Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Alternaria and *Curvularia* leaf spot pathogens show high aggressivity on watermelon, and are emerging pathogens in cucurbit production

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Summary. Fungal leaf spot pathogens of cucurbits cause significant yield losses. They cause extensive leaf necroses and defoliation, reducing host photosynthesis. They increase risks of fruit sunscald, and can cause substantial crop damage. *Alternaria cucumerina* has been recognized as the causal agent of leaf spot disease of cucurbits, and recent studies have identified other *Alternaria* species, and other emerging pathogens such as *Curvularia*. This study characterized 25 isolates obtained from infected watermelon and cucumber leaves from Hungary, Spain, and Kosovo. Morphological characterization and molecular analyses using *TEF1-α, HIS3,* and ITS gene regions identified *Alternaria alternata* and *A. arborescens*, and for the first time on this host, the genus *Curvularia*. Detached leaf assays of ten isolates on 73 watermelon accessions showed variation in isolate pathogenicity. The tested *Curvularia* isolate was the most aggressive, followed by the *A. arborescens* and *A. alternata* isolates, although *A. alternata* was the most frequently identified species. These results highlight the potential for emerging fungal pathogens causing cucurbit leaf spot, such as *Curvularia* sp., and show that these fungi can cause damage on economically important plants. This study also showed differing resistance within the watermelon collection, indicating potential for the plant introduction (PI) accessions as sources of resistance breeding.

Keywords. *Alternaria alternata, Alternaria arborescens*, bar coding, leaf spot, *Citrullus* spp.

OPEN ACCESS

Citation: Paredes-Machado, C., Bogaj, V., Papp, V., Balázs, G. & Papp, D. (2025). *Alternaria* and *Curvularia* leaf spot pathogens show high aggressivity on watermelon, and are emerging pathogens in cucurbit production. *Phytopathologia Mediterranea* 64(1): 41-55. doi: 10.36253/phyto-15955

Accepted: March 11, 2025

Published: May 15, 2025

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Thomas A. Evans, University of Delaware, Newark, DE, United States.

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INTRODUCTION

Watermelon (*Citrullus lanatus*, *Cucurbitaceae*) is an important food crop which has been cultivated for at least 5000 years since domestication in northeastern Africa (Paris, 2015; Chomicki *et al*., 2020). In 2022, world watermelon production was approx. 100 million tons, while the total area of

watermelon cultivation was approx. 2.9 million hectares, highlighting the extensive land use dedicated to this crop (FAO, 2022).

Watermelon plants are susceptible to several important fungal diseases, such as powdery mildew (*Podosphaera xanthii*, *Erysiphe cichoracearum*), downy mildew (*Pseudoperonospora cubensis*), anthracnose (*Colletotrichum orbiculare*), Fusarium wilt (*Fusarium oxysporum*) and gummy stem blight (*Didymella bryoniae*) (Egel *et al*., 2022). Fungal leaf spot of cucurbits has also been a growing concern during the last decade, resulting in significant economic losses for farmers due to reduced yields and increased production costs (Abu-Nasser and Abu-Naser, 2018; Ma *et al*., 2021; Shanthi Avinash *et al*., 2021).

Alternaria cucumerina has been identified as the causal agent of the Alternaria leaf spot of cucurbits, including watermelon (AA.VV., 2021; Kucharek, 1985). In recent decades, new *Alternaria* species have been reported as pathogens of cucurbits, including *A. alternata* f. sp. *cucurbitae* (Vakalounakis, 1990; Zhou and Everts, 2008; Ahmed *et al*., 2021), *A. tenuissima, A. infectoria,* and *A. gaisen* (Kwon *et al*., 2021; Ma *et al*., 2021).

The importance of these pathogens is indicated by *Alternaria* being among the ten most cited pathogenic fungal genera. This diverse genus includes more than 600 species, many of which are major plant pathogens causing leaf spots, blights, rots, and seed infections of a wide range of agricultural plants, including cereals, fruit crops, vegetables, and ornamentals (Bhunjun *et al*., 2022, 2024). New cucurbit host species and outbreaks of *Alternaria* diseases are being reported, highlighting the increasing threats of these pathogens to cucurbit production, especially in regions with extended periods of high humidity (Abu-Nasser and Abu-Naser, 2018; Matić *et al*., 2020; Shanthi Avinash *et al*., 2021). Alongside Alternaria leaf spot, more recent reports have shown that members of the related *Curvularia* genus can also affect cucurbits, including *Trichosanthes dioica* (pointed gourd), *Cucurbita argyrosperma* (kershaw, or silver-seed gourd), and *Cucumis melo* (melon) (Sarkar *et al*., 2018; Ayvar-Serna *et al*., 2022; Vanitha *et al*., 2024).

The *Pleosporaceae* includes 23 genera with *Alternaria* and *Curvularia* as two of the most important genera (Torres-Garcia *et al*., 2022; Hyde *et al*. 2024). *Curvularia* spp. are ubiquitous as pathogens and saprobes of plants, animals, and humans (Sivanesan, 1987; Marin-Felix *et al*., 2017), and many are known as causes of diseases of grasses and staple food crops, including rice, maize, wheat, and sorghum (Khan *et al*., 2023). More than 200 *Curvularia* species are recognized (Hyde *et al*. 2024). *Curvularia* and *Alternaria* have been described as "sister genera" within *Pleosporaceae*, due to their close evolutionary relationship (Bao and Roossinck, 2013). Therefore, they share morphological traits (Torres-Garcia *et al*., 2022), and cause similar symptoms on host plants (Rabaaoui *et al*., 2022), making species identification difficult. Results showing different capabilities of *Alternaria* species to cause disease on a given host have highlighted the importance of accurate species level diagnoses, for plant protection and resistance breeding (Fontaine *et al*., 2021).

Phylogenetic analyses utilizing barcoding markers are important for identification of fungi. In the last decade, *Alternaria* (Peever *et al*., 2004; Woudenberg *et al*., 2015; Dettman and Eggertson, 2021) and *Curvularia* (Manamgoda *et al*., 2012a; 2015; Marin-Felix *et al*., 2020; Connally *et al*., 2022) have undergone substantial taxonomic changes, with reclassification of several species and identification of new species based primarily on molecular genetic analyses. Considerable progress has been made in systematic revision of both genera through multi-locus sequence analyses, particularly using the genetic regions of Internal Transcribed Spacer (ITS), Translation Elongation Factor 1- α *(TEF1-α),* Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH),* RNA polymerase II second largest subunit *(RPB2),* Histone H3 *(HIS3),* Small Subunit ribosomal RNA *(SSU, 18S rRNA),* and Large Subunit ribosomal RNA *(LSU, 28S rRNA)* (Dettman and Eggertson, 2021; Aung *et al*., 2024). Those phylogenetic analyses have often been refined, by using endopolygalacturonase gene *(endoPG)* and two anonymous genome regions (OPA1-3 and OPA2-1), which are suitable for functional studies and species delimitations (Woudenberg *et al*., 2015; Aung *et al*., 2024). Species delimitation within *Alternaria* has challenges, and is often considered to be difficult, emphasizing the need for integrated approaches combining morphological, ecological, and molecular data for species identifications.

Selection of host plants that are tolerant or resistant gene sources for the breeding requires accurate identification of the respective pathogens. Among germplasm collections, plant introductions (PIs, USDA) of watermelon offer a valuable source of genetic diversity for resistance breeding programmes, that contribute to reducing the reliance on fungicides, and can improve fruit quality and yield to increase crop profitability (Grumet *et al*., 2021). Currently, little is known about resistance watermelon towards leaf spot diseases caused by *Alternaria* or *Curvularia* spp.

The present study reports occurrence of a *Curvularia* sp. on watermelon. Furthermore, *A. alternata, A. arborescens,* and *Curvularia* isolates were characterized as causal agents of leaf spot disease in cucurbits, combining

morphological and phylogenetic analyses, and evaluation of their pathogenicity on a diverse collection of watermelon accessions.

MATERIALS AND METHODS

Collection and culturing of fungal isolates

Diseased watermelon and cucumber (*Cucumis sativus*) leaves were collected from plants grown in open fields and greenhouses in Hungary, Spain, and Kosovo, between June and September of 2024. A total of 25 single conidium isolates were obtained from collected leaves. Isolations were carried out according to Venkatesagowda *et al*. (2012), with some modifications. Fungal lesions were cut out from leaves, and were surface-sterilized in 4% sodium hypochlorite solution for 60 sec, then rinsed with distilled water for 10 sec. Leaf fragments were then dried on sterile paper, and placed on Potato Dextrose Agar (PDA) in Petri plates. After 24 h, hyphal tips resembling *Alternaria* sp. were aseptically cut under a stereo microscope, and placed on new PDA plates. From the developing cultures, after 4–5 d, conidia were harvested by mixing a small piece of sporulating mycelium with sterile distilled water containing 0.03% Tween 20, and filtering the suspension through double cheesecloth. Resulting conidium suspensions were then spread on the surface of 1.6% water agar, (and individual conidia were each picked up with a needle and transferred to a new PDA plate. Single conidium isolates were maintained on 25°C, and were subcultured every 10 d.

To obtain conidium suspensions for morphological characterization and for host inoculations, isolates were grown on a calcium carbonate-based sporulation medium as described by Shahin (1979), with sucrose omitted from the medium formulation. Seven-day-old conidia were collected from the surfaces of abundantly-sporulating isolates, by washing with sterile distilled water containing Tween 20, or using the tape touch method for microscope slide preparations (Harris, 2000).

Culture and conidium morphology

Photographs of 1-week-old PDA cultures of the isolates were taken to evaluate culture appearance, including colour according to RAL code system (RAL GmbH), and texture following the description of Nobles (1948). Colony growth rates (cm d^{-1}) were calculated from the age of cultures, and their final colony diameters measured with a vernier caliper.

Conidium length (µm), width (µm), and numbers of conidia in chains or clusters were assessed using a Zeiss Axio Imager A2 (Carl Zeiss Microscopy). Conidium characteristics were measured for at least 20 replicates per species, with isolates selected randomly, using the software ImageJ2 (Rueden *et al*., 2017).

Molecular analyses

To identify species of the collected isolates, their genetic sequences were analysed and a phylogenetic tree was constructed. To extract DNA, mycelia of each monospore isolate grown on PDA were cut and stored at -80 °C. Frozen samples were then ground in a mortar, and DNA was extracted using the E.Z.N.A. plant DNA kit (Norcoss), according to the manufacturer's recommendations. DNA concentrations were verified by staining and running the extracted DNA on 1% agarose gels (BioReagent, Sigma-Aldrich).

Three barcoding markers were used for the amplifications: the Internal Transcribed Spacer (ITS) region was amplified using the primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (3'-TCCTCCGCTTATTGATATGC-5') (White, 1990), Histone 3 *(HIS3)*, using HIS3-F (5'-ACTAAGCAGACCGCCGCGAAG-3'), and (HIS3-R (3'-GCGGGCGAGCTGGATGTCCTT-5') (Steenkamp *et al*., 2000), and the Translation Elongation Factor 1-alpha *(TEF1-α)* using TEF1-F (5'-CATCGAGAAGTTCGAGAAGG-3') and TEF1-R (5′-TACTTGAAGGAACCCTTACC-3′) (Wu *et al.,* 2013).

The polymerase chain reaction (PCR) was carried out for DNA samples and a negative control (without DNA), using a Thermal Cycler 2720 PCR machine (Applied Biosystems). The PCR mixture (final volume of 16 µL) was prepared with the DreamTaq Green PCR Master Mix (Fermentas). The PCR program was set according to the specified temperatures, times, and number of cycles as described in Table S1**.** Integrity of amplicons was evaluated by running them on 1% agarose gel (BioReagent, Sigma-Aldrich). Amplicons were sequenced using an ABI 3100 automatic sequencer. All generated sequences were uploaded to GenBank (Table S2).

The quality of the resulting chromatograms was checked using CodonCode Aligner 7.0.1 (CodonCode Corporation). Reference sequences for *Alternaria* and *Curvularia* from the GeneBank database were included in the analyses (Table S2). *HIS3* was only used for Blast analyses. Sequences of ITS and *TEF1-α*, together with sequences of related species downloaded from GenBank, were aligned separately with the online MAFFT v. 7.0, using the L-INS-i strategies (Katoh and Standley, 2013). The alignments were checked and edited by manual adjustment in Multiple sequence alignment, and manual corrections (cutting of low-quality edges, indels) were

achieved using AliView (Larsson, 2014). Maximum Likelihood (ML) analysis was performed using RAxML in raxmlGUI2.0 (Stamatakis, 2014; Edler *et al*., 2021), with 1,000 rapid bootstraps and the GTRGAMMA substitution model. The resulting phylogenetic tree was visualized in MEGA11 (Tamura *et al*., 2021).

Pathogenicity assays

Healthy watermelon leaf samples were collected from an open greenhouse located in Soroksár, Hungary (47°23′49″ N, 19°09′10″ E), containing a collection of 73 watermelon varieties. The collection included 71 plant introduction (PI) accessions from the United States of America, and the commercial *C. lanatus* cultivars 'Black Diamond' and 'Calhoun Grey'. The plant accessions originated from a wide geographic range, including Turkey, Spain, Zimbabwe, Democratic Republic of Congo, Senegal, Iran, Japan, Syria, Ghana, Zambia, South Africa, and the United States of America.

The leaf samples were put between wet sterile tissue for transport to the laboratory. Leaves were then washed gently in 1% sodium hypochlorite solution for 60 sec and immediately placed in sterile water for a further 60 sec. Young undamaged leaf sections (each of 6 mm) were cut with a cork borer and surface water was removed by placing these on sterile paper towels. the leaf sections were then placed adaxial surface upwards on 1% water agar.

Following identification of isolates to genus or species, pathogenicity tests were carried out using six representative isolates from watermelon in Spain, identified as *A. arborescens*, one isolate from watermelon in Hungary, identified as *Curvularia*, and three isolates from watermelon in Hungary, identified as *A. alternata*. These assays were each conducted using a conidium suspension (1×10^6 conidia mL^{-1}, with 5 µL of the suspension applied on each leaf disc. Each plate contained three inoculated leaf discs per plant accession, for biological replicates and one leaf disc mock-inoculated with 5 µL of sterile water. The plates were incubated at 25°C in dark conditions. Relative disease severity (DS) was estimated visually at 5 d post-inoculation (DPI), using a 0 to 9 scale (Singh *et al*., 2020) and an Olympus BX41 stereomicroscope (Olympus Corporation). Development of new conidium chains, hyphae, and the presence of necrotic tissues were considered compatible reactions (Figure S1).

Statistical analyses

Morphological features of conidia were evaluated using the Wilks's lambda MANOVA model to compare the mean lengths, widths, and conidium counts per chain, among the different species. This model had the assumptions of homogeneity of covariance matrices, multivariate normality, and homogeneous variances. Assumption of homogeneity of covariance matrices was tested by Box's M-test and multivariate normality with the Shapiro-Wilk test, while homogeneity of variances was tested using Bartlett's test. MANOVA was carried out with Wilk's Lambda test. After identifying statistically significant overall differences among species using Wilks' Lambda ($P < 0.05$), pairwise comparisons between species were subsequently made using estimated marginal means based on the fitted multivariate linear model. *P*-values were adjusted using the Bonferroni correction for multiple comparisons.

A Principal Component Analysis (PCA) was conducted on five morphological traits related for each culture (growth rate, appearance) and conidia (length, width, number per chain or cluster). Principal Components (PC) were computed, and proportion of variance explained by each function was assessed to determine their contributions to species discrimination. To visualize the relationship between the main two PC across species, a two dimension scatterplot was created.

For pathogenicity assessments, Disease Index (DI) was calculated from DS values using the formula described by Singh *et al*. (2020). A one-way ANOVA model was carried out to compare mean Disease Index (DI) across the different fungal species. This model had assumptions of independent samples, non-homogeneous variances, and normally distributed residuals. Normality was checked using the d'Agostino test, and graphically by histogram and QQ-plot, while homogeneity of variances was tested using Levene's test. Pairwise comparisons were calculated by the Games-Howell *post hoc* test. All statistical tests used were two-sided, and the significance level was set at $\alpha = 0.05$. All statistical analyses and visualizations were carried out using the software R (Version 4.4.1. R Foundation for Statistical Computing).

RESULTS

Based on morphology and genetic barcoding, three species of fungi were isolated from watermelon leaf spots from the sampled plant material (Table 1).

Morphology of isolates

The morphological analyses showed variations in culture colour and texture between the studied species (Table 2). *Alternaria alternata* isolates had colony colours

Table 1. Locations and host species for fungal isolates (n = 25) collected from cucurbit leaf spots.

Isolate species	Isolate code	Location of origin	Host species
Alternaria arborescens	cc_1[1]	Spain (La Puebla)	*Citrullus lanatus*
Alternaria arborescens	cc_2	Spain (La Puebla)	*Citrullus lanatus*
Alternaria alternata	cc_3[1]	Spain (La Puebla)	*Citrullus lanatus*
Alternaria arborescens	cc_4[1]	Spain (La Puebla)	*Citrullus lanatus*
Alternaria alternata	cc_6	Spain (La Puebla)	*Citrullus lanatus*
Alternaria alternata	cc_7[1]	Spain (La Puebla)	*Citrullus lanatus*
Alternaria arborescens	cc_8[1]	Spain (La Puebla)	*Citrullus lanatus*
Alternaria arborescens	cc_9	Spain (La Puebla)	*Citrullus lanatus*
Alternaria arborescens	cc_10	Spain (La Puebla)	*Citrullus lanatus*
Alternaria alternata	cc_11	Hungary (Buda)	*Cucumis sativus*
Alternaria alternata	cc_12	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_13	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_14	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_15	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_16	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_17	Hungary (Polgár)	*Cucumis sativus*
Alternaria alternata	cc_20	Hungary (Soroksár)	*Citrullus lanatus*
Alternaria alternata	cc_21	Hungary (Soroksár)	*Citrullus lanatus*
Curvularia sp.	cc_22[1]	Hungary (Soroksár)	*Citrullus lanatus*
Curvularia sp.	cc_24	Hungary (Soroksár)	*Citrullus lanatus*
Alternaria alternata	cc_25	Hungary (Soroksár)	*Citrullus lanatus*
Alternaria alternata	cc_36	Kosovo (Drenas)	*Citrullus lanatus*
Alternaria alternata	cc_41[1]	Hungary (Ócsa)	*Citrullus lanatus*
Alternaria alternata	cc_42[1]	Hungary (Ócsa)	*Citrullus lanatus*
Alternaria alternata	cc_45[1]	Hungary (Jászszentandrás)	*Citrullus lanatus*

[1] Isolates used for leaf disc assays.

Table 2. Culture and conidium morphologies of *Alternaria* and *Curvularia* species.

Species	Texture[a]	RAL colour[b]	Mean growth rate (cm d^{-1})	Mean conidium length (µm)	Mean conidium width (µm)	Number of conidia
Alternaria alternata	Cottony	RAL 7013	1.14 ± 0.10	23.51 ± 3.55	9.76 ± 1.59	7[1]
Alternaria arborescens	Cottony	RAL 7002	1.21 ± 0.04	21.83 ± 2.92	9.59 ± 1.16	5[1]
Curvularia sp.	Velvety	RAL 8000	1.23 ± 0.07	28.03 ± 3.57	8.99 ± 1.12	8[2]

[a] Predominant texture among the studied strains.
[b] Predominant RAL colour among the studied strains.
[1] Mean number (± standard deviation) of conidia per chain.
[2] Mean number (± standard deviation) of conidia per cluster.

ranging from grey to black, with cottony textures, and generally smooth regular margins. The *A. arborescens* isolates had colony colours of various tones of grey, with a predominantly cottony appearance, and regular and irregular colony margins. Colonies of *Curvularia* isolates were green-brown in colour, of velvety texture, and had smooth margins. Differences between *A. alternata*, *A. arborescens*, and *Curvularia* sp. were further quantified based on conidium features.

Alternaria alternata and *A. arborescens* had dark-coloured conidia, that were multicellular, ovoid or obclavate, and with short conical or cylindrical beaks. The average conidium dimensions of *A. alternata* were 23.5 ± 3.6 µm × 9.8 ± 1.6 µm, while those of *A. arborescens* were 21.8 ± 2.9 µm × 9.6 ± 1.2 µm. Conidia were produced in chains on conidiophores. The *Curvularia* sp. conidia were dark, ellipsoid, and each had three transverse septa. The average conidium dimensions of

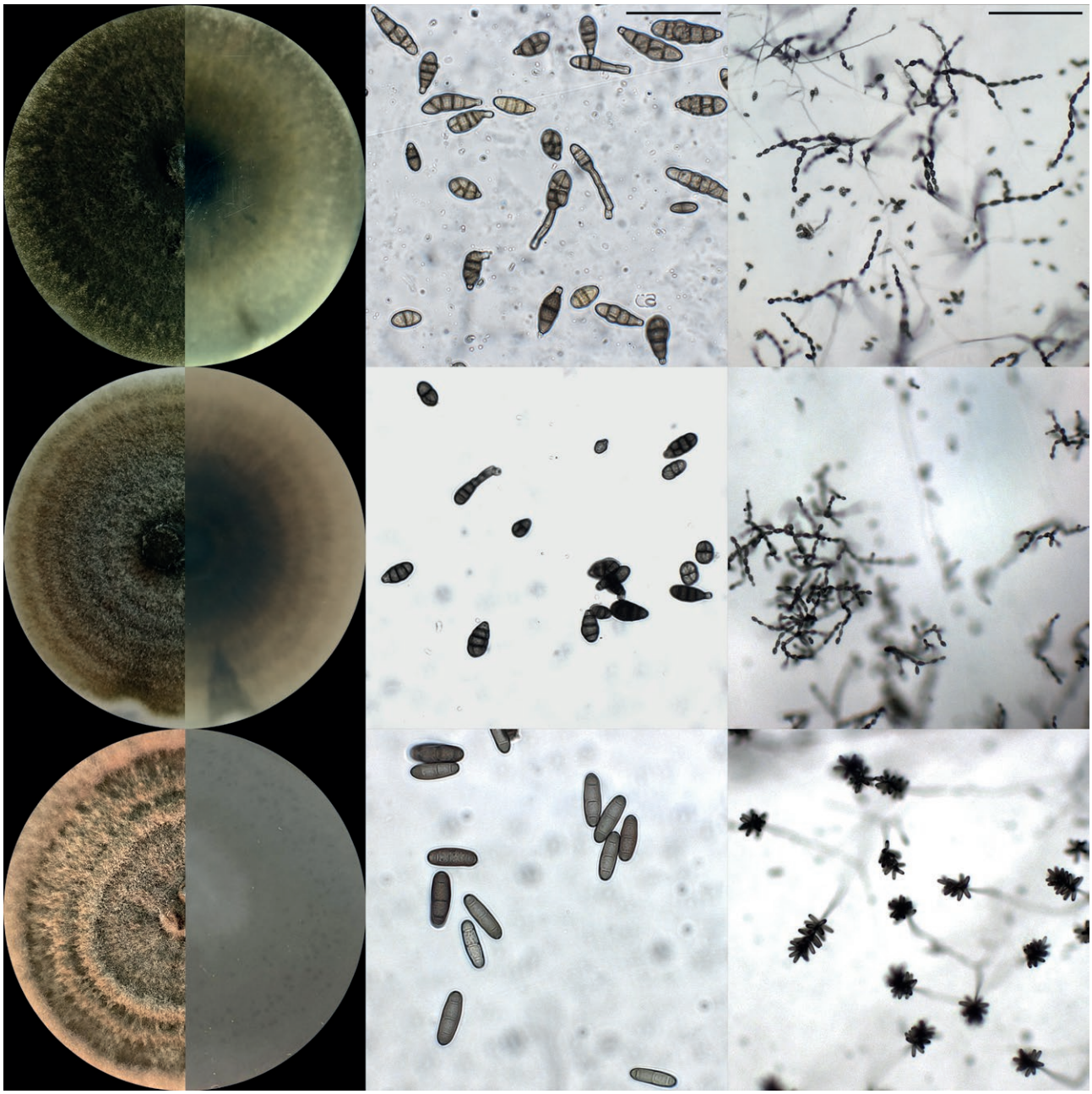

Figure 1. Cultures (left), conidia (middle), and conidium chains or clusters (right) of *Alternaria alternata* (upper images), *Alternaria arborescens* (middle), and *Curvularia* sp. (lower). Culture plates (right) were 9 cm diam. Scale bars = 50 μm (left) or 200 μm (right).

Curvularia sp. were 28.0 ± 3.6 μm × 9.0 ± 1.1 μm. These conidia developed close together in chains, appearing as clusters (Figure 1).

Evaluation of morphological traits of conidia using MANOVA Wilk's Lambda test showed statistically significant differences in mean lengths, widths, and numbers of conidia per chain across the three fungal species (Wilks' $F(6,168) = 14.60$; $P < 0.0001$). Pairwise comparisons using estimated marginal means, based on the multilinear model, showed significant differences in length, width, and number of conidia per chain between all pairs *Curvularia* sp. and *A. alternata* ($P < 0.01$), *Curvularia* sp. and *A. arborescens* ($P < 0.0001$), and *A. alternata* and *A. arborescens* ($P < 0.001$).

Morphological variations among the three species, *A. alternata*, *A. arborescens*, and *Curvularia* sp., based on

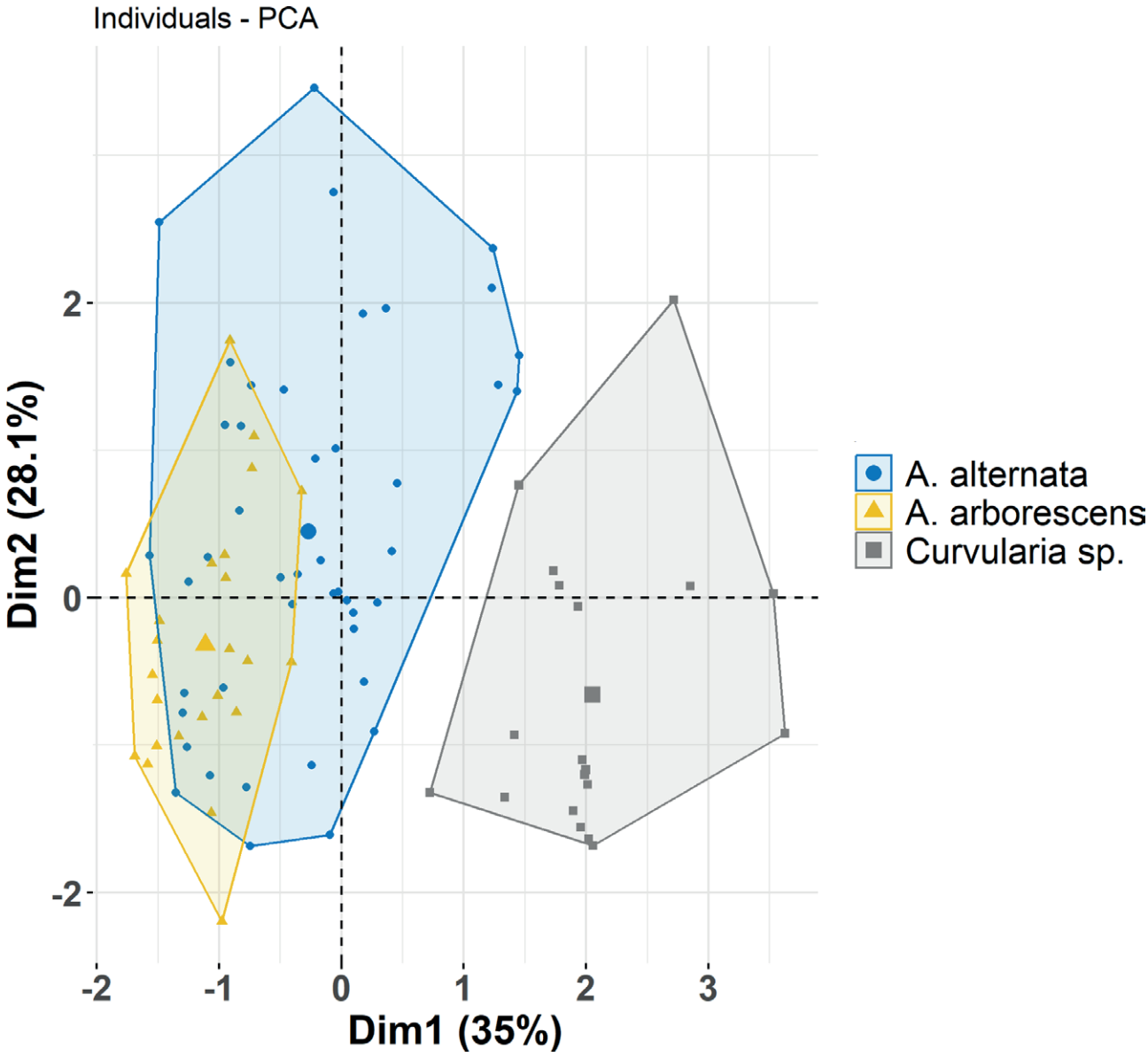

Figure 2. Principal component analyses of fungal isolates (n =25) obtained in this study, based on their morphological traits (colony growth rate, texture, conidium length, conidium width, and numbers of conidia per chain or cluster). The percentage of explained variability of the first two principal components (Dim 1 and Dim 2) is indicated in the parentheses.

the first two principal components (35.0% Dim. 1 and 28.1% Dim.2) are shown in Figure 2. *Curvularia* sp. was clearly distinct from the other two species, forming a separate cluster along the first principal component. In contrast, *A. alternata* and *A. arborescens* had overlapping clusters, indicating some morphological similarities and shared traits. Also, *A. alternata* had greater variability across both dimensions, reflecting high morphological diversity, while *A. arborescens* had a narrow cluster that indicated less variability within the species.

Phylogenetic analyses of fungal isolates

The phylogenetic analyses based on ITS sequences placed the studied isolates into two well-supported and clearly differentiated clades (Figure 3). Isolates cc_22 and cc_24 clustered with the type strains of *Curvularia buchloes, C. hawaiiensis, C. rouhanii,* and *C. spicifera.* BLAST analysis of *TEF1-α* sequences from the Hungarian *Curvularia* isolates (cc_24 and cc_24) identified the closest match with a *C. hawaiiensis* strain (GenBank no.

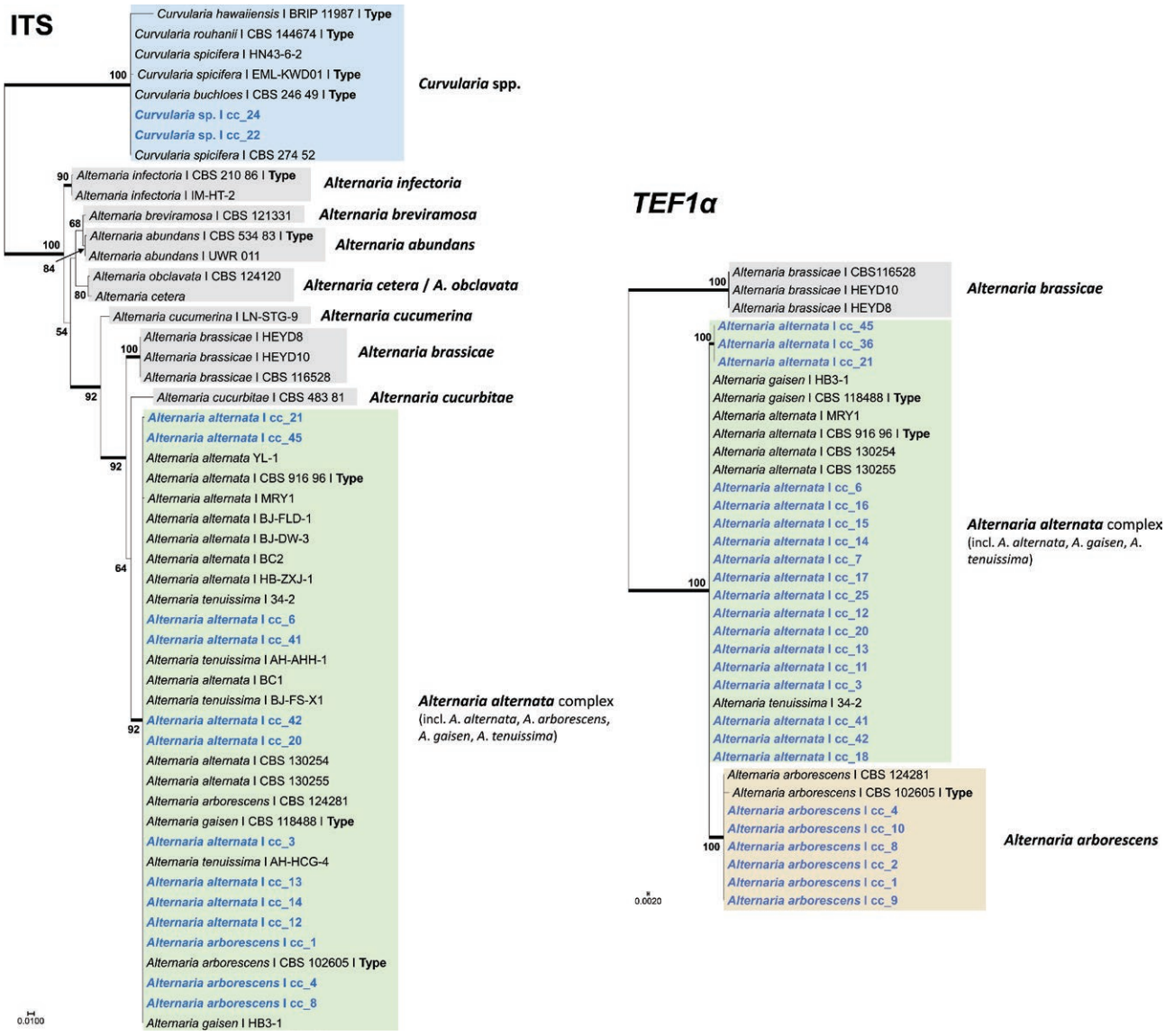

Figure 3. Internal Transcribed Spacer (ITS; left) and Translation elongation factor 1-α (*TEF1-α*; right) phylogenies of *Alternaria* and *Curvularia* isolates obtained in this study. Coloured boxes indicate different taxonomic groups. The obtained isolates are highlighted by blue font, and numbers on branches indicate bootstrap probabilities.

OR841149.1), showing 96% sequence similarity. In contrast, no relevant *Curvularia* sequences for *HIS3* were available in GenBank. Consequently, the Hungarian *Curvularia* isolates (cc_22 and cc_24) were reliably identified only to genus level.

In addition to the two *Curvularia* isolates, all the other isolates examined in this study were classified within the *Alternaria alternata* species complex, based on ITS sequence analysis (Figure 3). To achieve more precise species-level identifications within the complex, *TEF* sequence analysis was conducted. Six isolates (cc_1, cc_2, cc_4, cc_8, cc_9, and cc_10) formed a strongly supported clade (ML = 100%) with the type strain of *A. arborescens*, clearly separating them from other members of the *A. alternata* species complex (Figure 3). To further differentiate *A. alternata* s. str. from *A. tenuissima*, *HIS3* sequences were also analysed. However, none of the examined isolates showed sequence identity with *A. tenuissima*, indicating their affiliation with *A. alternata* s. str.

For species frequencies, 68% of the isolates were identified as *A. alternata*, 24% as *A. arborescens*, and 8% as *Curvularia* sp. The majority of *A. alternata* isolates were obtained from Hungary (77%), with smaller proportions from Spain (18%) and Kosovo (6%). In contrast,

A. arborescens isolates were all (100%) collected in Spain, and *Curvularia* sp. isolates were all (100%) collected in Hungary.

Pathogenicity assays

Leaf disc assays showed variations in host resistance to the pathogens, expressed by the disease index (DI) for the 73 different plant accessions and the ten fungal isolates (Figure 4). A one-way ANOVA showed differences in mean DI between the investigated groups of fungal species ($F(2,77.22) = 521.7$; $P < 0.0001$). According to Welch's ANOVA *post hoc* test, DI evaluated upon infection by *A. alternata*, *Curvularia* sp., and *A. arborescens* differed between all species ($P < 0.0001$). *Curvularia* sp. was the most aggressive with a meanDI of 63.69±26.0, followed by *A. arborescens* 50.75±26.04, and the least severe infection was caused by *A. alternata* 42.59±20.89 (Figure S2).

To focus on the pathogenicity of *Alternaria* and *Curvularia* species, and to draw conclusions about their aggressiveness to two reference cultivars with practical relevance, statistical analyses were narrowed to evaluate pathogenicity of *Alternaria* and *Curvularia* species using 'Calhoun Grey' and 'Black Diamond' as reference host cultivars. There was a statistically significant difference in mean DI between fungal species ($F(2,5.5) = 21.368$; $P < 0.01$). The Games- Howell *post hoc* test showed that *Curvularia* sp. (DI = 59.3 ± 6.4) and *A. arborescens* (DI 59.3 ± 26.1) were both more aggressive to watermelon than *A. alternata* (DI 33.3 ± 6.6) ($P < 0.05$). For 'Black Diamond', no statistically significant differences ($P > 0.05$) in mean DI were observed among the three fungal species. However, a consistent pattern was observed, with *Curvularia* sp. giving the greatest mean disease index (DI = 51.9, ± 12.8), followed by *A. arborescens* (DI = 46.9±16.4), and *A. alternata* (DI = 46.3 ± 16.7). Resistant plant accessions were identified with lower DI values than their commercial counterparts.

Based on the lowest recorded mean DI, which falls within the first quartile of the assessed plant accessions, the most resistant watermelon PIs were identified with a mean DI of 37.3 ± 2.9. These included accession PI512398 (from Spain), PI271775 (South Africa), PI512388 (Spain), PI167125 (Turkey), PI512346 (Spain), PI512350 (Spain), PI167059 (Turkey), PI482283 (Zimbabwe), PI512400 (Spain), PI175657 (Turkey), PI165002 (Turkey), PI167219 (Turkey), PI512401 (Spain), PI512383 (Spain), PI169292 (Turkey), PI169294 (Turkey), and accession PI512397 (from Spain).

No statistically significant differences ($P > 0.05$) in mean DIs were detected across the plant accessions orig-

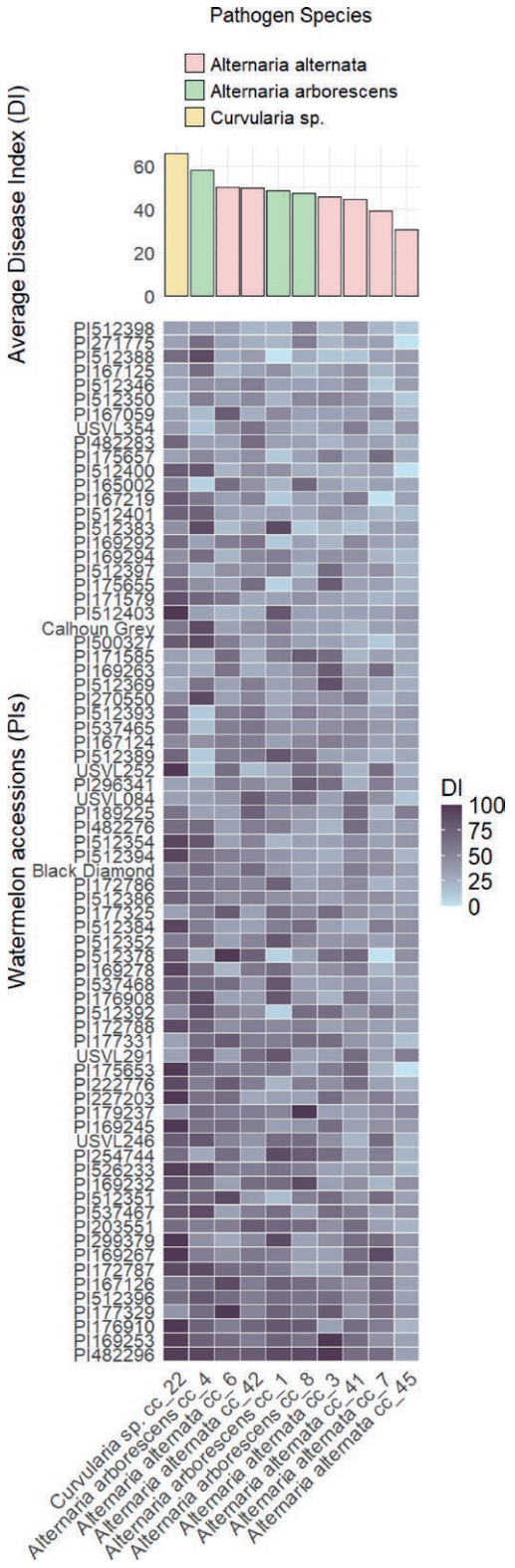

Figure 4. Mean Disease Indices (DIs) on leaf discs after inoculations of 73 watermelon accessions with *Curvularia* and *Alternaria* isolates (histogram), and heatmap of DI distribution across the watermelon accessions.

inating from different countries , indicating that country of origin did not affect resistance to leaf spots caused by *Alternaria* and *Curvularia* isolates among the studied watermelon collection.

DISCUSSION

Fungal leaf spot diseases pose increasing threats to cucurbit production (Abu-Nasser and Abu-Naser, 2018; Ma *et al*., 2021; Shanthi Avinash *et al*., 2021). Theses diseases cause premature losses of green leaf area through necroses and defoliation, as the infections progress, reducing photosynthetic rates, increasing fruit susceptibility to sunscald, and ultimately leading to important yield losses (Singh *et al.,* 2011; Kaniyassery *et al.,* 2024). Identifying the causal agents of these diseases is often challenging, as it requires distinction between closely related pathogen taxa that cause similar host symptoms and share similar morphological features (Rabaaoui *et al*., 2022; Torres-Garcia *et al*., 2022). Consequently, understanding the differences in pathogenicity among these fungi has been limited. The present study aimed to evaluate the morphology, genetic backgrounds, and pathogenicity of *Alternaria* and *Curvularia* species associated with leaf spot diseases in cucurbits collected from different geographical regions during 2024.

The most frequently isolated fungal leaf spot pathogen from infected leaves in the sampled locations was *A. alternata*, followed by *A. arborescens*, and *Curvularia* sp. The majority of *A. alternata* isolates were obtained from Hungary, while most *A. arborescens* isolates were collected from Spain, and *Curvularia* sp. isolates were exclusively from Hungary. Detection of *Curvularia* sp. in Hungary is notable since this is the first report of *Curvularia* sp. as a causal agent of fungal leaf spot on watermelon.

In the last decade, there have been few reports of *Curvularia* species affecting the cucurbits pointed gourd (Sarkar *et al*., 2018), kershaw (Ayvar-Serna *et al*., 2022), and melon (Vanitha *et al*., 2024). This limited number of reports suggests that the impacts of *Curvularia* on cucurbits may not be widespread, or may not have been consistently studied. In the present study, the observed symptoms of leaf spot diseases caused by *Curvularia* sp. on watermelon were similar to those described by Ayvar-Serna *et al*. (2022) and Sarkar *et al*. (2018), rather than blight symptoms reported by Vanitha *et al*. (2024).

The general morphological description of *Curvularia* sp. in the present study fits with other descriptions, for culture appearance and conidium size, septum number, and shape, and colony growth pattern (Ayoubi *et al*., 2017; Cui *et al*., 2020; Garganese *et al*., 2015). *Curvularia* species have been previously described considering only morphology, including conidium hila, septa, ontogeny, and wall structure, as well as culture colony appearance (Sivanesan, 1987). More recent studies have documented *Curvularia* spp. features including colony growth rates and conidium size and shape, which can depend on the culture conditions (dos Santos *et al*., 2018; Sun *et al*., 2003), which might lead to inaccurate pathogen identification. In the present study, species identification based on morphological characterization was challenging. To overcome these limitations multi-locus sequence analyses targeting ITS, *TEF1-α, GAPDH, RPB2, HIS3, SSU, LSU, endoPG,* OPA1-3, and OPA2-1 have been recommended (Dettman and Eggertson, 2021; Woudenberg *et al*., 2015; Aung *et al*., 2024). ITS, as the main barcoding marker for fungi, has been frequently employed for identifying *Curvularia* species (Ayoubi *et al*., 2017). However, recent studies have highlighted limitations in the use of ITS for species-level identification within *Pleosporales* (Bhunjun *et al*., 2020), suggesting the need for additional markers for more accurate delimitation of *Curvularia* species. Therefore, both *HIS3* and *TEF1-α* were included in the present study, based on previous research indicating their suitability to delineate *Curvularia* species (Manamgoda *et al*., 2011; 2012a; 2012b; 2015). However, in the present case, the two barcoding markers were not sufficient to determine the species identity of the isolates. BLAST results for ITS suggested *C. hawaiensis*, while *TEF1-α* suggested *C. spicifera* as the species, but with low support for both species. To confirm this identification or to describe our isolates as a new species, multilocus sequencing is recommended.

During sampling in Soroksár, Hungary, morphologically similar symptoms caused by *Curvularia* sp. and *Alternaria* spp. were observed co-occurring on the same leaves, raising the possibility that *Curvularia* may cause opportunistic infections. Recent studies have also reported the occurrence of *Curvularia* spp. along with other fungal pathogens suggesting that *Curvularia* spp. may be secondary pathogens infecting stressed plants, especially under environmental conditions of high temperatures and poor water quality (Katushova *et al*., 2021; Bessadat *et al*., 2023).

The present study fulfilled Koch's postulates for *Curvularia* as a pathogen of watermelon, using assays with 'Black Diamond' leaf disk, indicating pathogenicity. Furthermore, pathogenicity assessments were carried out for 73 watermelon accessions originating from 12 different countries. These assays showed statistically significant differences in DIs among the studied fungi, with *Curvularia* sp. having greatest pathogenicity, followed by *A. arborescens*, and *A. alternata* being the least pathogenic.

Several new host species has been recently reported for *Curvularia* spp. causing leaf blight and leaf spot diseases, many of which are important crops including sorghum (Akram *et al*., 2014), strawberry (Ayoubi *et al*., 2017), Chinese fir (Cui *et al*., 2020), maize (Garcia-Aroca *et al*., 2018; Manzar *et al*., 2024), citrus (Garganese *et al*., 2015), tomato (Huang *et al*., 2023), coffee (Nam *et al*., 2024), lettuce (Pornsuriya *et al*., 2018), rice (Majeed *et al*., 2015; Ren *et al*., 2022), vetiver (Sari *et al*., 2023), and dates (Rabaaoui *et al*., 2022). Previous reports have reported that within *Pleosporaceae,* the pathogenicity profiles and host ranges often depend on the production of host-specific toxins (HSTs) and non-host-specific toxins (nHSTs) (Akimitsu *et al*., 2014; Gao *et al*., 2014; Meena *et al*., 2017). *Alternaria* isolates produce several nHSTs, and low molecular weight HSTs that have host specificity. In contrast, *Curvularia* isolates have only been associated with nHSTs, which affect broader ranges of hosts (Meena *et al*., 2017; Rabaaoui *et al*., 2022). Understanding these differences will provide insights into the pathogenicity mechanisms and host-pathogen interactions of these fungi.

The present study results highlight the different host resistances within watermelon germplasm accessions, which could be utilized to select promising PI accessions, such as the Spanish PI512398, PI512350, and PI512388 which showed greater resistance than other accessions to *Alternaria* and *Curvularia*. The accession PI512398 has been used as a source of resistance to gummy stem blight (caused by *Stagonosporopsis cucurbitacearum*), so could be utilized as having multiple disease resistance (Gusmini *et al*., 2005; Rivera-Burgos *et al*., 2021). Additionally, during field sampling in the present study, PI512350 and PI512388 (both from Spain) were strongly tolerant to gummy stem blight under greenhouse conditions in Hungary (unpublished data, 2024). This suggests that some watermelon accessions classified as resistant in the present study may also possess resistance to a broader range of pathogens.

CONCLUSIONS

This study investigated morphology, phylogeny, and pathogenicity of *Alternaria* and *Curvularia* isolates associated with fungal leaf spots of cucurbits. *Alternaria alternata* occurred most frequently in the sampled locations, but was less aggressive in leaf disc assays with a diverse watermelon collection than the other fungi assessed. In contrast, only a relatively low number of *Curvularia* sp. isolates were detected, but the assessed isolate was the most aggressive in the leaf disc assays. This study is the first report of *Curvularia* sp. as a causal agent of fungal leaf spot disease on watermelon. This highlights the importance of considering species diversity and pathogenicity when managing leaf spot diseases of cucurbits. The present study also identified promising watermelon germplasm accessions among the plant material studied, that had resistance against *Alternaria* and *Curvularia* pathogens. Therefore, future research should focus on screening the watermelon germplasm collection under controlled and field conditions, and to further select resistant plant accessions. This knowledge will aid development of sustainable management strategies aimed at protecting cucurbit production from these emerging pathogens.

ACKNOWLEDGMENTS

Dr Maxim Byatets and Jeszenszky Szilvia, of the Watermelon Breeding Team, Syngenta Research Station, Ócsa, Hungary, donated samples and plant material, and strongly supported this research. Their expertise and guidance were instrumental in completion of this study.

AUTHOR CONTRIBUTIONS

C.P.; writing (original draft), visualization, validation, software, methodology, investigation, formal analysis, data curation, and conceptualization; V.P.; software, investigation, and formal analysis; V.B.; methodology, investigation; G.B.; methodology, resources, D.P.; supervision, writing, visualization, validation, software, methodology, investigation, formal analysis, data curation, conceptualization, resources, project administration, and funding acquisition. All the authors have read and agreed to the published version of the manuscript.

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