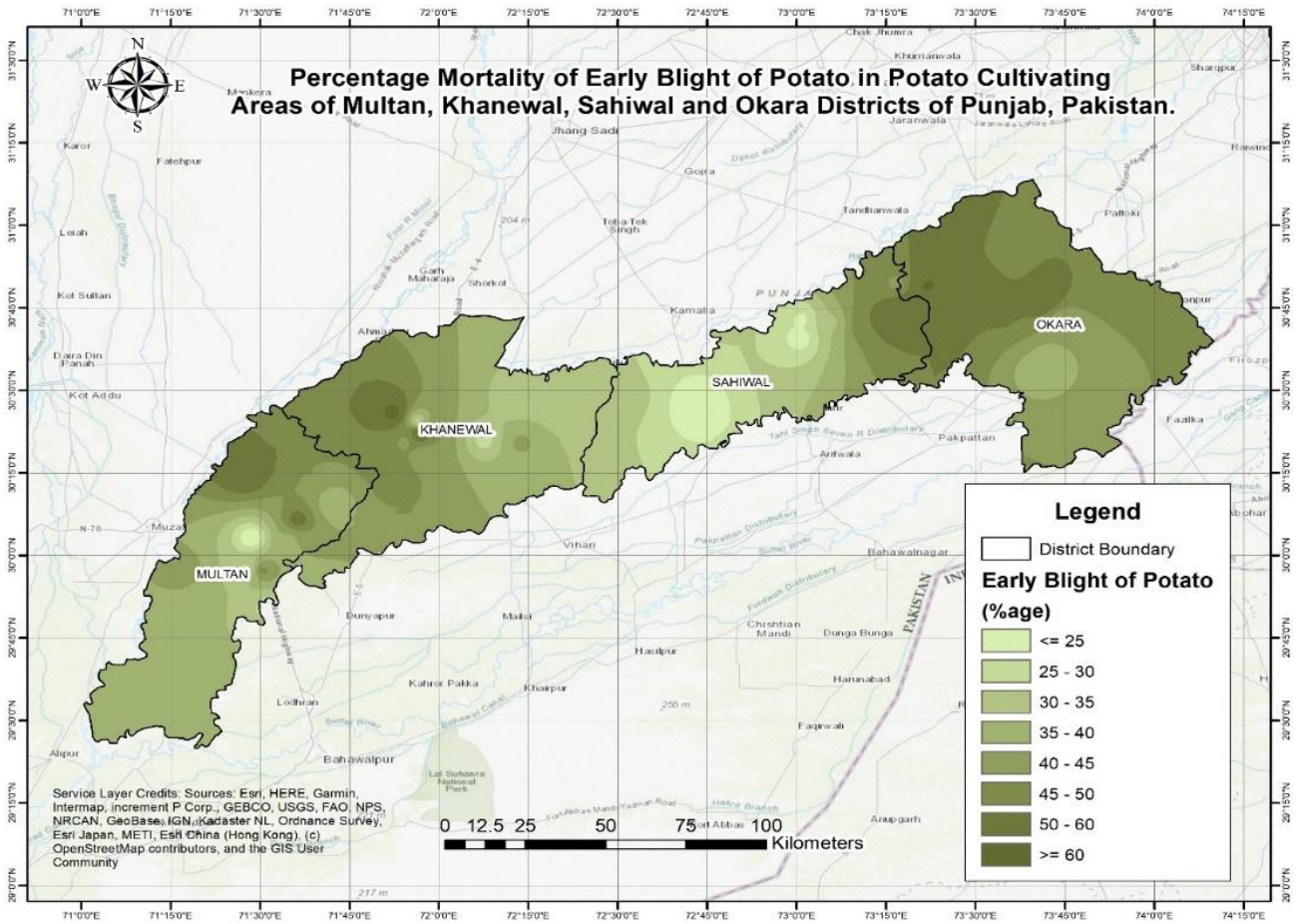


Figure 4. GIS map of Plant Mortality (%) due to Early Blight of Potato in selected potato-cultivating areas of Multan, Khanewal, Sahiwal, and Okara districts of Punjab, Pakistan.

Morphological Characteristics of *Alternaria solani* Isolates

Colony Morphology of *A. solani* Isolates: The morphological variations in *A. solani* isolates in PDA media were distinct at various periods of incubation until the ninth day. Isolate A3 was the largest producer of the colony against A1, A5, A2, and A4. Colors also varying in the colonies and were whitish, brownish-grey, and blackish-grey. The color of the colony changed slowly to darker with an increase in the time of growth (Figure 5; Table 7).

Table 7. Details of Colony Morphology of the isolates of *Alternaria solani*

Sr. No.	Isolates	Colony margin	Colony Colour	Zones	Growth Pattern	Colony Size (mm)
1	A1	Regular with grey brown	Grey-Brown	Concentric	Cottony	92.71
2	A2	Regular with grey brown	Grey-Brown	Concentric	Cottony	85.54
3	A3	Regular with grey brown	Grey-Brown	Concentric	Cottony	95.56
4	A4	Regular with grey brown	Grey-Brown	Concentric	Cottony	83.50
5	A5	Regular with grey brown	Grey-Brown	Concentric	Cottony	90.20

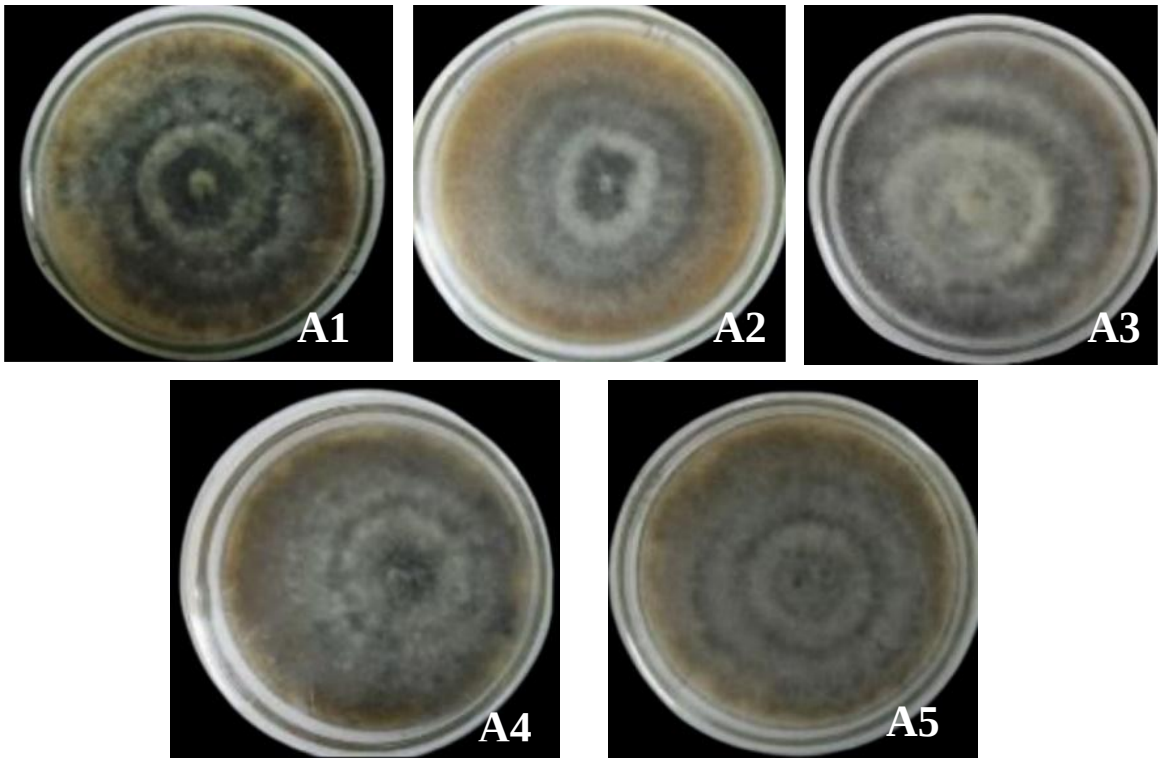


Figure 5. Colony Morphology among different isolates of *A. solani* isolated from infected potato

Microscopic Features of *A. solani* Isolates: It was clearly seen that the various isolates of *A. solani* had distinct differences in their microscopic characteristics. Generally, the conidia length (not including the beak) of the five isolates was between 15.28 and 41.24 µm and the width between 7.56 and 17.34 µm. The horizontal septa were 3 to 7 branches, but the vertical were less frequent (Figure 6; Table 8).

Table 8. Different microscopic characteristics of five *A. solani* isolates

Sr. No.	Isolates	No. of horizontal septations	No. of vertical septation	Length of conidia (µm)	Width of conidia (µm)
1	A1	3-6	1-3	15.60-41.24µm	9.53-15.92µm
2	A2	3-5	0-2	20.46-36.46µm	8.34-17.16µm
3	A3	3-7	0-1	15.28-31.56µm	7.56-15.10µm
4	A4	3-6	0-1	18.43-38.17µm	10.12-17.34µm
5	A5	3-7	1-3	15.46-36.58µm	8.29-17.34µm

Figure 6. Microscopic characterization of *A. solani* isolates at 100X

Molecular Characterization of *A. solani* isolates: All the *A. solani* isolates were capable of being amplified using the primer sets ITS4 and ITS5. The DNA fragments that were amplified had a size of about 1200 base pairs (bp). The amplification results are presented in Figure 7, with lane 1 (M) marking the molecular size marker to compare the size of the fragments. Clearly, DNA bands of approximately 1200 bp were observed in lanes 2 (A1) and 3 (A2) corresponding to *A. solani* isolates. Similarly, lanes 4 (A3), 5 (A4) and 6 (A5) had amplified DNA fragments with sizes of about 1200 bp. These results prove the amplification of the target gene region to be successful in all the tested isolates.

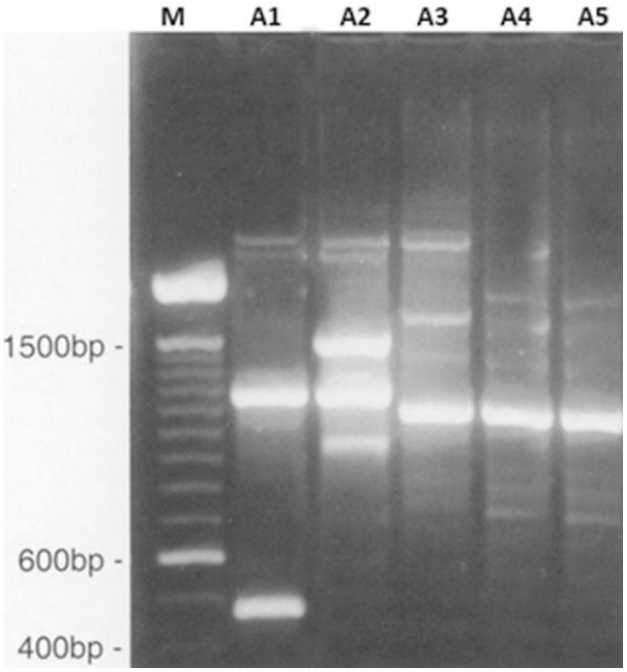


Figure 7. PCR amplification of the ITS region from *Alternaria solani* isolates. Lane 1 (M): DNA size marker; lanes 2–6: isolates A1, A2, A3, A4, and A5, respectively. All isolates produced an amplicon of approximately 1200 bp.

The ITS sequences were subjected to BLAST analysis to identify the fungi, with sequence identities ranging from 99.1 to 99.8 per cent to the reference sequences (Table 9) that were *Alternaria solani* from Pakistan and India. These results gave molecular evidence which correlated with the morphological identification of the isolates. However, because ITS alone may not fully resolve closely related *Alternaria* species, multilocus analysis using additional markers, such as GAPDH, TEF1-α, RPB2, and Alt a 1, would provide stronger species-level confirmation.

Table 9. Comparison of current 5 isolates with reference sequences from NCBI BLAST results

Isolates	Closest Match	Reference Accession No.	Similarity	Country of Origin
A1	*Alternaria solani*	OQ295864	99.5%	Pakistan
A2	*Alternaria solani*	OK036371	99.1%	India
A3	*Alternaria solani*	OQ295870	99.7%	Pakistan
A4	*Alternaria solani*	OK036375	99.8%	India
A5	*Alternaria solani*	OQ295875	99.4%	Pakistan

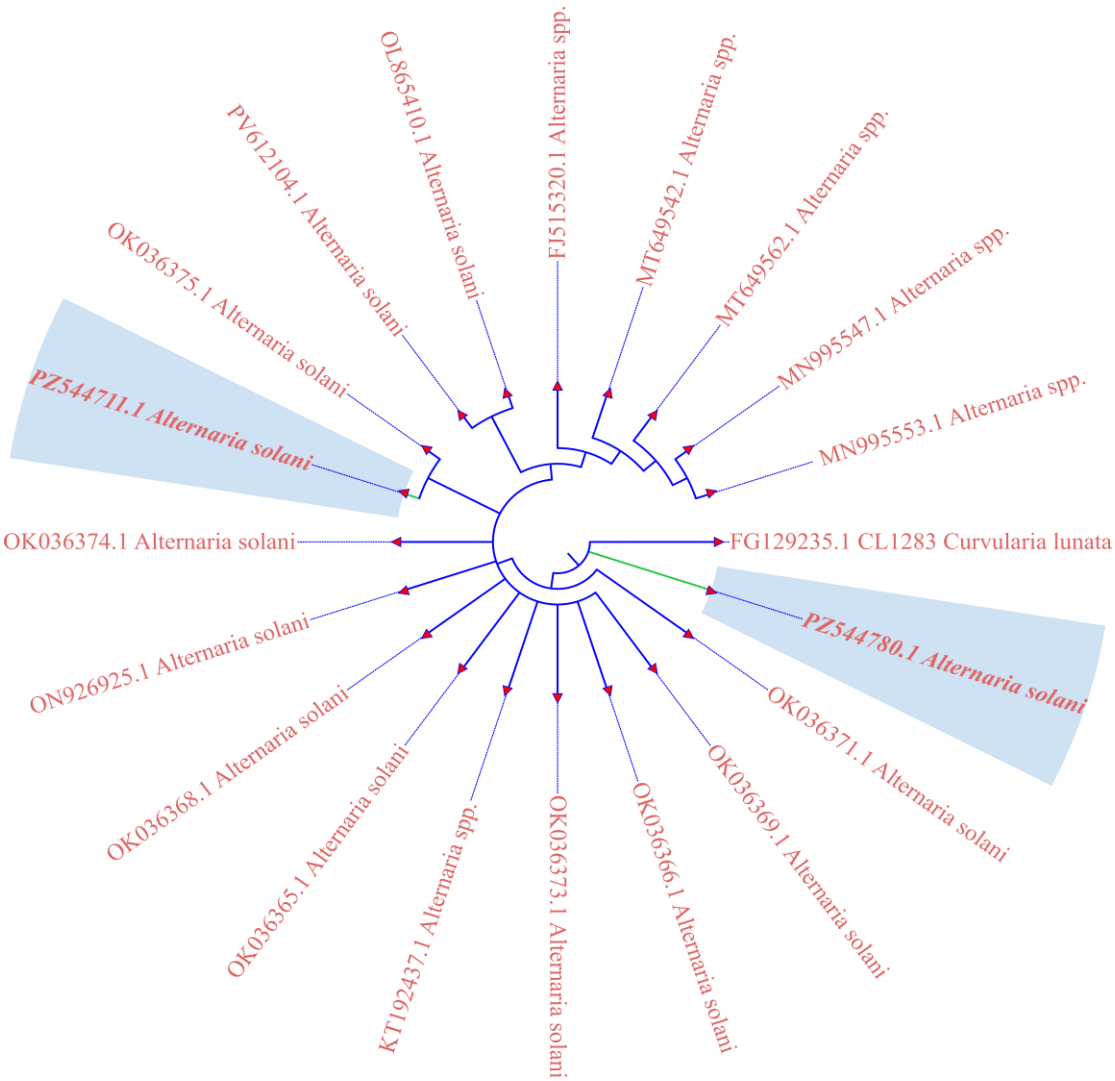


Figure 8. Phylogenetic tree derived from the Maximum Likelihood method displaying the relationship of the sequences obtained from the isolates of *Alternaria* collected during the study and the sequences of the reference *Alternaria* isolates from Genbank. MEGA 11 software was used for phylogenetic analysis with substitution model: Tamura–Nei, with 1,000 bootstrap replicates. *Curvularia lunata* (FG129235) was used as the outgroup. Study isolates are highlighted in red.

DISCUSSION

This study provides a comprehensive evaluation of the distribution, intensity, and pathogen diversity of potato early blight in key growing regions of Punjab, Pakistan, integrating GIS-based epidemiological mapping with morphological, microscopic, and molecular characterization of *A. solani* isolates. GIS-based spatial analysis revealed marked variability in early blight disease incidence, prevalence, and plant mortality across the four surveyed districts of Punjab. Okara and Khanewal consistently emerged as high-risk districts, while Multan and parts of Sahiwal exhibited comparatively lower disease pressure. This spatial heterogeneity in disease distribution is consistent with patterns documented in other regions for potato and tomato crops using spatial analysis (Hussain *et al*., 2019; Ding *et al*., 2021). Such variation likely reflects local interactions among host susceptibility, pathogen inoculum pressure, and environmental conditions. High disease incidence at Moza Nabipur Kabirwala (68%) and Adda Gambir (62–67%) indicates early establishment and rapid spread of *A. solani*, which is characteristic of this pathogen under conducive conditions (Chaudhary *et al*., 2021).

In the current study, prevalence was more than 55% in some places of Okara and Khanewal, which implies that the pathogen was established widely. The prevalence is high, which implies the *A. solani* inoculum can stay in crop remnants and soil, which is also in line with its saprophytic survival strategy (Schmey *et al.,* 2024). On the other hand, due to the small prevalence in Gopalpur and Chichawatni, this could be due to a positive cultural practice, or varietal resistance or unfavorable microclimatic conditions. The values of plant mortality in hotspots in areas which are considered to be 60-64% demonstrate the devastating nature of early blight in cases where it is not controlled. Extensive defoliation and loss of plants have a direct impact on reducing photosynthetic capacity and tuber bulking, leading to significant loss of yield. Losses of 5-50% of the yield have been commonly reported in periods of severe epidemics of early blight (Chaudhary *et al.,* 2021). The fact that high incidence, prevalence, and mortality in this study share a strong spatial overlap also goes to the extent of verifying the suitability of GIS mapping as a decision-support tool in targeted disease intervention. The maps of spatial interpolation help identify clear areas that need priority management, which approve earlier research that underlines an effective use of GIS as the effective method of plant disease surveillance (Hussain *et al.,* 2019; Ding *et al.,* 2021). These tools are especially important in agricultural systems with a limited number of resources, as they can help to control diseases more precisely and implement fungicides rationally, which will help minimize the costs of production and minimize the impact on the environment.

The morphological differences distinctly existed between the *A. solani* isolates cultivated on the PDA medium, especially in the size and growth rate of the colonies. Isolate A3 exhibited the largest colony diameter (95.56 mm), followed by A1 (92.71 mm), A5 (90.20 mm), A2 (85.54 mm), and A4 (83.50 mm). These differences indicate variation in colony growth among the studied isolates under the experimental conditions (Ayad *et al.,* 2019; El-Ganainy *et al.,* 2021). Morphological character alone as a species identifying character is, however, becoming deemed unreliable since there is an overlap between large-spored *Alternaria* species. Further microscopic characterization indicated that there was a difference in conidium size, septation and shape among isolates. The obtained values of conidial length (15.28-41.24 um) and width (7.56-17.34 um) are in line with classical descriptions of *A. solani* (El-Ganainy *et al.,* 2021). This variability can potentially affect the efficiency of the spore dispersal and capacity to infect, and lead to differences in the severity of the disease between places.

Although ITS sequence analysis provided molecular evidence consistent with the morphological identification of the studied isolates as A. solani, this marker alone may not adequately distinguish closely related Alternaria species. Future investigations incorporating additional molecular markers, including GAPDH, TEF1-α, RPB2, and Alt a 1, would improve species-level resolution and provide a more robust assessment of genetic diversity (Woudenberg et al., 2014). This result is consistent with previous morphological and molecular studies of *Alternaria* isolates associated with potato and other solanaceous crops (El-Ganainy *et al.,* 2021). Specifically, isolate PZ544711 showed 99.81% sequence similarity with *A. solani* isolate OK036375 from India, whereas isolate PZ544780 showed 99.15% similarity with *A. solani* isolate OK036371 from India. In the Maximum Likelihood phylogenetic tree, both study isolates clustered with their respective closely related *A. solani* reference sequences (Figure 8). The low ITS sequence variation observed among the study isolates and selected reference sequences is broadly consistent with previous reports of genetic variation within *A. solani* populations (Van der Waals *et al.,* 2004; Chaerani *et al.,* 2017). However, because ITS alone may not fully resolve closely related *Alternaria* species, multilocus analysis using additional markers, such as GAPDH, TEF1-α, RPB2, and Alt a 1, would provide stronger species-level confirmation. Although ITS sequence analysis, together with morphological characteristics, provided preliminary support for the identification of the study isolates as *A. solani*, ITS alone may not provide sufficient resolution to distinguish closely related *Alternaria* species. Multilocus phylogenetic analysis incorporating additional markers, such as GAPDH, TEF1-α, RPB2, and Alt a 1, would provide more robust species delimitation and phylogenetic inference in future studies (Woudenberg *et al.,* 2014).

Areas like Khanewal and Okara, which are susceptible to fungal attacks, need to be given more vigilance, use of fungicides at the right time and use of resistant cultivars where feasible. Additionally, the reported genetic variation of *A. solani* isolates underscores the need to change fungicides with varying modes of action in order to postpone the emergence of resistance. The lack of completely resistant potato varieties highlights the necessity of the use of integrated disease management strategies that combine the cultural practice, resistant germplasm, and the use of rationally applied fungicides.

The findings underscore the need for region-specific, integrated disease management approaches in Punjab's potato sector. Areas such as Khanewal and Okara require enhanced surveillance, timely fungicide application with rotations among different modes of action to mitigate resistance development, and deployment of resistant cultivars where available. The calendar-based fungicide application currently practiced in the region is economically unfeasible and environmentally unsustainable (Khan *et al*., 2003; Ganie *et al*., 2013). GIS-based disease maps provide a spatially explicit decision-support framework to guide targeted, rational interventions. The observed genetic similarity among isolates does not preclude intraspecific variation in aggressiveness, which should be investigated in future studies alongside multi-season disease monitoring

Conclusions: The current study combined the spatial assessment and morphological, microscopic and ITS-molecular characterization of potato early blight in four major potato growing districts of Punjab, Pakistan and implemented it using a GIS-based approach. Spatial analysis revealed Okara and Khanewal as comparatively high level of disease intensity (site-level disease incidence of 68%, disease prevalence of 62% and plant mortality of 64%). Variation between the fungal isolates studied was found by morphological and microscopic examinations. The molecular evidence of 99.1–99.8% similarity with the reference sequences of Alternaria solani as determined by ITS sequence analysis confirmed the identification of the isolates. Maps of disease distribution using GIS provide a spatial context to assist in the identification of disease management priorities for disease surveillance. A detailed multilocus molecular characterisation and a multi-season epidemiological investigation are suggested to aid in pathogen identification and the development of disease-management strategies in Punjab.

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