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Molecular identification of ascochyta blight of *Cicer montbretii* Jaub. & Spach

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ABSTRACT

Molecular detection of ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. is important for effective control of the ascochyta blight and efficient chickpea (*Cicer arietinum* L.) breeding program. The present research was therefore aimed to diagnose ascochyta blight of *C. montbretii* Jaub. & Spach via molecular techniques. Infected plant samples were collected and placed on potato dextrose agar (PDA) medium for 1 week at 20-24℃, and colonies with typical ascochyta blight symptoms were transferred to new PDA medium and incubated for 1 week at 25℃. DNA was isolated from small parts of fungus isolates via the CTAB method. Internal transcribed spacer (ITS) regions (ITS-1, 5.8S rDNA subunit, ITS-2) were amplified with ITS 5 and ITS 4 primers for molecular characterization. Based on the BLAST analysis, the sequence had 99 and 100% nucleotide identity with the corresponding sequence of *A. rabiei* in GeneBank. To our knowledge, this is the first report of ascochyta blight of *C. montbretii* in Turkiye. The pathogen is considered to be co-evolved with *C. montbretii*. Molecular techniques, as in the present study, can be diagnosed with great accuracy, in a short time, and with relatively little effort and expense.

1. Introduction

The genus *Cicer* L. consists of 39 *Cicer* species, including 31 perennials and 8 annuals including cultivated chickpea (*C. arietinum* L.), reported by van der Maesen (1972). From 1972 to 2007, the number of *Cicer* taxa was updated to 44 taxa consisting of 9 annuals and 35 perennials (van der Maesen et al. 2007). The following perennial species, subspecies and varieties were added as new *Cicer* taxa: *C. uludereensis* Donmez (2011), *C. floribundum* Fenzl. var. *amanicola* M. Ozturk & A. Duran, *C. heterophyllum* Contandr., Pamukc. & Quezel var. *kassianum* M. Ozturk & A. Duran and *C. incisum* (Willd.) K. Maly subsp. *serpentinica* M. Ozturk & A. Duran were more recently added (Ozturk et al. 2011, 2013). *C. turcicum* Toker, Berger & Gokturk has been more recently introduced to the scientific world. With new annual *Cicer* species, the number of taxa in the genus has reached 50 taxa, and the most distribution of annual *Cicer* species are found in the Anatolia (Asia minor) region including progenitor, *C. reticulatum* Ladiz. (Toker et al. 2021).

Cicer species have been reported all over the world, from the Canary Islands and the Atlas Mountains, in the west, to South and Central Asia in the east, and from the Ethiopian highlands in the south to the Balkans and Caucasia in the north (van der Maesen 1972, 1987; van der Maesen et al. 2007).

The well-known cultivated species do not only possess high levels of protein and vitamin content in their seeds (Ahlawat et al. 2007; Jukanti et al. 2012), but they can also fix atmospheric nitrogen into soil for the following crop and reduce some diseases, insects and weeds if the plant is used as a rotation crop with small grain cereal-based cropping systems (Kantar et al. 2007). Both cultivated chickpeas, including cream coloured large-seeded chickpeas (*macrosperma* or 'kabuli') and small-seeded chickpeas (*microsperma* or 'desi'), are not only grown for edible dry seeds all over the world, but are also grown for green fresh seeds in some countries including Türkiye. In addition to food usage, dry seeds and hays after harvest have been evaluated for animal feed. In 2020, the total sowing area and production quantity of chickpeas was recorded as 14.8 million ha and 15.1 million tons, respectively. According to the harvested area in the world, chickpeas are known to be the first ranked among cool season food legumes (FAOSTAT 2023). The yield of chickpeas suffers from a/biotic stresses including diseases, insect pests, weeds, heat, cold, drought and salinity as well (Singh and Saxena 1993; Muehlbauer and Kaiser 1994). Among biotic stresses, diseases of chickpea are considered to be the most important biotic stresses (Singh et al. 2007).

Ascochyta (Mycosphaerella) blight is caused by *Ascochyta rabiei* (Pass.) Labr., teleomorph, *Didymella rabiei* (Kovacheski) var. Arx (Syn. *Mycosphaerella rabiei* Kovacheski) (Akamatsu et al. 2012). It is one of the most important devastating foliar diseases of chickpeas worldwide (Shahid et al. 2008). At the same time, it has been reported in most of the chickpea-growing fields (Nene et al. 1996; Singh et al. 2007; Pande et al. 2010). Ascochyta blight of chickpea has caused considerable yield losses of up to 100% in susceptible areas, especially in the years when epidemic conditions occur in producing countries (Verma et al. 1981; Singh et al. 1982; Hawtin and Singh 1984; Kaiser and Muehlbauer 1988; Udupa et al. 1998; Chen et al. 2004; Pande et al. 2010). The fungus generally reproduces in cool (15-25 ℃) and humid (>150 mm rainfall) areas (Pande et al. 2010) and needs at least 6-10 h of leaf wetness to infect (Khaliq et al. 2021). Three pathotypes, pathotype I (the least virulent), pathotype II

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(moderately virulent) and pathotype III (the most virulent) were determined but later the fourth pathotype (high degree of virulent) has already been reported (Turkkan et al. 2008; Nalcaci et al. 2021). It has not only been found in the cultivated chickpea, but also reported in wild species (Frenkel et al. 2010; Kafadar et al. 2019; von Wettberg et al. 2018). Molecular determination of ascochyta blight of chickpea is essential for effective disease control and efficient chickpea resistance breeding programs. The purpose of the present study is the identification of ascochyta blight of *Cicer montbretii* via molecular techniques.

The pathotyping system, which was described by Udupa et al. (1998) and modified by Imtiaz et al. (2011), is logical and it is the most widely used system: pathotype I (least aggressive), pathotype II (aggressive), pathotype III (highly aggressive), and pathotype IV (most aggressive).

2. Materials and Method

2.1. Plant and pathogen

As plant material, perennial wild chickpea (*C. montbretii* Jaub. & Spach) was used in the present study (Figure 1). *C. montbretii* is called ‘deli nohut’ in Turkish (Guner et al. 2012). Ascochyta blight (Figure 2) was isolated from the surface of infected leaflets and pods of *C. montbretii* and then stored at 4℃ until use.

2.2. Collection sites

Plant samples of *C. montbretii* were collected in Kozak plateau, Bergama, Izmir in 2017 (Figure 1-2). Plant samples and infected plant parts were collected every 100 m and were controlled, respectively.

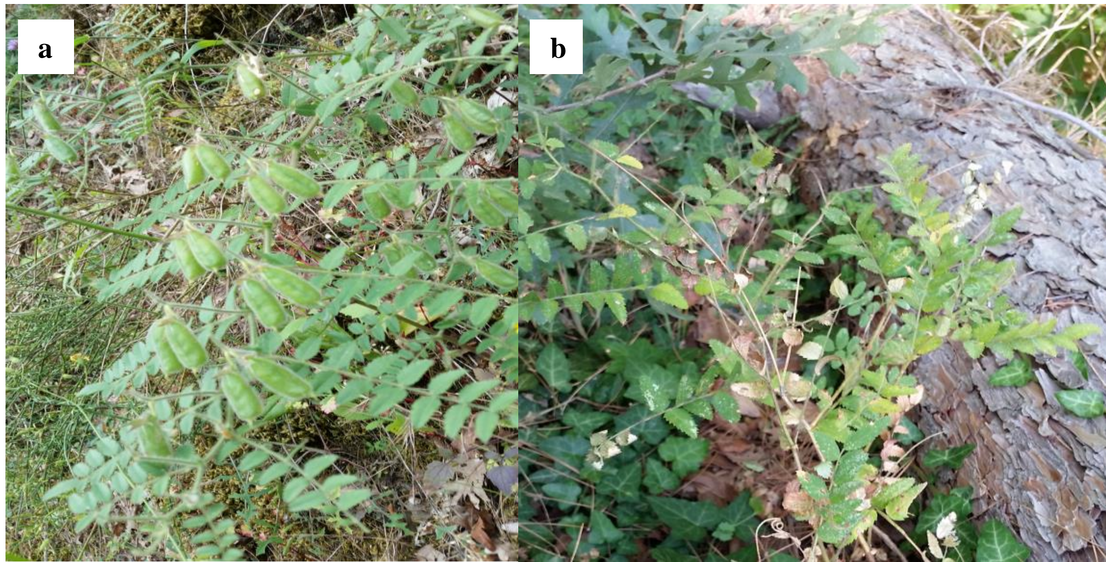

Figure 1. A healthy plant (a) and ascochyta blight infected plant (b) of *C. montbretii* in Kozak plateau road, Bergama, Izmir, Türkiye.

Figure 2. Ascochyta blight on leaf and leaflets of *C. montbretii* in Kozak plateau road, Bergama, Izmir, Türkiye.

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2.3. Isolation and reproduction of pathogen

Ascochyta blight fungus was collected from the surface of infected leaflets and pods of *C. montbretii*. Infected and dried plant tissues were applied with 95% ethanol 1 min, sterile distilled water 1 min, 0.5% NaClO 1 min, and sterile distilled water 1 min, and dried on sterile blotting paper respectively (Bahr et al. 2016). With the aid of a scalpel under stereo binocular (Nikon SMZ 460™), only the fungal pathogen was taken from the infected areas. Conidial suspension (1×10^{-6} conidia mL^{-1}) described by Frenkel et al. (2007) was retained on petri dishes consisting of potato dextrose agar (PDA) medium for 1 week at 20-24℃ for 12 h photoperiod under fluorescents lights in order to induce increase in sporulation (Walter 2009). Five isolates for each symptom type were grown as single-spore colonies and used for further studies.

After conidial culture was accomplished, it was placed in PDA medium to obtain single-spore culture. Fungi hyphal fragments were placed on PDA medium to determine single-spore culture and then cultures with single-spore were transferred on cellophane paper to petri medium and then covered with parafilm. These cultures were kept warm for 1 week at 25℃ in an incubator. After the incubation period, colonies including fungi were scraped and transferred to Eppendorf tubes for DNA isolation. Samples including fungal isolates were stored at -20℃ until DNA isolation.

2.4. Pathogen analyses

The total genomic DNA was isolated using fungal spore culture with the CTAB method (Doyle and Doyle 1990). Extracted DNA was manually tested for quality and concentration with 1% agarose gel electrophoresis using a DNA standard. After, the DNA was kept at -20℃ until use. The rDNA internal transcribed spacer (ITS) regions (ITS1, 5.8S rDNA subunit, ITS-2) of ascochyta blight were amplified using PCR with universal primers ITS 5 (forward) and ITS 4 (reverse) in Table 1 (White et al. 1990).

The following PCR procedures were applied: the total reaction mixture volume used was 15 µL containing 8.12 µL Milli-Q water, 1.5 µL 10 × PCR buffer, 1.5 µL $MgCl_2$, 1.5 µL of dNTPs mix, 0.4 µL each primer, 0.08 µL *Taq* DNA polymerase (Fermentas Life Sciences™, Burlington, Canada) and 1.5 µL fungal DNA template (Peever et al. 2007). Amplification was conducted in a thermocycler (Bioneer, MyGenie™) under the following conditions: 94℃ initial denaturation for 5 min, 30 cycles of 94℃ for 30 s, annealing temperature 55℃ for 30 s, 72℃ for 1 min, and then a final extension of 10 min at 72℃ (Barve et al. 2003). The amplified product was visualized under UV light after being dyed with ethidium bromide in 2% agarose gel in 1× TBE buffer. A single band was cleaned with the GeneJET Gel Extraction Kit (Thermo Scientific Ferments™, Vilnius, Lithuania) and sequencing was carried out at Macrogen Inc., Europe via BM Laboratories Ltd., with direct sequencing in both directions using the amplification primers. Sequences of ITS region were compared with the BLAST (Basic Local Alignment Search Tool) sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/) in databases using nucleotide-nucleotide BLAST (blastn) with default settings.

3. Results and Discussion

The purpose of the present study was to identify ascochyta blight of *C. montbretii* Jaub. & Spach via molecular techniques. Previously, molecular characterization studies on ascochyta blight in different chickpea species have been reported in various studies (Phan et al. 2002; Cho et al. 2005; Bahr et al. 2016; Baite et al. 2017; Kumar et al. 2020) including in Türkiye (Bayraktar et al. 2007). However, there were limited studies on the blight in wild *Cicer* species in the world and even in Türkiye (Abbo et al. 2007; Peever et al. 2007; Frenkel et al. 2010; Kafadar et al. 2019; von Wettberg et al. 2018). Also, a first report on ascochyta blight of *C. montbretii* was studied in Bulgaria by Kaiser et al. (1998). A similar approach on ascochyta blight was described for *C. isauricum* P.H. Davis and *C. anatolicum* Alef. (Guler 2018; Tekin et al. 2018). Due to the importance of the ascochyta blight of chickpea, more than 25 QTLs were detected for molecular assisted breeding (Sharma et al. 2012; Misra et al. 2016; Islam et al. 2017). Thanks to some of these QTLs, a chickpea ideotype has been improved as resistant or tolerant to ascochyta blight, heat tolerant, double-podded and about 58 g per 100 seed weight (Eker et al. 2022). In the present study, ascochyta blight isolated from infected leaflets and pods *C. montbretii,* were grown in potato dextrose agar (PDA) medium and developed single spore culture which was put into Eppendorf tubes for molecular characterization. PCR reaction was carried out with ITS5 and ITS4 primers with fungal DNA obtained as a result of DNA isolation. Sequencing of the PCR product was performed. The sequence of the rDNA ITS region of fungal DNA (ITS-1, 5.8S rDNA, ITS-2) with a length of 556 base pairs are presented in Table 2.

Table 1. Primers used in PCR, base sequences and annealing temperatures for diagnosis of ascochyta blight in *C. monbretii* in Kozak plateau road, Bergama, Izmir, Türkiye

No	Primers	Base length	Sequence (5’→3’)	Temperature (°C)
1	ITS4	20	TCCTCCGCTTATTGATATGC	58
2	ITS5	22	GGAAGTAAAAGTCGTAACAAGG	63

Table 2. Sequence of the rDNA ITS region of fungal DNA of ascochyta blight in of *C. montbretii* in Kozak plateau, Bergama, Izmir, Türkiye

Line	Sequence of the rDNA ITS region
1	TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTA CCTAGAGTTT GTGGGCTTTG
61	CCCGCTACCT CTTACCCATG TCTTTTGAGT ACTTACGTTT CCTCGGCGGG TCCGCCCGCC
121	GATTGGACAA AATCAAACCC TTTGCAGTTG CAATCAGCGT CTGAAAAACA TAATAGTTAC
181	AACTTTCAAC AACGGATCTC TTGGTTCTGG CATCGATGAA GAACGCAGCG AAATGCGATA
241	AGTAGTGTGA ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGCGCCCCT
301	TGGTATTCCA TGGGGCATGC CTGTTCGAGC GTCATTTGTA CCTTCAAGCT TTGCTTGGTG
361	TTGGGTGTTT GTCTCGCCTC TGCGTGTAGA CTCGCCTTAA AACAATTGGC AGCCGGCGTA
421	TTGATTTCGG AGCGCAGTAC ATCTCGCGCT TTGCACTCAT AACGACGACG TCCAAAAGTA
481	CATTTTTACA CTCTTGACCT CGGATCAGGT AGGGATACCC GCTGAACTTA AGCATATCT

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The BLAST analysis was performed with the obtained sequence result, having a high degree of similarity with the ITS region (ITS1-5.8S-ITS2) sequences available on the National Center for Biotechnology Information (NCBI) (Figure 3). As a result of the BLAST analysis, the sequence of ITS region was found to overlap 100% with 37 different ascochyta blight isolates. On the other hand, a 99% identity with 34 isolates from all over the world was observed. The ITS sequence studied showed a single nucleotide difference in three different positions with an isolate from East Azerbaijan (MK100148.1) while one cytosine deletion was detected at position 17 compared to the sequence of an isolate from China (KP859584.1).

The importance of using DNA sequences as a primary source of information for species identification of many organisms is increasing day by day (Savolainen et al. 2005; Toker et al. 2021). These sequences are used as species genetic barcodes and are stored in the International Nucleotide Sequence Databases (INSDC) GenBank, EMBL, and DDBJ. Species identifications made in this way have many advantages, such as identifying taxa that are not easy to detect according to morphological diagnosis and preventing false definitions due to phenotypic flexibility.

4. Conclusion

Based on the BLAST analysis, the sequence had 99 and 100% nucleotide identity (Table 2) with the corresponding sequences in GeneBank for *A. rabiei* (Table 3). According to available literature, this is the first report of ascochyta blight of *C. montbretii* in Türkiye. The pathogen is considered to be co-evolved with *C. montbretii*. As is generally known, molecular techniques, as in the present study, can be diagnosed with great accuracy, in a short time, and with relatively little effort and expense. The accurate detection of the disease will provide an insight to chickpea breeders in disease management and improvement of resistant chickpea cultivars.

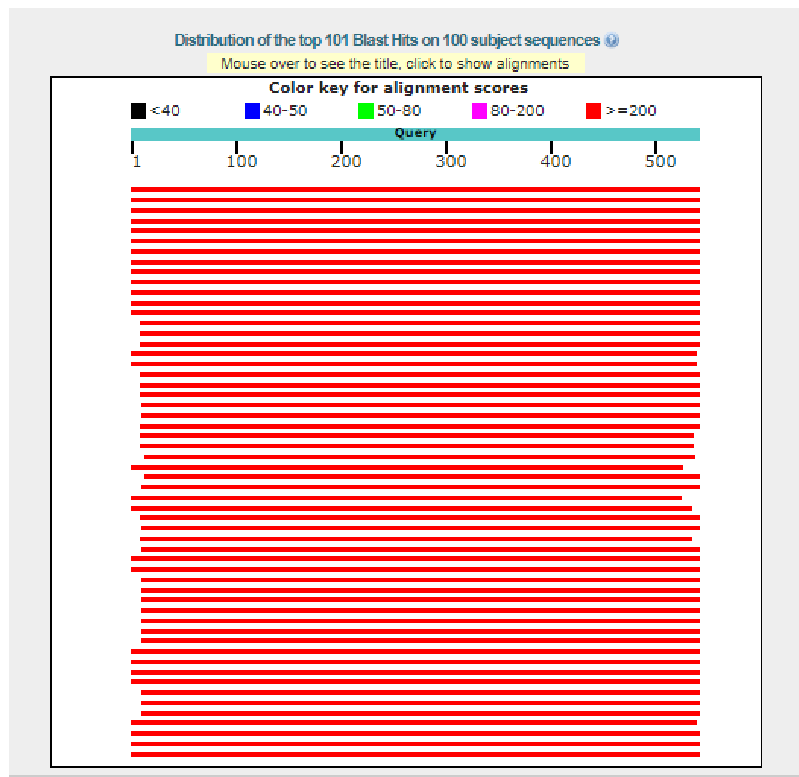

Figure 3. BLAST analyses of ascochyta blight in *C. monbretii* in Kozak plateau, Bergma, Izmir, Türkiye.

Table 3. BLAST analysis of ITS region of ascochyta blight in *C. monbretii* in Kozak plateau road, Bergama, Izmir, Türkiye (NCBI)

Similar sequence region	Similarity (%)
Ascochyta rabiei strain CISA1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	100
Didymella rabiei strain CAr03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100
Ascochyta rabiei strain CBS 237.37 small subunit ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	100
Didymella rabiei strain CAr04 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99
Didymella rabiei strain CAr02 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99
Didymella rabiei strain CAr01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99
Ascochyta rosae culture MFLUCC:15-0063 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	99

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