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Curvularia Lunata as New Causal Pathogen of Tomato Early Blight Disease in Egypt

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Short Report

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Abstract

Tomato plants displaying early blight symptoms were collected from different localities in the provinces of Assiut and Sohag, Egypt. The causal pathogens were isolated on potato dextrose agar plates. Pathogenicity tests with 48 isolates were carried out under greenhouse conditions on tomato cultivar (CV 844). All tested isolates caused symptoms of early blight disease with different degrees. The highest disease severity on tomato plants was found after inoculation with isolate No. 6 followed by isolates No. 20 and No. 31. The most pathogenic isolates were identified by sequence analysis using ITS1 and ITS4 primers. The analysis of the amplified sequences from fungal isolates No. 6, 20 and 31 displayed 99 - 100% nucleotide identity with *Alternaria solani, Curvularia lunata* and *A. alternata*, respectively. To our knowledge, this is the first report of *Curvularia lunata* as one of the causal pathogens of early blight disease of tomato plants in Egypt.

Introduction

Tomato (*Solanum lycopersicum* L.) is an important horticultural crop and grown worldwide. It is the world's second most consumed vegetable crop after potato [1]. In 2008, Egypt ranked fifth in the world in the production of tomato [2]. In 2018, the cultivated tomato area in Egypt was approximately 175 Thousand hectares, producing about 6.8 Mill. tons of tomatoes [3]. Early blight (EB) is a destructive fungal disease of tomato and potato plants [1, 4]. As the plants get older, the symptoms of EB disease first develop on the lower leaves. They appear as small, dark, necrotic lesions which typically have target-like concentric circles and are often surrounded by a yellowing zone. The pathogen releases enzymes that degrade the host cell wall, and toxins that destroy the host cells, thereby making nutrients accessible to the pathogen [5]. Under favorable conditions, the disease spreads over all parts of the plant (leaves, stems, branches and fruits), leading to detaching of leaves, drying of branches and premature fruit drop. The resulting fruit yield losses often amount to 50 to 86 % [6-8]. For the development of an effective disease management strategy it is essential to understand the pathogen population. Therefore, efficient and conclusive identification of the inciting pathogens is important [4, 9]. The previously employed classical methods (mycelial growth rates, colony morphology, microscopic features) to identify fungal species are not as specific as the genotyping methods [10]. Many of the modern identification methods are based on molecular genetic relationships among fungal groups [11]. Molecular techniques are effective instruments for speedy detection of fungal species [12]. Characterization based on sequence analysis of genes of the internal transcribed spacer regions (ITS) of the rDNA is a useful tool to identify and characterize fungal isolates as presumed new species [13-15]. It has generally been found beneficial for research at species level and within species [15, 16].

The objective of the present study was to characterize the causal pathogens of tomato early blight disease in Upper Egypt (Provinces Assiut and Sohag). For this purpose, candidate strains were isolated from diseased plants and screened in pathogenicity tests. The most pathogenic isolates were then identified by PCR with ITS primers and sequencing of the PCR products.

Materials And Methods

Isolation of pathogens

Naturally diseased early blight tomato plants were obtained from open fields in various localities in Assiut and Sohag. Diseased leaves were cut into small pieces, put in 0.5 % sodium hypochlorite for 2 min. and then rinsed 3 times in sterilized distilled water for 2 min. to eliminate residues of the disinfectant. The leaf parts were placed on potato dextrose agar (PDA) in 9 cm Petri plates, and the plates were incubated at 27 ± 1 °C for 4 days. After the incubation period, tips of outgrowing hyphae were singly picked from the lesions and transferred to PDA plates. The plates were incubated at 27 ± 1 °C for 15 days [4]. Isolates were held at 4 °C on a PDA medium until used.

Pathogenicity tests

Pathogenicity tests were carried out under greenhouse conditions at the Department of Plant Pathology, Faculty of Agriculture, Assiut University. Thirty day-old tomato seedlings (cv 844) were replanted in sterilized pots (30 cm diameter) filled with autoclaved sandy clay soil (3 kg / pot; two seedlings / pot), and 3 g fertilizer NPK 12:4:6 were added to each pot. The pots were kept at 30 ± 5 °C with 68 – 80 % relative humidity and watered whenever needed [4]. Altogether, 48 isolates from diseased tomato leaves were employed in the pathogenicity test. To sporulating cultures on PDA, obtained after incubation at 27 ± 1 °C as described above, distilled water was added at 10 ml per plate, and the cultures were gently rubbed with a sterile needle. The resulting spore suspensions were adjusted to $4x10^6$ CFU/ml and sprayed on 30-day old tomato plants using an atomizer. Control plants were sprayed with water [17]. The plants were then covered for 48 hours with polythene bags. Each treatment had three replicates, each with three pots. Thirty days after inoculation the disease severity was rated numerically using a 0 - 4 scale [7, 9], with 0 = leaves without any spot (controls), 1 = spots occupying < 25 %, 2 = 26 - 50 %, 3 = 51 - 75 %, and 4 = 76 - 100 % of the leaf area. The formula was then modified as follows: Disease severity (%) = Σ (n x r) / NR x 100, where n = number of infected leaves on the plant, r = numerical rate of infected leaves, N= total number of leaves on the plant and R= maximum numeric rate.

In order to fulfill Koch`s postulates, the pathogens were re-isolated from tomato plants showing the typical symptoms of early blight and compared by microscopy to the material used for inoculation.

Molecular characterization of pathogenic isolates

The three fungal isolates causing the highest disease severity of early blight disease were identified using molecular methods (ITS-rDNA). The isolates were cultured for 7 days at 27 °C on Czapek`s yeast extract agar with 20% sucrose (CY20S) [18] and sent to Biology Research Unit, Assiut University for DNA extraction using the Patho gene-spin™ DNA / RNA extraction kit (Intron Biotechnology, Inc., South Korea). For polymerase chain reaction (PCR) and gene sequencing, extracted DNA was sent to SolGent Co., Ltd., Daejeon, South Korea. The PCR was conducted using two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were integrated in the reaction mixture. Primers are composed according to the

following, ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') [11]. Using a size nucleotide marker (100 base pairs), the purified PCR products were reconfirmed by electrophoreses on 1% agarose gel. The bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs). Each sample was sequenced using ITS1 and ITS4 primers in the sense and antisense directions. DNA sequences of the three isolates were assembled using the DNA STAR computer package (version 5.05). Assembled sequences were aligned with those downloaded from GenBank using MAFFT [19]. Alignment gaps and parsimony uninformative characters were treated by BMGE [20]. Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using PhyML 3.0 [21]. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications [22]. The best optimal model of nucleotide substitution for the ML analyses was determined using Smart Model Selection (SMS) version 1.8.1 [23]. The phylogenetic tree was visualized using Fig Tree version 1.4.3 [24].

Statistical analysis

Statistical variance (ANOVA) of data was performed using MSTAT-C program version 2.10 (1991). According to the procedures described by Gomez et al. [25] means were compared by Duncan’s multiple range tests and statistical significance was determined at 5% level.

Results

Pathogenicity tests

With 48 fungal isolates, pathogenicity tests were carried out under greenhouse conditions (Table 1). All tested isolates were pathogenic and caused symptoms of early blight disease on tomato plants, although with different degrees. About 75 % of isolates were either highly or very highly pathogenic, whereas the remaining isolates were only low or medium in pathogenicity (Table 2). The highly pathogenic isolates constituted the largest group. Isolates No. 6, 20 and 31 caused the highest disease severity (88-90%). The symptoms of early blight disease were large spots (dark necrotic lesions, often surrounded by a yellowing zone) which spread over most parts of the plant (Fig.1). The spots coalesced and enlarged over time.

Identification of pathogenic isolates using ITS

Isolates No. 6, 20 and 31, that among the 48 isolates tested had shown the strongest pathogenicity on tomato plants, were selected for molecular identification using ITS-rDNA sequencing. The resulting sequences were compared using the BLAST program to those of the NCBI site. A 556 bp fragment from isolate No. 6 (synonym: strain AUMC 14485) showed 99% sequence similarity with four reference strains of *Alternaria solani*. Based on this, the isolate was identified as *A. solani* (Fig. 2). The ITS-rDNA sequence of *A. solani* strain AUMC 14485 was deposited in the GenBank under the accession number MT444991. Isolate No. 31 (synonym: strain AUMC 14486) was identified as *Alternaria alternata*, as sequencing of a 545 bp fragment from this isolate revealed 100 % identity with three reference strains of *A. alternata* (Fig. 2). The ITS-rDNA sequence of *A. alternata* AUMC 14486 was deposited in the GenBank nucleotide

sequence database under accession number MT777510. The sequencing data of a 562 bp fragment from isolate No. 20 (synonym: strain AUMC 14229) clustered together (100 % identity) with three reference strains of *Curvularia lunata* (Fig. 2). The isolate was thus named as *C. lunata* AUMC 14229 and registered in GenBank under the accession number MT444990.

Discussion

The objective of this study was to obtain information on the fungal pathogens causing early blight of tomato in Upper Egypt. Among the isolates studied, a large variability of the pathogenicity was observed. However, most isolates were grouped as highly or very highly pathogenic. In studies with different isolates of *Alternaria*, Remezani et al. [26] also observed that all isolates obtained from tomato early blight lesions were pathogenic, with substantial variations in pathogenicity levels between the isolates. Our observations on the disease symptoms incited by inoculation with the pathogenic strains are in agreement with the literature, both regarding shape and color as well as their first appearance on the lower leaves and spreading with increasing growth of plants [27]. *A. solani* and *A. alternata* [1, 4] as well as a number of other species of *Alternaria* [5, 28] are well known as causal pathogens of early blight of tomato. *Curvularia spicifera* has been described as a pathogen causing leaf spot of tomato in India [29]. *Curvularia lunata* was reported to cause fruit rot of tomato [30], but little is known about *C. lunata* as causal pathogen of tomato EB. We are only aware of a single report from Pakistan [31] describing the isolation of *C. lunata* var. *aeria* from tomato leaves with EB. According to the *Index Fungorum* [32] *C. lunata* var. *aeria* is synonym with *C. lunata.*

To summarize, fungal isolates from diseased tomato plants from Assiut and Sohag (Upper Egypt) were tested for ability to cause tomato EB. Using PCR with ITS primers and sequencing of the PCR products, the three most pathogenic of these isolates were identified as *A. solani*, *A. alternata* and *C. solani,* respectively. While species of *Alternaria* have been reported before as EB pathogens in Egypt [4], this is the first report for *C. lunata* as causal pathogen EB of tomato in Egypt.

Declarations

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Compliance with ethical standards

Ethical responsibility: Our manuscript is original research and it is not submitted to full or in parts to other journal for publication.

Conflict of interest: The authors declare that they do not have any actual or potential conflict of interest.

Informed consent: All authors have reviewed the manuscript and approved the final version of manuscript before submission.

AUTHOR CONTRIBUTIONS

S.N.M.A., and M.S.M. suggested the idea of the work and contributed to data curation and their validation as well as writing original draft. A.H.A.S. and B.H.M.K contributed to the formal analysis of the data S.N.M.A., A.H.A.S and B.H.M.K. contributed to the reviewing and editing the manuscript. All authors reviewed and approved the final version of the manuscript.

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Tables

Table1: Pathogenicity tests of 48 fungal isolates on tomato cultivar (CV 844) under greenhouse conditions.

Isolates No.	Disease severity %	Lacation	Isolates No.	Disease severity %	Lacation
1	30.76 ± 0.08 t	Assiut	25	65.00 ± 0.64 jk	Sohag
2	30.15±0.21 t	Sohag	26	34.26 ±0.66 s	Sohag
3	70.06±0.06 h	Sohag	27	60.89l ± 0.57 m	Sohag
4	41.79±1.26 r	Sohag	28	62.85 ±1.29 kl	Sohag
5	58.02±1.50 n	Sohag	29	76.29 ±0.74 fg	Assiut
6	94.04±1.12 a	Assiut	30	70.20 ±0.07 h	Assiut
7	59.47±0.57 mn	Assiut	31	87.98b±1.08 c	Assiut
8	51.73±0.46 op	Assiut	32	32.18 ±1.69 st	Assiut
9	39.50±0.67 r	Assiut	33	80.26 ±0.09 de	Assiut
10	60.28l±0.15 mn	Assiut	34	80.00 ±0.69 de	Assiut
11	30.50±0.21t	Sohag	35	66.67 ±0.93 ij	Sohag
12	50.52±0.36 p	Sohag	36	30.39 ±0.01 t	Sohag
13	42.20±1.13 qr	Sohag	37	44.59 ±0.58 q	Sohag
14	51.07±0.63 p	Sohag	38	54.29 ± 0.50 o	Sohag
15	50.52±0.29 p	Sohag	39	76.36 ±1.08 fg	Sohag
16	40.26±0.21 r	Sohag	40	66.96 ±1.19 ij	Sohag
17	69.00±1.23 hi	Sohag	41	84.33 ±0.42c	Sohag
18	65.11±0.86 jk	Sohag	42	59.89 ±0.17mn	Sohag
19	32.07±0.81 st	Sohag	43	74.26 ±1.66 g	Assiut
20	90.04±1.06 b	Sohag	44	61.39 ±0.84 lm	Assiut
21	31.26±0.88 t	Sohag	45	82.43 ±1.43cd	Assiut
22	54.27±2.14 o	Assiut	46	78.34 ±0.12ef	Assiut
23	62.28±1.26 lm	Assiut	47	70.96 ±0.35 h	Assiut
24	30.55±0.75 t	Assiut	48	67 ±1.67 u	-

Means defined in columns with the same letter(s) do not significantly according to the multiple range check by Duncan (P = 0.05). All values are the means of three replicates ±SE

Table 2: Grouping of isolates according to their ability to cause symptoms of early blight

Pathogenicity group (disease severity)	Number of isolates (n=48)
Low (10 – 35 %)	8
Medium (36 – 50 %)	5
High (51 – 69 %)	21
Very high (70 – 100 %)	14

Figures

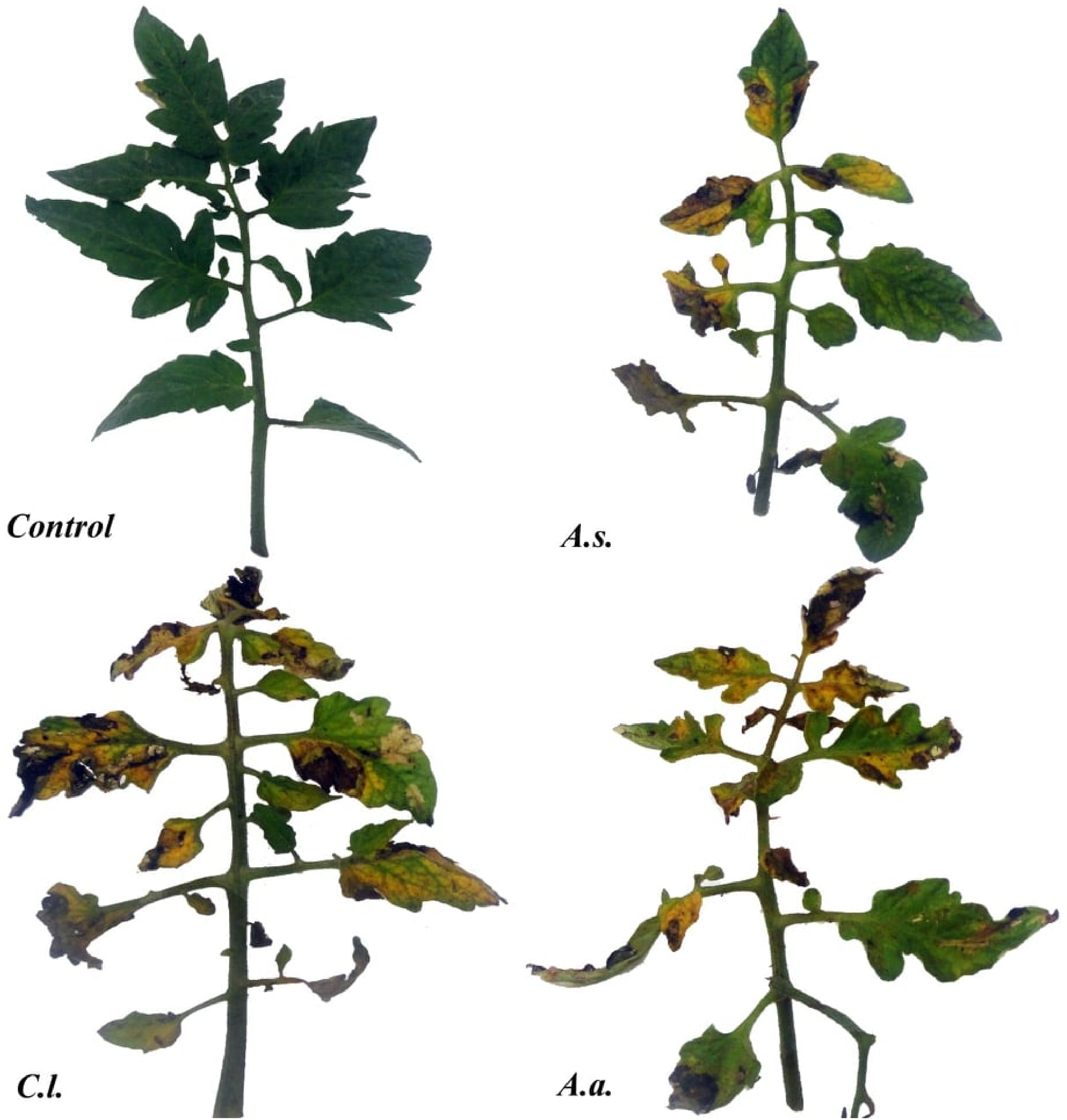

Figure 1

Symptoms of early blight disease on tomato leaves caused by Altrnaria solani (A.S.), Clvularia lunata (C.L.) and Altrnaria alternata (A.a.). Healthy plants (control) haven't any symptoms of the disease.

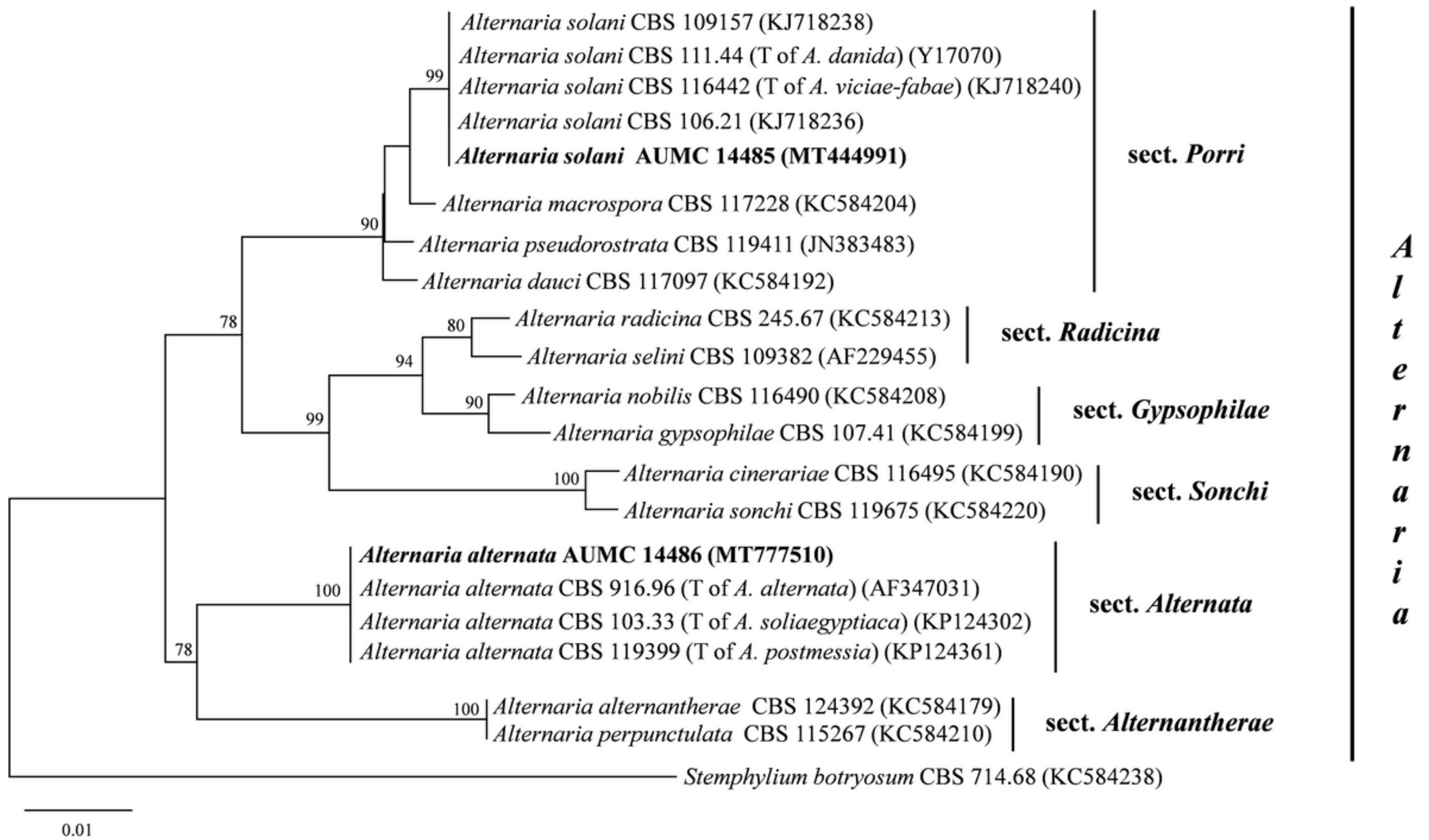

Figure 2

Maximum likelihood phylogenetic tree based ITS sequences and bootstrap support values > 60 (BS) are given at the nodes (BS). ITS sequences of rDNA of the isolated fungal strains (No. 6 and 31) in the present study were Alternaria solani (AUMC 14485) and Alternaria alternata (AUMC 14486) resp. which were aligned with closely related sequences accessed from the GenBank. The marker reflects the relative phylogenetic distance measurement.

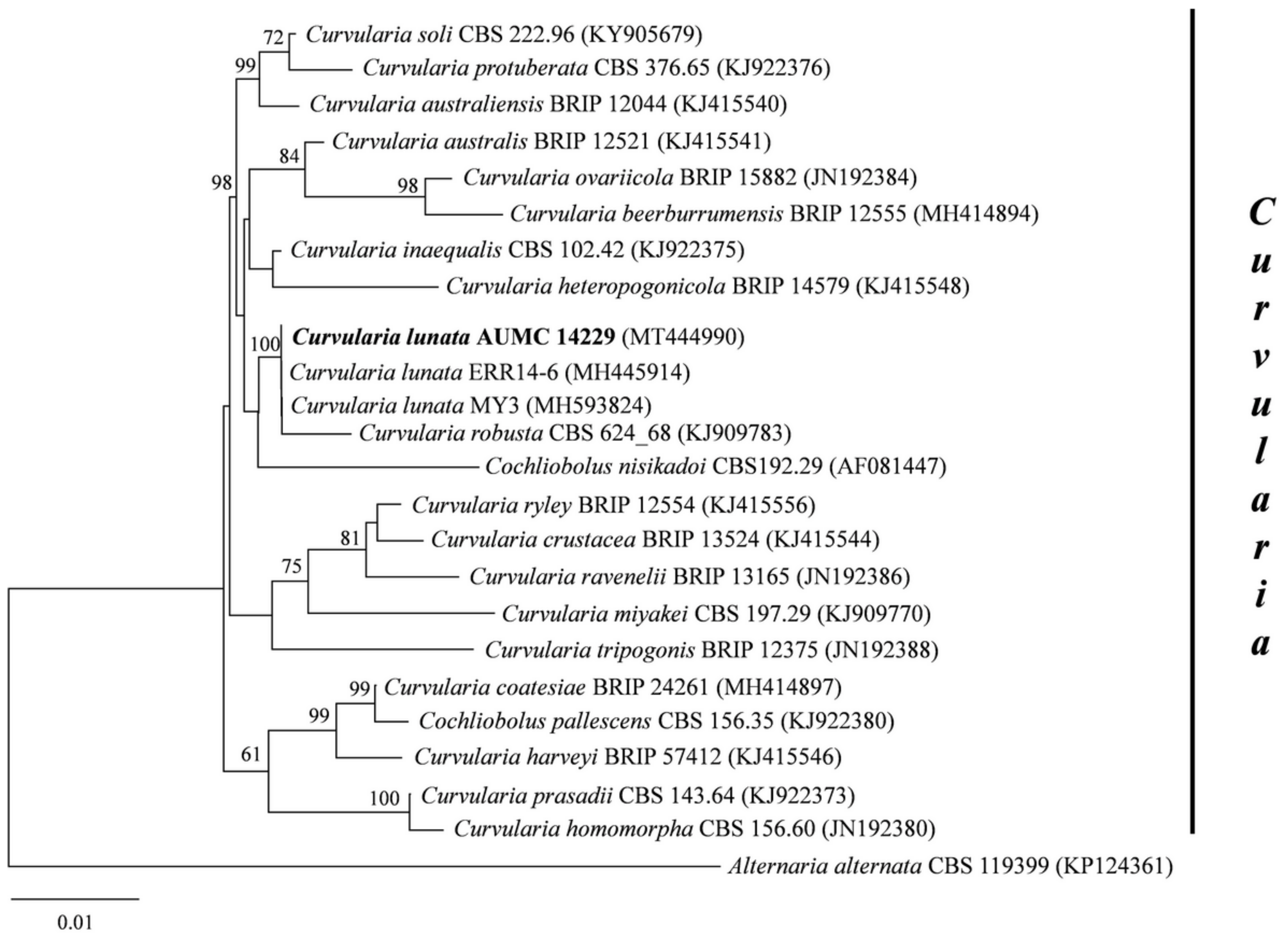

Figure 3

Maximum likelihood phylogenetic tree based ITS sequences and bootstrap support values > 60 (BS) are given at the nodes (BS) on ITS sequences of rDNA of the isolated fungal strain (No. 20) in the present study was Curvularia lunata (AUMC 14229) aligned with closely related sequences accessed from the Gen Bank. The marker reflects the relative phylogenetic distance measurement