Molecular Identification and Morphological Characterization of *Alternaria solani* Causing Potato Early Blight in the Southern Region of Kurdistan - Iraq

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Abstract

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Potato early blight caused by *Alternaria solani* is a common disease wherever potato crop is grown. This research is aimed to identify the early blight pathogen which infects potatoes in the Southern Region of Kurdistan and characterize the existing isolates. Sampling was conducted in different provinces of the Region where infected potato leaves were collected from which the fungal pathogen was isolated and then identified. The microscopic examination revealed that the causal fungus was *Alternaria solani*. Fungal conidia were multicellular, brown to pale, with 1-6 transverse septa and 1-2 longitudinal septa, with curved beaks of 4.5-11.25 µm long. The identity of the fungus was confirmed by molecular diagnosis using PCR amplification with the aid of species-specific primers, AS1 and AS2. The results revealed that the primers were highly specific for the detection of *A. solani*. DNA amplicon size generated by PCR was 289 bp for all 31 samples tested in this study. The results of morphological characterization showed the existence of 4 phenotypes. The isolates were different in their morphological and microscopical characteristics such as colony colour and length and width of conidia. Results obtained confirmed that the use of species-specific primers in PCR is an efficient tool for quick and accurate pathogen identification.

Keywords: Alternaria leaf blight, PCR, Phenotyping.

Introduction

Potato (*Solanum tuberosum* L.) is a worldwide cultivated tuber-bearing plant considered as the fourth main food crop in the world after wheat, rice, and maize, in terms of both cultivated area and total production (Douches *et al.*, 1996). The top three potato-producing countries in the world are China, India, and Ukraine. China is the largest potato producer in the world with 90 million tonnes, followed by India with 48 million tons and Ukraine with 22 million tons of annual production (Singha & Maezawa, 2019).

The potato production in Iraq was more than 190k tonnes with a total cultivated area of 7950 hectares at an average yield of 24 tons per hectare (Anonymous, 2019). According to the Ministry of Agriculture and Water Resources in the North of Iraq, potato production in the area was reported to be over 150,000 tons in 2016 (Barznjy e*t al.*, 2019). Over the past ten years, growers in the North of Iraq have paid more attention to planting potatoes. Potato annual production in this region has increased from 12,000 tons to 213,356 tons over the past eight years. The private sector has also helped to grow this crop by bringing in potato seeds from abroad, particularly from the Netherlands (Jongerden *et al.*, 2019).

Growing potatoes is challenging and marketable yield is threatened by many pests and diseases. *Alternaria* leaf blight is one of the major diseases of potato, known as early blight, which is characterized by typical symptoms of concentric rings on leaves favored by high temperature and alternating periods of dry weather and high humidity. The disease reduces yield and affects tuber size and quality. The common *Alternaria* leaf blight pathogen is *Alternaria solani*, which was primarily considered to be the causal agent of early blight in potatoes and other Solanaceae crops (Zhao *et al.*, 2016). However, various other *Alternaria* species have also been identified such as *A. alternata*, *A. tenuissima*, *A. dumosa*, *A. arborescent*, and *A. infectoria* which have been reported in major potato growing regions in Iran (Tahery *et al.*, 2010); *A. protect* in Algeria; *A. alternata*, *A. arborescent*, *A. protect*, and *A. grandis* in Europe; *A. longipes* in Pakistan; *A. arborescent*, *A. alternata*, and *A. arbusti* in the United States; and *A. tenuissima* and *A. alternate* in China (Zhao *et al.*, 2016).

A. solani has dark-colored mycelium in older diseased tissue, it produces conidia on short, simple, and erect conidiophores that bear a single and branched chain of conidia (Agrios, 2005). *A. solani* produces large conidia and elongated beaks, the typical conidia are ovoid or obclavate pale brown and multi-celled with transverse septa (Ghazanfar *et al.*, 2016).

This study aimed to identify the Altenaria species causing early blight of potatoes in the southern part of Kurdistan region based on morphological characterization and molecular techniques.

Material and Methods

Sample collection and fungal isolation

Potato leaf samples showing early blight symptoms were collected during May and June of 2021 from different fields in Erbil, Duhok, and Suleimani Provinces. Fourteen isolates were collected from Erbil, ten from Duhok and seven from Suleimani. The potato diseased samples were collected from fields grown with different potato cultivars. Collected

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samples were placed in polythene nylon bags, marked, and kept in cool boxes until they are brought to the laboratory. The samples were then stored in the refrigerator at 4°C for later use.

The fungus was isolated from infected leaf samples by cutting leaves into small pieces of 0.5 × 0.5 cm, surface sterilized with 70% ethanol for approximately 2 min, washed three times with sterilized distilled water, blotted dry, and placed on prepared fresh potato dextrose agar medium. To inhibit bacterial growth, the fungal growth medium was supplemented with streptomycin sulfate at a rate of 0.2 g/L. The Petri plates were incubated at 25±2°C for 7 days to check the growth and sporulation for further studies. The cultures were then purified by the hyphal tip method and the single spore isolation technique (Pathak, 1972; Pathak & Zaidi, 2013). As soon as the mycelial growth was observed in the Petri plates, advancing hyphal tips growing out of tissue segments were cut off with a sterilized inoculation needle and transferred to PDA slants for further growth (Waller *et al.,* 2002). The isolates of the pathogen were identified depending on cultural and microscopical characteristics and compared with standard diagrams (Watanabe, 2002).

Morphological and cultural characteristics

The morphological characteristics of *A. solani* isolates were studied on pure culture in the laboratory. Traditional identification was conducted by growing subcultures of the fungal isolates on PDA at 25ºC for one week. The fungal colonies were visually assessed for their color, margins, and margin growth. Conidial dimensions (length and width of conidia, and length of beak) were measured under the microscope. The microscopic features were examined with the aid of a compound microscope (Zeiss, Germany). To measure mycelial and conidial dimensions, a stage micrometre (Erma Stage, Japan) was used. The results of both cultural and microscopical characters were compared and categorized depending on the similarity or dissimilarity of the measured characters.

DNA extraction and PCR amplification

DNAs were extracted from 31 isolates of *A. solani* grown on PDA media according to the protocol set by the manufactures Genomic DNA Extraction Kit (FATG/Korea). The target DNA was amplified using species-specific primers: AS1 (5'- GCTCCCACTCCTTCCGCGC-3') and AS2 (5' GGAGGTGGAGTTACCGACAA-3') designed from β-tubulin gene and used earlier by Kumar *et al.* (2013). To confirm the specificity of the primers, blast analysis was used to compare sequences available at the GenBank database. For further confirmation of the specificity of the primers, the DNA of a different fungus (*Trichoderma harzianum*) was also amplified with the same primer pairs. DNA was amplified in a reaction volume of 25 µl in PCR tubes. The master mix was composed of 12.5 µl of 2x PCR master mix, 1µl of each forward and reverse primer, 2 µl of DNA template, and 8.5 µl of DNase-free water to complete the volume to 25 µl. The PCR protocol followed was an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 sec, primer annealing at 58°C for 45 sec, an extension at 72°C for 1 min and final step was an extra extension at 72°C for 5 min. To separate the amplified DNA, agarose gel electrophoresis was performed using 1% agarose gel and was electrophoresed at 100 volts for 30 min, and then photographed under ultraviolet light (UV).

Results and Discussion

Morphological identification of *A. solani* isolates

In this study, 31 isolates of *A. solani* collected during the spring growing season of 2021 were investigated, 14 isolates were from Erbil, 10 from Duhok and 7 from Suleimani. Generally, microscopical investigation revealed that the conidia of *A. solani* were multicellular, brown to pale. The number of transverse septa ranged from 1-6 with 1-2 longitudinal septa, and the beaks were curved with a length of 4.5-11.25 µm. Identification was based on conidial dimensions, including the length and width and the presence of beaks which were considered the most essential characteristics of *A. solani*. Spore size of fungal isolates ranged between 11.7-30.75 × 8.25-12.75 µm and 8-13.5 × 9.25-13.25 µm, with one curved beak of 4.5-9.25 µm. Based on the results obtained from the laboratory investigation, the causal agent of early blight disease in potatoes in Erbil Province was identified as *A. solani*.

Depending on the observed morphological types, the isolates were categorized into four groups (Table 1). Group one included the largest number of isolates (12 isolates) characterized by having greyish colony colour with irregular colony margins, rough-raised, and without zonation (Figure 1). The conidial length and width dimensions were 26-19.75 and 9.25-13.25 µm, respectively, with beak length of 5.00-11.25 µm. Group two (10 isolates) was characterized by olive colony color with circular margin growth and concentric zonation. The lengths and widths of conidia were 18.75-30.75 and 8.25-12.75 µm, respectively; with beak length of 4.5-9.25 µm. Group three (4 isolates) was characterized by colonies of olive to black color with irregular margin growth with smooth flat surface growth with zonation. The conidial length and width dimensions were 24.25-34.25 and 8.75-13.5 µm with beak lengths of 5.25-7.00 µm. Group four (5 isolates) was characterized by colonies with irregular margin growth and dark green color with rough-raised surface growth and without zonation. However, one isolate in this group had different surface growth which was smooth and flat with zonation. The conidial length and width were 11.7-26.5 and 8-11.5 µm, respectively, and the length of conidia beaks ranged between 6.5 and 8µm. Microscopical investigations confirmed that the pathogen under investigation was *A. solani*.

According to their microscopical and morphological and cultural characteristics, the fungal isolates were categorized into 4 phenotypes. Previous literature has described and characterized the species of *A. solani* by having brown to pale and multicellular conidia. The average number of transverse septa ranged from 1-6 with 1-2 longitudinal septa, and the beaks were curved with a length of 4.5-11.25 µm. These findings are in agreement with those reported by Marak *et al.* (2014) who described the fungus that had dimensions of conidia of 21.5-33.21 x 8.03-17.85µm

with a beak length of 2.18-7.40, having 3-4 horizontal and 1-2 vertical septa.

On the other hand, the collected isolates in the current study showed variation in colony color such as greyish, olive, olive to black, and green to black. The colony margins were regular or irregular, whereas the colonies' surface were either smooth flat or rough-raised. These features were close to those reported by Nuwamanya *et al.* (2022) who studied 96 *A. solani* isolates from three counties in Kenya and characterized them using cultural features and conidial morphology. They found that most colonies (45%) were greenish white in colour with diameter range of 65.5-85.0 mm with concentric zonation (63%) and margins were mostly regular (53%). Conidia was ellipsoidal in most isolates (54%) with lengths range of 16.72-20.48 µm. Conidial transverse septa number range was 2-5 and longitudinal septa range was 1-3 with beak septa of 1-4. Mugao *et al.* (2021) assembled *A. solani* isolates into 5 groups. Nikam *et al.* (2015) found that *A. solani* was the pathogen that caused potato early blight in India and stated that *A. solani* isolates had variability in their growth, sporulation, and pigment production on PDA, and they also differed in mycelial and conidial dimensions and septation.

The results of the current study revealed that the macroscopic features such as growth pattern, colony color, and growth margins color showed variability among *A. solani* isolates evaluated. Similar results were reported earlier by many researchers (Kumar & Singh, 2017; Weber & Halterman, 2012). Nevertheless, two isolates of *A. solani* were thick grey to black with scattered aerial mycelia, often branched, and simple conidiophores with multi-septate conidia of 0-7 transverse septa and 2-10 longitudinal septa (El-Ganainy *et al.,* 2021). In addition, microscopic features of mycelia and conidia of *A. solani* were variable but similar to those reported by Rahmatzai *et al.* (2016) who found that the average conidial size (L × W) was higher in the isolate As1 (25-44 × 7-15µm) and was comparatively lower in isolate As2 (20-30 × 5-13 µm). Furthermore, Alhussaen (2012) described the conidia of the *Alternaria solani* which had a length of 35-37 µm and a width of 10-20 µm with 2-7 transverse septa and 1- 4 longitudinal septa. The culture pigmentation on the PDA medium varied from yellow, brown, black, and brownish to green-black in isolates of *A. solani*, and our findings are similar to those reported by Kumar *et al.* (2008) who stated that the conidia of *A. solani* on PDA ranged from black to greenish-black, brown, and yellow. Similar results were obtained by Nikam *et al.* (2015).

Molecular identification of *A. solani* isolates

The traditional identification of *A. solani* was confirmed with the aid of PCR test. The species-specific primers amplified a 289 bp of a DNA fragment from the pathogen's genome for 31 fungal tested in this study. No bands were detected with the control (lane C, Figure 2) which was used to demonstrate species specificity of the primers used (Figure 2).

Molecular tools have been used by researchers to complement the morphology-based approaches used to detect and study fungal plant pathogens. The present investigation aimed to develop a rapid detection tool for *A. solani*, the causal agent of potato early blight diserase. For this purpose, the B-tubulin gene was chosen as a target for the specific detection because this gene is a highly conserved and represent a suitable region for designing specific primers (Nahimana *et al.*, 2000).

Similar to our results, the size of the band generated by PCR using AS1/AS2 specific primers was about 289 bp, was used earlier to detect 27 isolates of *A. solani* (Kumar *et al.*, 2013).

Table 1. Morphological characterization of *A. solani* isolates collected from the Southern Region of Kurdistan, Iraq.

Isolate	Colony color	Margin	Surface	Zonation	Conidia length (µm)	Conidia width (µm)	Beak length (µm)
Group 1							
As7, As9, As11, As13, As27	Greyish	circular	rough-raised	-	26.00-19.75	9.25-13.25	5.00-11.25
As15, As17, As20, As22	Greyish	irregular	rough-raised	-	19.00-25.50	9.25-11.50	5.25-7.50
As18, As25, As29	Greyish	irregular	rough-raised	+	24.25-34.00	9.75-12.25	5.00-25.00
Group 2							
As8, As23, As28,	Olivaceous	irregular	smooth-flat	+	18.75-27.00	8.00-12.25	5.00-9.25
As10, As12, As14, As16, As19, As21, As30	Olivaceous	circular	rough-raised	+	18.75-30.75	8.25-12.75	4.50-9.25
Group 3							
As6, As24, As26, As31	Olivaceous to black	irregular	smooth-flat	+	24.25-34.25	8.75-13.50	5.25-7.00
Group 4							
As1, As3, As4, As5	dark green	Irregular	rough-raised	-	11.70-26.00	8.00-11.50	6.50-8.00
As2			smooth-flat	+			

(+) with zonation, (-) without zonation

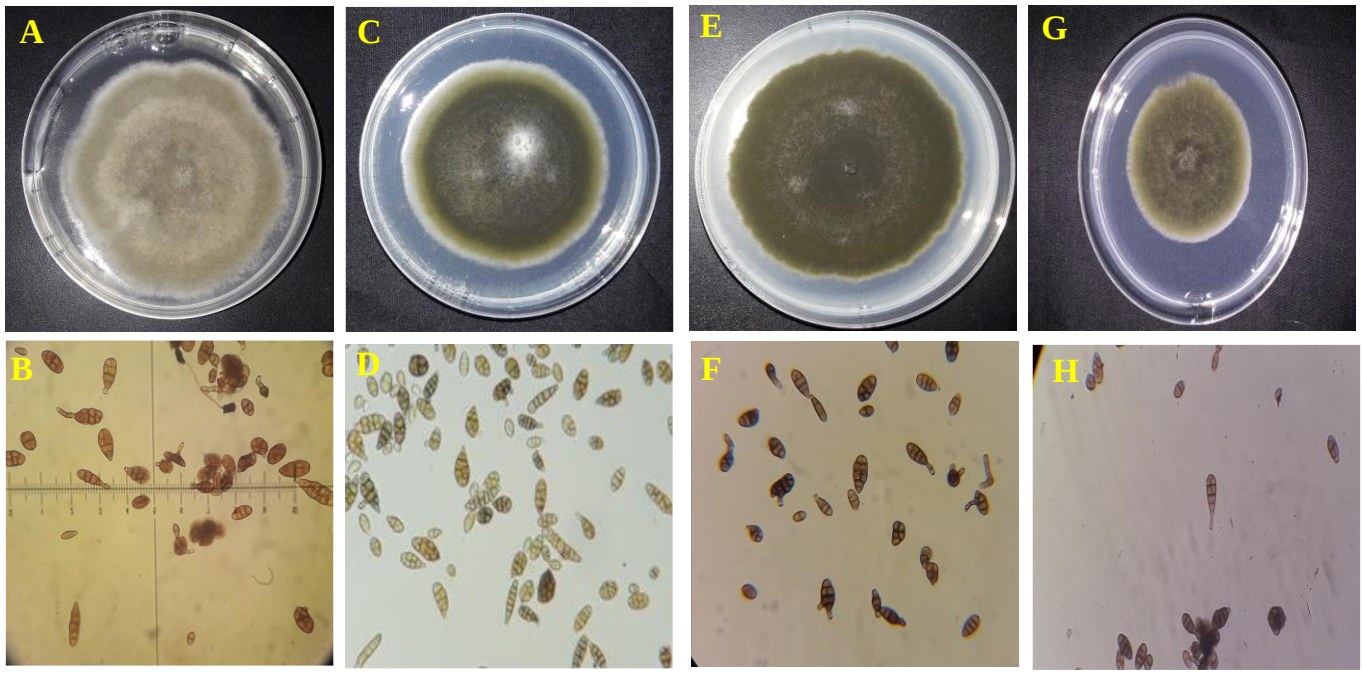

Figure 1. Colonies and conidia of different groups of *A. solani* on PDA plates: (A and B) represent group one; (C and D) represent group two; E and F represent group three, and G and H represent group four.

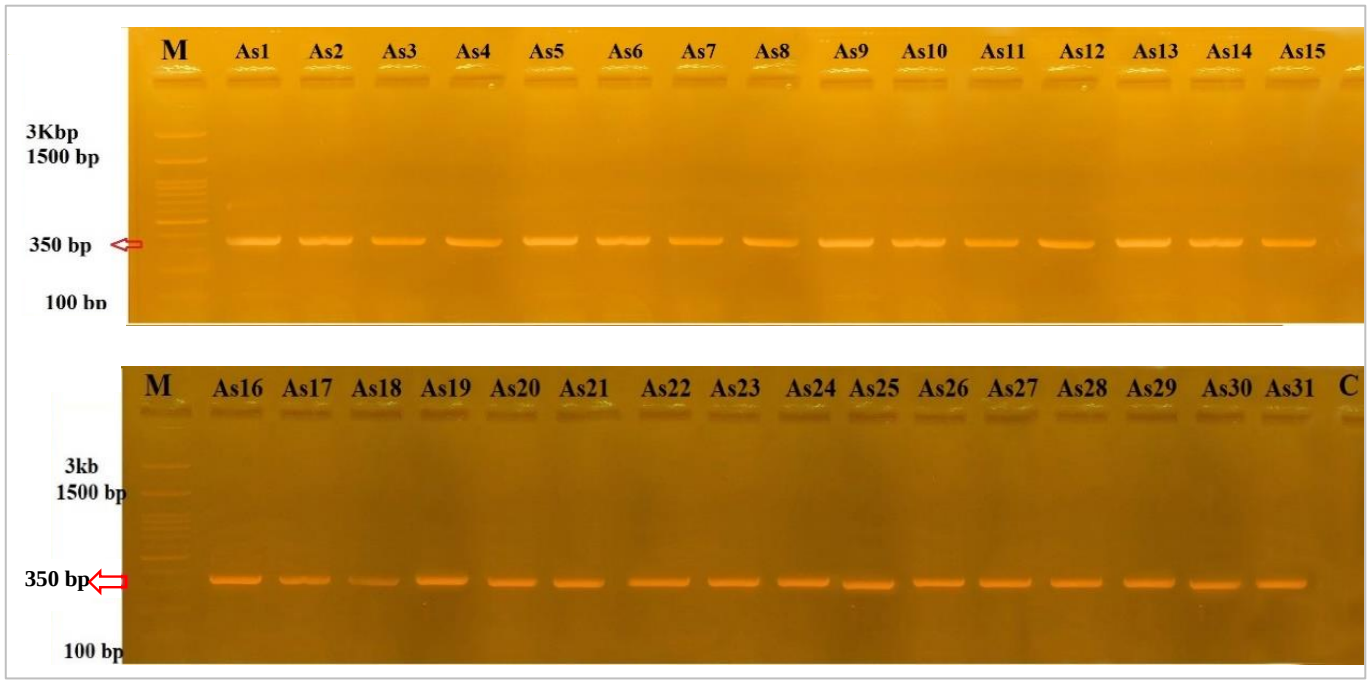

Figure 2. Polymerase chain reaction amplification products using a specific primer for *A. solani* where lanes As1–As31 represent the amplicon of 31 isolates with generated band size of 289 bp, and C is the positive control which represented the amplification of *Trichoderma harzianum* DNA run with the same primer pairs.

الملخص

حسن، أزين محمد وقاسم عبد الله مرزاني. 2024. التعريف الجزيئي والخصائص المزرعية للفطر *Alternari solani* المسبب للفحة المبكرة على البطاطا/البطاطس في الإقليم الجنوبي من منطقة كردستان العراق. مجلة وقاية النبات العربية، 42(1): 13−18.
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تعدّ اللفحة المبكرة على البطاطا/البطاطس المتسببة عن الفطر *Alternaria solani* مرضاً شائعاً في جميع مناطق زراعة البطاطا. هدف البحث إلى تشخيص العامل المسبب للفحة المبكرة على البطاطا/البطاطس في الإقليم الجنوبي من كردستان العراق ومعرفة صفات عزلات الفطر. أخذت العينات المدروسة من مناطق مختلفة من الإقليم، وتمّ العزل من الأوراق المصابة بغرض التشخيص. أظهر الفحص المجهري بأن الفطر المسبب هو *Alternaria solani*. أظهرت النتائج بأن أبواغ الفطر متعددة الخلايا، بنية إلى شاحبة، مع وجود 1−6 مقسم عرضي و 1−2 مقسم طولي مع منقار منحني ذو قياس 4.5−11.25 مم. تم التأكد من هوية الفطر بالاستناد إلى الفحص الجزيئي باستعمال تقنية PCR واستخدام بادئات متخصصة من نوع AS1 وAS2. أظهرت النتائج بأن هذه البادئات كانت عالية التخصص بتشخيص الفطر المسبب *A. solani*. كان حجم ناتج التفاعل 289 زوج قاعدي لجميع العزلات (31 عزلة) المدروسة. أظهرت نتائج الصفات المظهرية وجود 4 اشكال مظهرية (Phenotypes). كانت العزلات مختلفة فيما بينها من حيث الشكل المظهري والصفات المجهرية، مثل لون المستعمرة وطول الأبواغ وعرضها. أثبتت النتائج بأن استعمال بادئات متخصصة بالنوع يعدّ تقنية فعالة ودقيقة لتشخيص المسبب.

كلمات مفتاحية: لفحة الألترناريا المبكرة، PCR، تنميط ظاهري.

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