horticulturae

MDPI

Article

Diversity of *Colletotrichum* spp. on *Aristolochia grandiflora*: A Case Study in an Italian Botanical Garden

Federico Brugneti [1], Silvia Turco [1,*], Luca Rossini [2], Diana Martignoni [3] and Angelo Mazzaglia [1,*]

[1] Dipartimento di Scienze Agrarie e Forestali, Università degli Studi della Tuscia, 01100 Viterbo, Italy; federico.brugneti@unitus.it
[2] Service d'Automatique et d'Analyse des Systèmes, Université Libre de Bruxelles, 1050 Brussels, Belgium; luca.rossini@ulb.be
[3] Orto Botanico "Angelo Rambelli", Università degli Studi della Tuscia, 01100 Viterbo, Italy; artemis@unitus.it
* Correspondence: silvia.turco@unitus.it (S.T.); angmazza@unitus.it (A.M.)

Abstract: During a survey conducted in autumn 2022 and spring 2023, extensive leaf spots were observed on *Aristolochia grandiflora* plants in the Botanical Garden "Angelo Rambelli" (Viterbo). To preserve the botanical garden collection and avoid disease spread, morphological and molecular identification of the causal agent were carried out. The results revealed three distinct *Colletotrichum* species belonging to the *Colletotrichum boninense* and *Colletotrichum orchidearum* species complexes, which have never been reported together within the same host and, for two of them, never in Italy. These findings may contribute to further extend the state of the art on leaf anthracnose, as well as provide new insights and molecular data for further phylogenetic studies.

Keywords: tropical plant pathology; pathogen identification; phylogenetics

check for updates

Citation: Brugneti, F.; Turco, S.; Rossini, L.; Martignoni, D.; Mazzaglia, A. Diversity of *Colletotrichum* spp. on *Aristolochia grandiflora*: A Case Study in an Italian Botanical Garden. *Horticulturae* **2024**, *10*, 1215. https://doi.org/10.3390/horticulturae10111215

Academic Editors: Manuel Anguita Maeso, Zhi Li and Min Gao

Received: 9 October 2024
Revised: 8 November 2024
Accepted: 10 November 2024
Published: 17 November 2024

1. Introduction

Aristolochia (Kingdom: Plantae; Phylum: *Tracheophyta*; Class: *Magnoliopsida*; Order: *Piperales*; Family: *Aristolochiaceae*; Genus: *Aristolochia* L.) is a genus of flowering plants in the Aristolochiaceae family, encompassing around 400 species of herbaceous perennials, undershrubs, or shrubs, many of which are rich in essential oils. These plants are widely distributed across tropical regions of Asia, Africa, and South America, where they thrive in a variety of ecosystems, from tropical rainforests and deciduous forests to more arid environments [1]. Renowned for their distinctive flower shapes, resembling pipes, Dutch shoes, or pelicans, *Aristolochia* plants have a long history of medicinal use. These plants have been long used to treat snake bites, fevers, and digestive issues [2]. However, several species contain aristolochic acids, compounds known for their toxic and carcinogenic effects, and, for these reasons, their use in herbal medicine has significantly declined in modern times, leaving more room for their ornamental function [3]. *Aristolochia grandiflora* stands out for its ornamental value, featuring enormous, velvety flowers that can be up to 30 cm in length and that are often mottled or striped in hues of white, purple, and green. Because of their ornamental value and aesthetic appeal, these plants are highly appreciated and requested by botanical gardens worldwide. These institutions not only showcase the unique beauty of species such as *A. grandiflora*, but they also play a pivotal role in biodiversity preservation [4,5]. Accordingly, frequent monitoring to ascertain the presence of symptoms becomes a crucial aspect of species preservation. Introducing plant species from diverse global regions into a controlled environment significantly raises the risk of non-native phytopathogen spread, and this plant congregation also facilitates the spread of pathogenic agents (or insect pests) to new host plants, such as Aristolochia, that may not be present in their native ranges. Early detection of threats through frequent screening of potential symptoms is, therefore, essential to prevent the spread of infections and ensure the health of native and non-native plants. Thus, regular inspections, proper sanitation,

and careful isolation of infected plants can help mitigate the risk of disease outbreaks in botanical gardens [5].

This frequent screening is also applied at the Botanical Garden "Angelo Rambelli", Tuscia University in Viterbo (Lazio, Italy). During regular inspections of the ornamental plants preserved, severe anthracnose-like symptoms were observed on *A. grandiflora* leaves.

Anthracnose is caused by fungal pathogens belonging to different groups, including the genus *Colletotrichum*. Different studies carried out over the years showed the harmfulness of many *Colletotrichum* species on annual crops of economic relevance, forest, and ornamental plants worldwide [6–10]. Damage can be observed during both pre- and post-harvest stages, and it is concentrated mainly on leaves, stem tubers, and seedlings [10–13].

Aristolochia plants inspected at the botanical garden showed symptoms extremely close to anthracnose caused by *Colletotrichum* species. These clues were the main inspiration for this study, which aimed to identify the causal agent of the observed symptoms to adopt precise control strategies and to prevent spread of the disease.

2. Materials and Methods

2.1. Sampling and Fungal Isolation

During two seasonal surveys conducted in autumn 2022 and spring 2023, anthracnose-like symptoms were observed in 70% of *A. grandiflora* leaves, whose plants are located in the tropical greenhouse of the Botanical Garden "Angelo Rambelli" of Tuscia University in Viterbo, Italy (Figure 1). Leaves were collected with sterile scissors, sealed in plastic bags, and brought to the laboratory for downstream processing.

Figure 1. *Aristolochia grandiflora* leaves showing typical anthracnose symptoms.

To identify the symptom's causal agent through fungal isolation, ten symptomatic leaves were rinsed with distilled water, sterilized with 70% ethanol for 30 s, rinsed twice with sterile water, and dried under a laminar flow. Leaf pieces of 3–4 mm were excised from the transition zone between diseased and healthy tissue, placed on Potato Dextrose Agar plates (PDA), and incubated at 25 °C. After 7 days of incubation, the obtained fungal colonies were further purified and classified in three different morphotypes according to their morphological traits. Among a total of twenty, eight representative isolates were selected and maintained in pure culture for further analysis.

2.2. DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from 100 mg of fresh mycelium from each of the twenty obtained isolates using the NucleoSpin® Plant II Midi DNA extraction kit (Macherey-Nagel, Dueren, Germany), following the manufacturer's instructions, and stored at −20 °C.

Molecular characterization was initially carried out by PCR amplification and Sanger sequencing of the ribosomal internal transcribed spacer (*ITS*). Then, eight isolates were selected and five additional genes were amplified and sequenced for a more detailed phylogenetic analysis: β-tubulin (*TUB2*), actin (*ACT*), partial chitin synthase (*CHS-1*), histone 3 (*HIS3*), and a 200 bp intron of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) regions. The sequence of the primers used and the size of the amplicons are reported in Table S1. Forty nanograms of template DNA were incubated with 1x GoTaq Green MasterMix (Promega Corporation, Madison, WI, USA) and 0.5 µM of both forward and reverse primers in a final volume of 25 µL. PCR reactions were carried out according to [14]. The thermal cycle consisted of an initial step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C for the *ITS* regions using the ITS1-ITS4 primers, 30 s at 51 °C for the *TUB2* using primers T1 and BT2b, 30 s at 54 °C for the actin gene with ACT-512F and ACT-738R primers, 30 s at 58 °C for the *CHS* gene using CHS-79F and CHS-354R primers, 30 s at 57 °C for the *HIS3* gene using CYLH-3F and CYLH-3R primers, followed by 30 s of elongation at 72 °C and a final extension at 72 °C for 5 min. An aliquot of the amplified products was visualized on a 1.2% agarose gel and the rest sent to Eurofins Genomics (Konstanz, Germany) for Sanger sequencing.

2.3. Identification and Multi-Locus Phylogenetic Analysis

The electropherograms obtained from Sanger sequencing were visually inspected with FinchTV v.1.4 (available at https://digitalworldbiology.com/FinchTV, accessed on 4 October 2023). A BLASTn search with the obtained *ITS* sequences suggested that the isolates belonged to *C. boninense* and *C. orchidearum* species complexes. *ITS* sequences of *Colletotrichum* isolates belonging to the same species complexes according to [10,12,13] and whose sequences were available on the GenBank database (Table S2) were used for phylogenetic analysis. Three strains of *C. gloeosporioides* (CBS 112999, Col-41, and Col-69) were included as outgroups. The sequences were adjusted using UGENE v48.1 [15], concatenated, and further aligned with MUSCLE v3.8.31 (Edgar 2004). The alignment file was then used as input to build a maximum likelihood (ML) phylogenetic tree using RAxML-HPC v8.2.12 [16], and set with a GTRCATI algorithm as a substitution model and 1000 bootstraps. The trees were visualized using FigTree v1.4.4 (available at http://tree.bio.ed.ac.uk/software/figtree/, accessed on 4 October 2024) and further edited with Inkscape v0.92 (available at https://inkscape.org, accessed on 4 October 2024).

Based on these results, a phylogenetic analysis was carried out using the six selected loci of eight representative isolates. Two separated phylogenetic trees were built as described previously, one for the *C. orchidearum* species complex and one with the isolates belonging to the *C. boninense* species complex.

2.4. Morphological Characterization and Growth Rates

2.4.1. Morphological Characterization

The eight selected isolates were maintained in pure culture by transferring single hyphae to plates containing various substrates: standard PDA and two media specific for morphological characterization, Synthetic Nutrient-poor Agar medium (SNA, [17]), and Oatmeal Agar (OA, [18]). These plates were then incubated at 25 °C and 100% humidity in the dark. After ten days, the following morphological characteristics were observed: colony shape and color, conidiomata, conidial shape and size, hyphae, and appressoria. The mean lengths and widths of 30 randomly selected conidia from each isolate were measured using 40× magnification in a microscope (Leitz, Wetzlar, Germany).

2.4.2. Mycelium Growth and Data Analysis

Four millimeters diameter plugs (∅) of each *Colletotrichum* isolate, grown on PDA at 25 °C for one week, were transferred to the center of four new 9 cm diameter (∅) per each PDA, SNA, and OA media plates. After seven days at 25 °C and a relative humidity of 100% RH, the fungus did not reach the border of the dishes, and the mycelium radial growth rate was measured with a ruler (±1 mm) in all four directions from the center of the plug, according to the protocols of Drais et al. [19] and Brugneti et al. [6].

The collected data were imported within the R environment (v.4.2.3), where the isolate and the substrate were considered as factors, while the plate and the orthogonal directions of the measured radii were considered as a random variable. Three different analyses were carried out: the first one to identify differences between the three *Collettothricum* species regardless of the media, the second one to check the differences among media regardless of the species, and the third one to analyze the mycelium growth differences among the different species on the three different media. Before each analysis, the normality of the dataset was checked through a Shapiro–Wilk test using the *shapiro.test()* function and through a visual inspection of the Quantile–Quantile (Q–Q) plot using the *qqmath()* function within the *lattice* R package. In case the dataset was not normally distributed, as for the first and second analyses, the best expression to transform the dataset was chosen using the *bestNormalize()* function within the *bestNormalize* R package.

After transformation, for the first and second analyses, the dataset was analyzed through a Linear Model (LM), considering the radii as numeric values, the species and the substrate as independent variables, and the plate and the orthogonal direction (N-W-S-E) as random variables. For the third analysis, instead, the measured radii were considered as numerical values, the substrate as a factor, and the plate and the orthogonal direction as a random effect. Calculations were carried out using the *lmer()* function within the *lme4* R package. The linear model was followed by a Bonferroni *post hoc* test (=0.05), carried out through the *emmeans()* function within the R package *emmeans*, the *pairs()* function within the R package *multcompView*, and the *cld()* function within the R package *multcomp*.

The script and the dataset to fully reproduce the results of the present work are publicly available at https://github.com/lucaros1190/Colletotrichum-VTBGarden (accessed on 3 August 2024).

2.5. Pathogenicity Tests

Pathogenicity tests were conducted following the method described by Moral et al. [20] on healthy, lesion-free leaves collected from healthy plants. For the inoculum, a conidial suspension (10^5 conidia/mL) of each isolate was prepared in sterile distilled water. Detached asymptomatic *A. grandiflora* leaves were surface sterilized by immersion in 3% NaClO for 2 min, washed twice with sterile-distilled water, and air dried on sterile filter paper under a laminar flow hood. Ten leaves for each isolate were then inoculated with 15 µL of conidial suspension at four different sites on the leaf surface. Control leaves were inoculated with sterile distilled water using the same method. The inoculated leaves were covered with a plastic box to maintain high relative humidity, and incubated in the dark at 25 °C.

An additional pathogenicity test was conducted by inoculating a mixture of the three *Colletotrichum* species together. One isolate for each *Colletotrichum* species was chosen to prepare a pooled conidia suspension (10^5 conidia/mL), following the method used by Garcia-Lopez (2023) [21], and inoculation was carried out as described previously. Symptoms were observed 7 days after inoculation, and to fulfill Koch's postulates, the three *Colletotrichum* pathogens were re-isolated from symptomatic leaves, and their identity was confirmed both morphologically and molecularly.

3. Results

3.1. Fungal Isolates Identification

A total of twenty isolates were obtained from ten symptomatic leaves of *A. grandiflora* and, based on their morphological traits, recognized as *Colletotrichum* spp. The BLASTn

results of the twenty *ITS* sequences on the GenBank database indicated that the isolates belonged to two specific complexes. The isolates C2, C3, C4, C5, C6, C9, C12, C13, C14, C16, and C19 belonged to the Orchidearum species complex and were grouped in the *C. cattleyicola* cluster (Figure S1), while the isolates C1, C7, C8, C10, C11, C15, C17, C18, and C20 clustered within the Boninense species complex, and the isolates clustered in the *C. karstii* and *C. boninense* group (Figure S2). Among those, eight representative isolates were chosen for phylogenetic analysis and for a detailed morphological characterization based on colony shape, color, growth rate, and conidial morphology, and for phylogenetic analysis.

Colletotrichum karstii Y.L. Yang, Zuo Y. Liu, K.D. Hyde and L. Cai, Cryptogamie Mycologie 32: 241. 2011 [22] Figure 2: The C1 isolate on PDA produced gray and pale-orange aerial mycelium and appeared orange/reddish from the bottom, with orange conidial masses submerged in the mycelium. Conidiogenous cells were cylindrical, hyaline, straight with rounded ends, with contents appearing granular, and 12–18 × 5.5–7.5 µm in size. Ascospores were allantois and pear-shaped, inequilateral, and often straight on the inner side, had rounded apices, tapered towards base, and were 12–19 × 3.5–7 µm. Colonies on OA were flat with an entire margin, buff to rosy buff to pale salmon, covered with orange to off-white conidiomata, lacking aerial mycelium, reverse buff, and orange to rosy. Colonies on SNA were flat with an entire margin and hyaline.

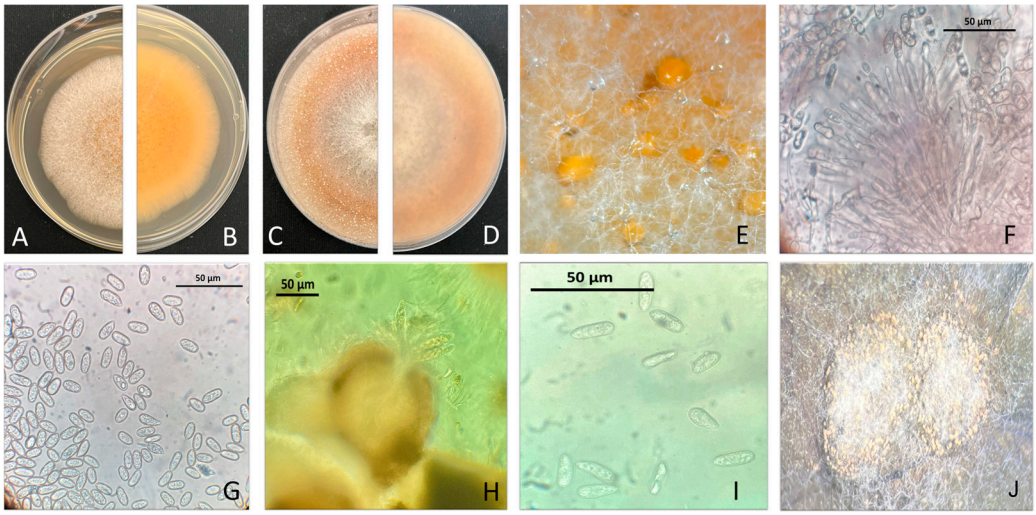

Figure 2. *Colletotrichum karstii* C1 isolate. (**A**,**B**) Front and back view, respectively, of 10 d old PDA culture; (**C**,**D**) front and back view, respectively, of 10 d old OA culture; (**E**) conidiomata on PDA medium; (**F**) conidiophores on PDA medium; (**G**) conidia on PDA medium; (**H**) ascomata with asci on PDA medium; (**I**) ascospores on PDA medium; and (**J**) conidiomata on *Aristolochia* leaf.

Colletotrichum cattleyicola Damm and Toy Sato, MycoBank MB824220 [12], Figure 3: All five isolates (C2, C3, C4, C5, and C19) formed gray-white aerial mycelium and appeared gray to yellow from the bottom on PDA. Conidiophores were cylindrical and hyaline. Conidia were in yellowish white masses, 14–21 × 3.5–7 µm, one-celled, smooth-walled, hyaline, cylindrical, straight, and rounded at the ends. Sexual morphs were not observed. Colonies on OA were flat with an entire margin, covered with whitish-gray aerial mycelium, with the same colors on the reverse, and abundant black conidiomata on the surface of the medium. Conidial appeared in whitish masses. Colonies on SNA were flat with entire margins, and had agar medium partially covered by hyaline, whitish slightly floccose mycelium, and the same colors on the bottom.

Colletotrichum boninense Moriwaki, Toy, Sato and Tsukib., Mycoscience 44(1): 48. 2003 [23], Figure 4: Two isolates (C7 and C10) showed an aerial mycelium that was felty to light gray, abundantly slimy, and had orange conidial masses, with the same colors on the bottom of the PDA medium. Conidia were 14–19 × 3.5–6.5 µm, hyaline, smooth-walled, aseptate, straight, and cylindrical, with a round apex and round base with two big polar

guttules. Ascomata (perithecia) were observed after 4 weeks of culture on PDA, varying from subglobose to pyriform, and were glabrous and medium brown. Asci were cylindrical-clavate, 40–58 × 11–17 µm, eight-spored, and with a rounded or slightly tapered, slightly curved apex. Colonies on OA were flat with an entire margin; the surface was covered with felty white, rosy buff, or very pale orange aerial mycelium; the center aerial mycelium was white to gray; they were reverse, light brown or pale orange. Colonies on SNA were flat with a slightly undulate margin, and were hyaline with a felty white aerial mycelium on filter paper.

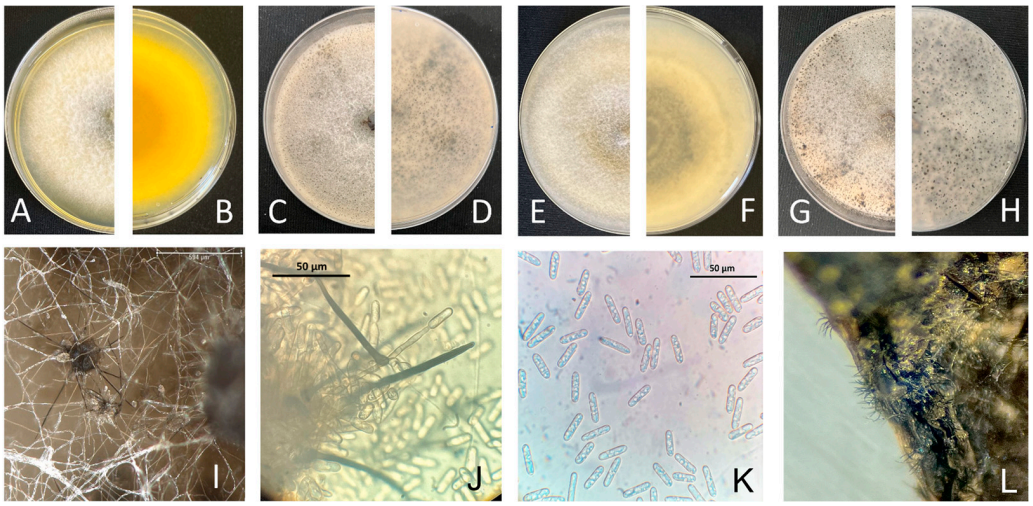

Figure 3. *Colletotrichum cattleyicola* C2 and C3 isolates as representatives of the species. (**A**,**B**) Front and back view, respectively, of 10 d old PDA culture of isolate C2; (**C**,**D**) front and back view, respectively, of 10 d old PDA culture of isolate C3; (**E**,**F**) front and back view, respectively, of 10 d old OA culture of isolate C2; (**G**,**H**) front and back view, respectively, of 10 d old OA culture of isolate C3. (**I**) Conidiomata on SNA medium; (**J**) conidiophores and setae on SNA medium; (**K**) conidia on SNA medium; and (**L**) conidiomata on *Aristolochia* leaf.

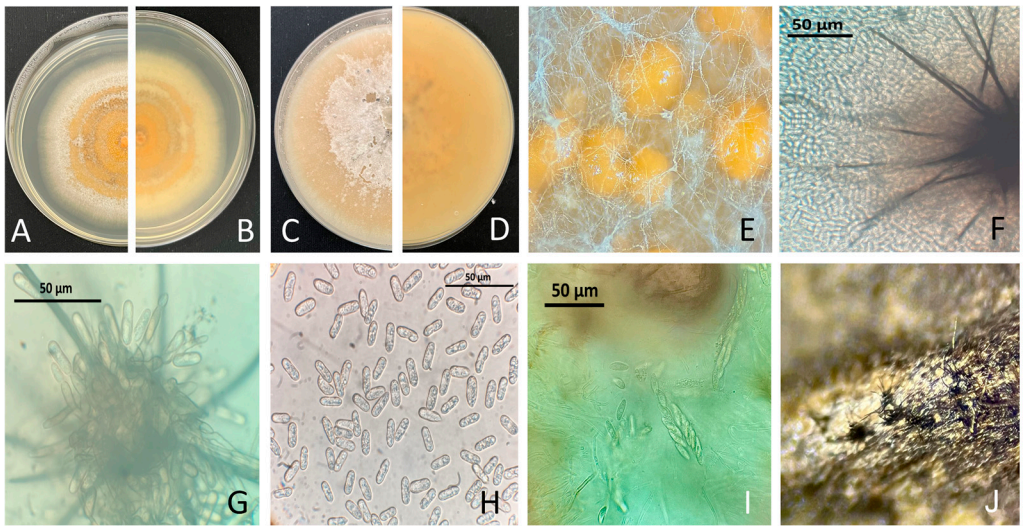

Figure 4. *Colletotrichum boninense*. (**A**,**B**) Front and back view, respectively, of 10 d old PDA culture of isolate C10; (**C**,**D**) front and back view, respectively, of 10 d old OA culture of isolate C10; (**E**,**F**) conidiomata on SNA medium; (**G**) conidiophore on SNA medium; (**H**) conidia on PDA medium; (**I**) asci with ascospores on OA medium; and (**J**) conidiomata on *Aristolochia* leaf.

3.2. Phylogenetic Analysis

For a deeper taxonomic classification, five additional genes (*GAPDH*, *CHS-1*, *HIS3*, *ACT*, and *TUB2*) were amplified, Sanger sequenced, and all the related sequences deposited on the NCBI Genbank database (Table S3). The sequences were concatenated and aligned to other *Colletotrichum* isolates belonging to the *C. boninense* species complex or the *C. orchidearum* species complex, respectively. In both ML phylogenetic trees (Figures 5 and 6), each isolate clustered within its own expected group: the C1 isolate among *C. karstii*; C7 and C10 isolates among *C. boninense*; and C2, C3, C4, C5, and C19 among *C. cattleyicola*. Both trees were rooted on the *C. gloeosporioides* species complex. The robustness of each clustering is further supported by the high bootstrap values indicated on the trees.

3.3. Mycelium Growth over Species and Substrates

Overall, the species showed a different mycelium growth rate, independent from the substrate: the isolate *C. boninense* was slower, while *C. cattleyicola* was the fastest (Figure S3). All species were statistically different from each other according to the LM analysis, all with a $p < 0.0001$. Statistical differences were assessed on the substrates as well (Figure S4), with no distinction among the species: mycelium extension was significantly different between the PDA and OA substrates (LM, df = 376, t = 6.319, $p < 0.0001$) and the SNA and OA substrates (LM, df = 376, t = 4.749, $p < 0.0001$). No statistical differences were observed between PDA and SNA substrates (LM, df = 376, t = −1.570, $p = 0.3516$).

An in-depth analysis of the dataset provided interesting results on the different responses of the species to the substrates (Figure 7). The highest and lowest mycelium extensions of *C. boninense* were observed on the OA and PDA substrates, respectively. Overall, all growth was statistically different in all the substrates (LM, df = 89: OA-PDA, t = 7.685, $p < 0.0001$; OA-SNA, t = 4.229, $p = 0.0002$; PDA-SNA, t = −3.456, $p = 0.0025$). The highest and lowest mycelium extension rates of *C. karstii*, instead, were observed on the OA and SNA substrates, respectively. Statistical differences in growth were assessed between the OA and PDA substrates (LM, df = 45, t = 6.070, and $p < 0.0001$), and the OA and SNA substrates (LM, df = 45, t = 6.193, and $p < 0.0001$). No differences in growth on the substrates PDA and SNA were observed (LM, df = 45, t = 0.123, and $p = 1$). A similar scenario was observed for *C. cattleyicola*: statistical differences in mycelium growth were assessed between the OA and PDA substrates (LM, df = 230, t = 5.841, and $p < 0.0001$) and the OA and SNA substrates (LM, df = 230, t = 5.817, and $p < 0.0001$), while there were no differences between the PDA and SNA substrates (LM, df = 230, t = −0.024, and $p = 1$). *C. cattleyicola* grew faster in the OA and slower in the PDA substrate.

3.4. Pathogenicity Tests

Pathogenicity tests were carried out on ten healthy *A. grandiflora* leaves for each of the eight *Colletotrichum* isolates. Seven days post-inoculation, all leaves inoculated with one of the *Colletotrichum* isolates showed necrotic spots, more or less extended depending on the isolate and the species (Figure 8). Abundant acervuli formation was observed on the necrotic tissue (Figures 2J, 3L, and 4J), while control leaves remained healthy (Figure 8). The same results and same symptoms were obtained from the pathogenicity test carried out with the pooled inoculation (Figure S5). Nonetheless, the morphology of the fungal colonies re-isolated from the symptomatic leaves was close to the original isolates used for inoculation, satisfying Koch's postulates.

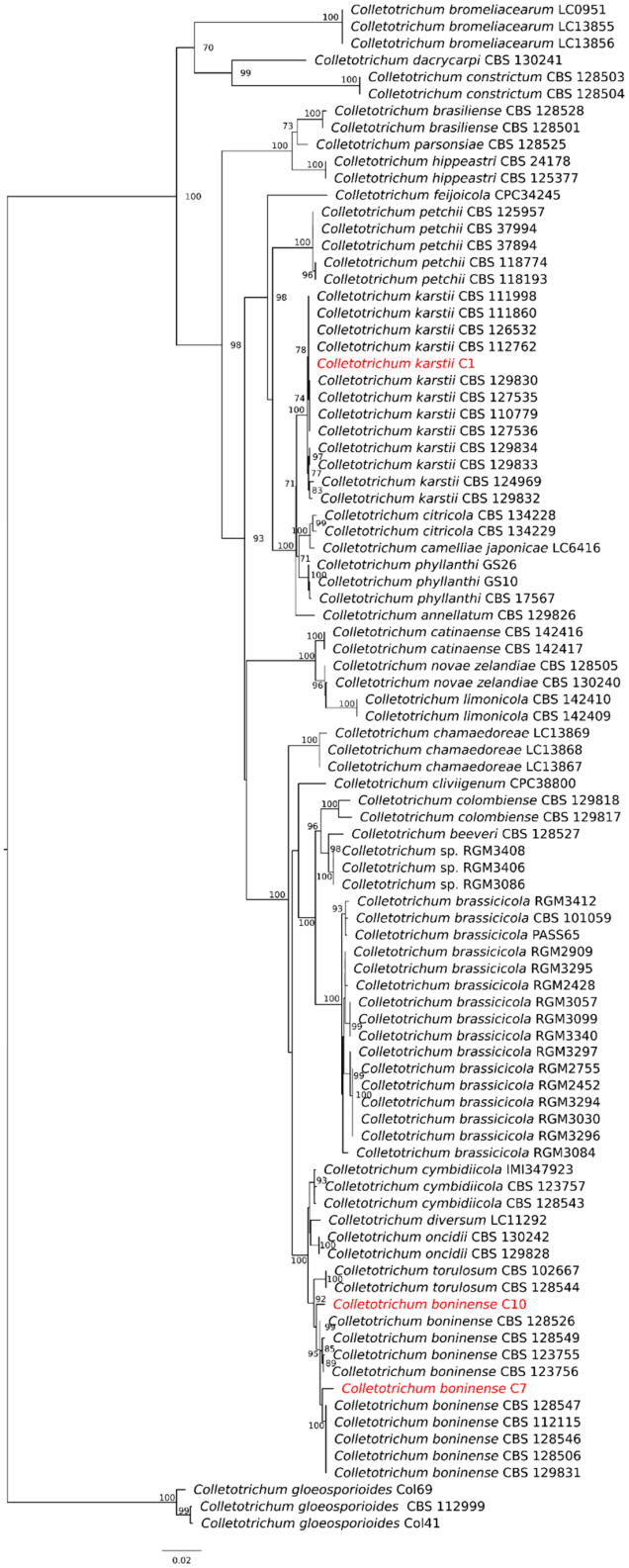

Figure 5. Maximum likelihood phylogenetic tree of the concatenated sequences of six housekeeping genes among isolates of the *Colletotrichum boninense* species complex. The tree was rooted on the *Colletotrichum gloeosporioides* species complex. Bootstrap values indicating the robustness of the clustering are reported as node values. The isolates indicated in red are the ones identified and characterized in this study.

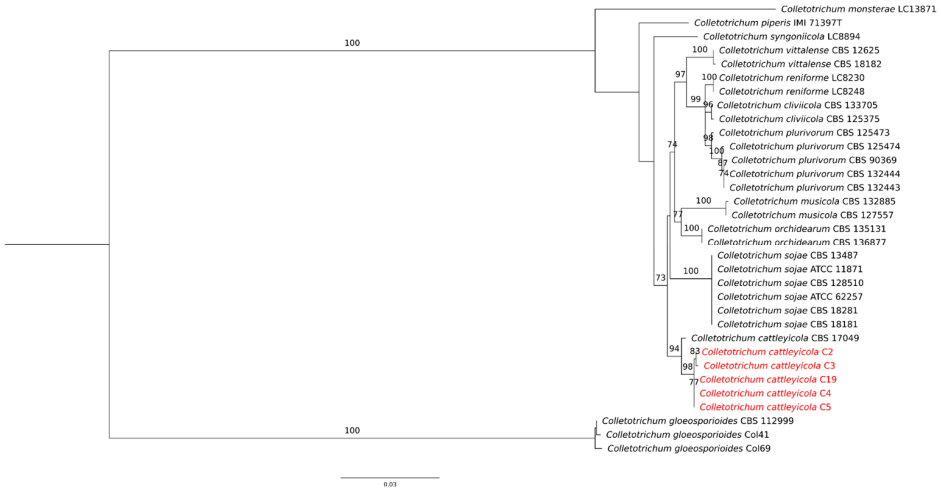

Figure 6. Maximum likelihood phylogenetic tree of the concatenated sequences of six housekeeping genes among isolates belonging to the *Colletotrichum orchidearum* species complex. The tree was rooted on the *Colletotrichum gloeosporioides* species complex. Bootstrap values indicating the robustness of the clustering are reported as node values. The isolates indicated in red are the ones identified and characterized in this study.

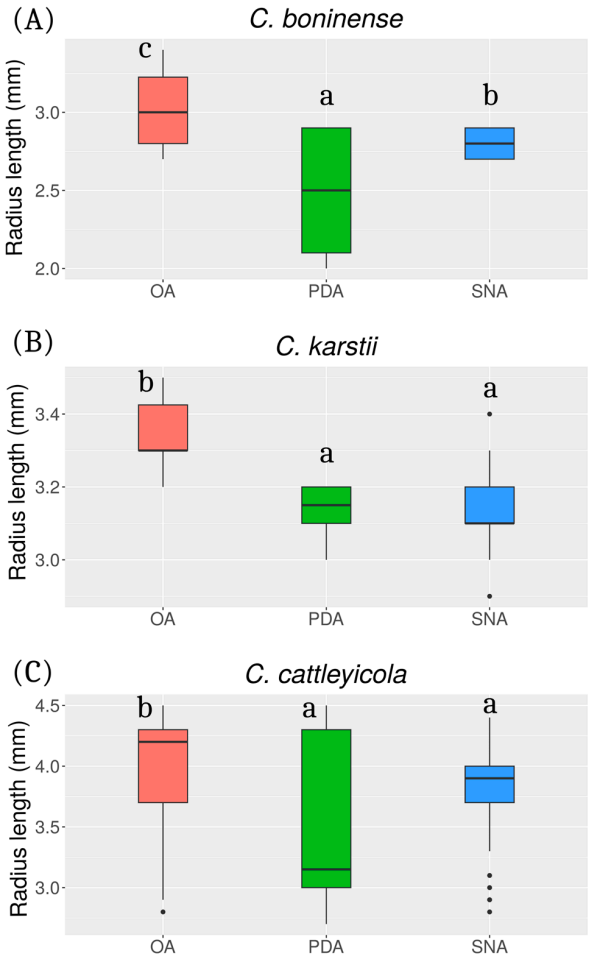

Figure 7. Mycelium growth rate of the different species at 25 °C on different substrates: (**A**) *Colletotrichum boninense*, (**B**) *Colletotrichum karstii*, and (**C**) *Colletotrichum cattleyicola*. Different letters mean statistical differences assessed by a linear model with random effects (dish and orthogonal direction) followed by a Bonferroni post hoc test = 0.05.

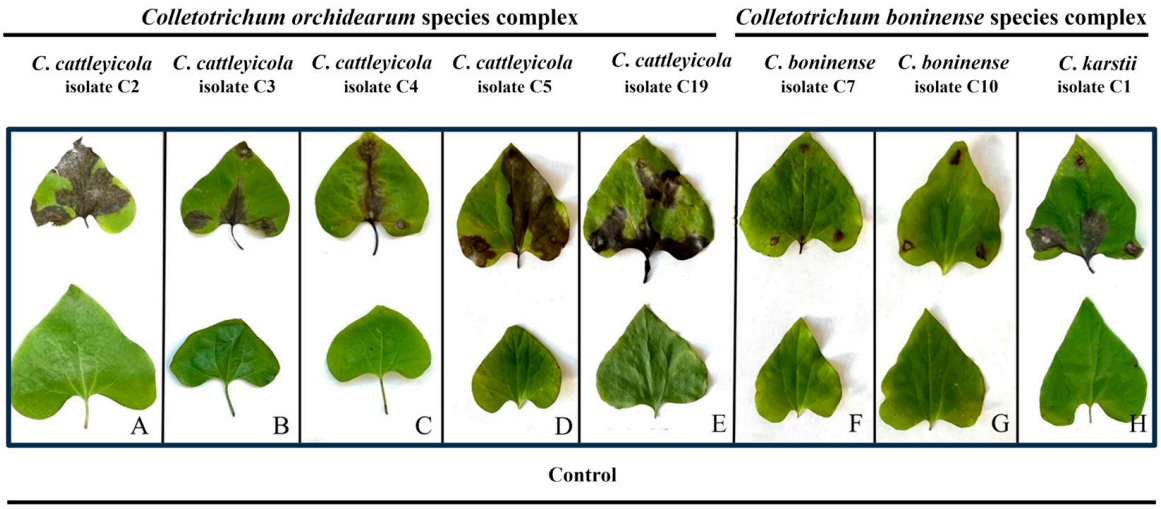

Figure 8. Necrotic spots on *Aristolochia grandiflora* leaves after inoculation with a spore suspension of isolates and incubation at 25 °C for seven days; leaves inoculated with spore suspension (**above**) and control (**below**). Labels A–H refer to the different isolates inoculated for the pathogenicity test and indicated on top of the figure.

4. Discussion

The *Colletotrichum* genus is among the top ten fungal genera encompassing 340 currently recognized species, grouped in 20 different species complexes, and able to infect 3400 different host species. In this study, we report the coexistence of three *Colletotrichum* species (*C. karstii*, *C. boninense*, and *C. cattleyicola*) on a single host plant, the ornamental tropical plant *Aristolochia grandiflora*. These isolates belong to two distinct *Colletotrichum* species complexes, the *boninense* species complex (*C. karstii* and *C. boninense* isolates) and *orchidearum* species complex (*C. cattleyicola*), respectively. The simultaneous coexistence of different *Colletotrichum* species within the same host is common, and usually there is one species that is predominant over the others [9,20].

From a morphological point of view, the identified species showed a high similarity with the ex-type isolates described in previous studies [12]. However, relying solely on morphological traits or on molecular identification through *ITS* region is not sufficient for a proper identification, especially for complex fungal genera such as *Colletotrichum* or *Fusarium* [24–26]. Conversely, a multi-locus phylogenetic analysis based on a combination of six loci (*ITS*, *GAPDH*, *CHS-1*, *HIS3*, *ACT*, and *TUB2*) provided a more reliable classification of the isolated species, showing that the isolates formed three well-supported clades with known species.

C. cattleyicola has been previously reported only on *Cattleya* spp. [12]. The only sequences available on the GenBank database refer to one isolate from Belgium from *Cattleya* sp. roots (CBS 170.49), and one isolate from *Cattleya* sp. from Japan, Mie Prefecture (MAFF 238321). Thus, our study represents the first report of this species in Italy and on *Aristolochia* plants, expanding the host range of this still poorly known species.

Conversely, species belonging to the *C. boninense* complex, *C. karstii*, and *C. boninense*, are known to infect a large range of hosts from numerous different plant families [9,11]. Although *C. karstii* have been repeatedly reported in several species in Italy [9,27–29], to date, there are no reports of *C. boninense* s.s. in any hosts.

The genus *Colletotrichum* has been reported as an endophyte of *Aristolochia* by Zhi-jun et al. [30] and in Brazil in 2017 (*C. gloeosporioides*, GenBank accession numbers MF076612–MF076615, unpublished data) but without causing any visible disease symptoms, while Tekade and Mohod [31] observed leaf blight caused by *C. dematium* on *A. bracteata* leaves.

5. Conclusions

To the best of our knowledge, this study represents the first report of *C. karstii*, *C. boninense*, and *C. cattleyicola* together within the same host, *A. grandiflora*, as well as the first report of *C. boninense* and *C. cattleyicola* in Italy. Our findings underline even more the importance of botanical gardens not only for plant species preservation but also to better understand the harmfulness of autochthonous and alien pathogens and pests on different host species. As shown by this study, frequent screening of plants can be extremely helpful to gather novel information that can be further helpful for plant nurseries and ornamental plant sellers.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10111215/s1: Table S1: Primers sequences used in this study and amplicon lengths; Table S2: Strains used for the phylogenetic analyses of *Colletotrichum* spp. with details about host, location, and GenBank accession numbers; Table S3: List of selected *Colletotrichum* isolates and GenBank accession numbers; Figure S1: Maximum likelihood phylogenetic tree on the *ITS* sequences among isolates belonging to the *Colletotrichum orchidearum* species complex. The tree was rooted on the *Colletotrichum gloeosporioides* species complex. Bootstrap values indicating the robustness of the clustering are reported as node values; Figure S2: Maximum likelihood phylogenetic tree of the *ITS* sequences among isolates of the *Colletotrichum boninense* species complex. The tree was rooted on the *Colletotrichum gloeosporioides* species complex. Bootstrap values indicating the robustness of the clustering are reported as node values. Figure S3: Mycelium growth rates of the eight isolates at 25 °C among the species. Different letters mean statistical differences assessed by a linear model with random effects (dish and orthogonal direction) followed by a Bonferroni post hoc test $\alpha = 0.05$; Figure S4: Mycelium growth rates of the eight isolates at 25 °C on different substrates, regardless of species distinction. Different letters mean statistical differences assessed by a linear model with random effects (dish and orthogonal direction) followed by a Bonferroni post hoc test $\alpha = 0.05$; Figure S5: Symptoms on leaves of *Aristolochiagrandiflora* seven days (25 °C) after inoculation with a pooled spore suspension of isolates C3, C1, and C10 (above, from left to right) and control (below).

Author Contributions: Conceptualization, F.B., S.T., L.R., D.M. and A.M.; methodology, F.B., S.T. and L.R.; software, S.T. and L.R.; validation, F.B. and S.T.; formal analysis, F.B., S.T. and L.R.; investigation, F.B. and D.M.; resources, D.M. and A.M.; data curation, S.T. and L.R.; writing—original draft preparation, F.B., S.T. and L.R.; writing—review and editing, F.B., S.T., L.R., D.M. and A.M.; visualization, S.T., F.B. and L.R.; supervision, S.T., L.R., D.M. and A.M. All authors have read and agreed to the published version of the manuscript.

Funding: The research was carried out within the framework of the Ministry for University and Research (MUR) initiative "Department of Excellence" (Law 232/2016) DAFNE Project 2023-27 "Digital, Intelligent, Green and Sustainable" (acronym: D.I.Ver.So). L.R. is funded by the European Commission under the Marie Sklodowska Curie Actions Postdoctoral Fellowship (MSCA-PF-2022) project "PestFinder" Grant n. 101102281.

Data Availability Statement: The script and the dataset to fully reproduce the results of the present work are publicly available at https://github.com/lucaros1190/Colletotrichum-VTBGarden (accessed on 3 August 2024). The nucleotide sequences related to this work have been deposited and are available on the NCBI GenBank database under the Accession Numbers: OR607089-OR607096 (*ITS*); OR636336-OR636343 (*TUB2*); OR636328-OR636335 (*ACT*); OR636344-OR636351 (*CHS-1*); OR636312-OR636319 (*HIS3*); and OR636320-OR636327 (*GAPDH*).

Acknowledgments: The authors would like to thank staff members of the Botanical Garden "Angelo Rambelli" (Viterbo) for their collaboration. All the bioinformatics calculations and analyses were carried out at the DAFNE HPC scientific computing center of the Università degli Studi della Tuscia.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Aliscioni, S.S.; Achler, A.P.; Torretta, J.P. Floral Anatomy, Micromorphology and Visitor Insects in Three Species of *Aristolochia* L. (Aristolochiaceae). *N. Z. J. Bot.* **2017**, *55*, 496–513. [CrossRef]
2. Scarborough, J.; Fernandes, A. Ancient Medicinal Use of *Aristolochia*: Birthwort's Tradition and Toxicity. *Pharm. Hist.* **2011**, *53*, 3–21. [PubMed]
3. Heinrich, M.; Chan, J.; Wanke, S.; Neinhuis, C.; Simmonds, M.S. Local Uses of *Aristolochia* Species and Content of Nephrotoxic Aristolochic Acid 1 and 2—A Global Assessment Based on Bibliographic Sources. *J. Ethnopharmacol.* **2009**, *125*, 108–144. [CrossRef] [PubMed]
4. Voronkova, N.M.; Kholina, A.B.; Koldaeva, M.N.; Nakonechnaya, O.; Nechaev, V.A. Morphophysiological Dormancy, Germination, and Cryopreservation in *Aristolochia contorta* Seeds. *Plant Ecol. Evol.* **2018**, *151*, 77–86. [CrossRef]
5. Wondafrash, M.; Wingfield, M.J.; Wilson, J.R.; Hurley, B.P.; Slippers, B.; Paap, T. Botanical Gardens as Key Resources and Hazards for Biosecurity. *Biodivers. Conserv.* **2021**, *30*, 1929–1946. [CrossRef]
6. Brugneti, F.; Rossini, L.; Drais, M.I.; Turco, S.; Mazzaglia, A. Effect of Temperature on in Vitro Germination and Growth of *Colletotrichum fioriniae*, a New Emerging Pathogen of Olive Fruits. *Environ. Microbiol. Rep.* **2024**, *16*, e13275. [CrossRef]
7. Brugneti, F.; Iezzi, E.; Turco, S.; Drais, M.I.; Giubilei, I.; Mazzaglia, A. First Report of *Colletotrichum fioriniae* Causing Hazelnut Defects in Italy. *J. Plant Pathol.* **2024**. [CrossRef]
8. Giubilei, I.; Brugneti, F.; Turco, S.; Drais, M.I.; Mazzaglia, A. First Report of Anthracnose on *Fagus Sylvatica* Caused by *Colletotrichum fioriniae* in Italy. *New Dis. Rep.* **2023**, *48*, e12226. [CrossRef]
9. Talhinhas, P.; Baroncelli, R. Hosts of *Colletotrichum*. *Mycosphere* **2023**, *14*, 158–261. [CrossRef]
10. Zapata, M.; Rodríguez-Serrano, E.; Castro, J.F.; Santelices, C.; Carrasco-Fernández, J.; Damm, U.; Palfner, G. Novel Species and Records of *Colletotrichum* Associated with Native Woody Plants in South-Central Chile. *Mycol. Prog.* **2024**, *23*, 18. [CrossRef]
11. Damm, U.; Cannon, P.F.; Woudenberg, J.H.C.; Crous, P.W. The *Colletotrichum acutatum* Species Complex. *Stud. Mycol.* **2012**, *73*, 37–113. [CrossRef] [PubMed]
12. Damm, U.; Sato, T.; Alizadeh, A.; Groenewald, J.Z.; Crous, P.W. The *Colletotrichum dracaenophilum*, *C. magnum* and *C. orchidearum* Species Complexes. *Stud. Mycol.* **2019**, *92*, 1–46. [CrossRef] [PubMed]
13. Liu, F.; Ma, Z.Y.; Hou, L.W.; Diao, Y.Z.; Wu, W.P.; Damm, U.; Song, S.; Cai, L. Updating Species Diversity of *Colletotrichum*, with a Phylogenomic Overview. *Stud. Mycol.* **2022**, *101*, 1–56. [CrossRef] [PubMed]
14. Weir, B.S.; Johnston, P.R.; Damm, U. The *Colletotrichum gloeosporioides* Species Complex. *Stud. Mycol.* **2012**, *73*, 115–180. [CrossRef]
15. Okonechnikov, K.; Golosova, O.; Fursov, M.; Team, U. Unipro UGENE: A Unified Bioinformatics Toolkit. *Bioinformatics* **2012**, *28*, 1166–1167. [CrossRef]
16. Stamatakis, A. RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* **2014**, *30*, 1312–1313. [CrossRef]
17. Nirengerg, H.I. Untersuchungen Uber Die Morpholoigische Und Biologische Differnzierung in Der Fusarium-Sektion Liseola. *Mitt. Biol. Bundesanst. Land-U. Forstwirtsch. Berl.-Dahl.* **1976**, *169*, 1–117.
18. Crous, P.W.; Verkley, G.J.; Groenewald, J.Z.; Samson, R.A. *Fungal Biodiversity*; APS Press: Saint Paul, MN, USA, 2009.
19. Drais, M.I.; Rossini, L.; Turco, S.; Faluschi, A.; Mazzaglia, A. Modelling Germination and Mycelium Growth Rates of *Monostichella coryli* under Constant Temperature Conditions. *Fungal Ecol.* **2023**, *61*, 101201. [CrossRef]
20. Moral, J.; Agustí-Brisach, C.; Raya, M.C.; Jurado-Bello, J.; López-Moral, A.; Roca, L.F.; Chattaoui, M.; Rhouma, A.; Nigro, F.; Sergeeva, V.; et al. Diversity of *Colletotrichum* Species Associated with Olive Anthracnose Worldwide. *J. Fungi* **2021**, *7*, 741. [CrossRef]
21. Garcia-Lopez, M.T.; Serrano, M.S.; Camiletti, B.X.; Gordon, A.; Estudillo, C.; Trapero, A.; Diez, C.M.; Moral, J. Study of the Competition between *Colletotrichum godetiae* and *C. Nymphaeae*, Two Pathogenic Species in Olive. *Sci. Rep.* **2023**, *13*, 5344. [CrossRef]
22. Yang, Y.L.; Cai, L.; Yu, Z.N.; Liu, Z.Y.; Hyde, K.D. Colletotrichum species on Orchidaceae in southwest China. *Cryptogam. Mycol.* **2011**, *32*, 229–253.
23. Moriwaki, J.; Sato, T.; Tsukiboshi, T. Morphological and molecular characterisation of *Colletotrichum boninense* sp. nov. from Japan. *Mycoscience* **2003**, *44*, 47–53. [CrossRef]
24. Lücking, R.; Aime, M.C.; Robbertse, B.; Miller, A.N.; Ariyawansa, H.A.; Aoki, T.; Cardinali, G.; Crous, P.W.; Druzhinina, I.S.; Geiser, D.M. Unambiguous Identification of Fungi: Where Do We Stand and How Accurate and Precise Is Fungal DNA Barcoding? *IMA Fungus* **2020**, *11*, 14. [CrossRef]
25. Bhunjun, C.S.; Phukhamsakda, C.; Jayawardena, R.S.; Jeewon, R.; Promputtha, I.; Hyde, K.D. Investigating Species Boundaries in *Colletotrichum*. *Fungal Divers.* **2021**, *107*, 107–127. [CrossRef]
26. Turco, S.; Grottoli, A.; Drais, M.I.; De Spirito, C.; Faino, L.; Reverberi, M.; Cristofori, V.; Mazzaglia, A. Draft Genome Sequence of a New *Fusarium* Isolate Belonging to *Fusarium tricinctum* Species Complex Collected from Hazelnut in Central Italy. *Front. Plant Sci.* **2021**, *12*, 788584. [CrossRef]
27. Schena, L.; Mosca, S.; Cacciola, S.O.; Faedda, R.; Sanzani, S.M.; Agosteo, G.E.; Sergeeva, V.; Magnano di San Lio, G. Species of the *Colletotrichum gloeosporioides* and C. Boninense Complexes Associated with Olive Anthracnose. *Plant Pathol.* **2014**, *63*, 437–446. [CrossRef]

28. Aiello, D.; Carrieri, R.; Guarnaccia, V.; Vitale, A.; Lahoz, E.; Polizzi, G. Characterization and Pathogenicity of *Colletotrichum gloeosporioides* and *C. Karstii* Causing Preharvest Disease on Citrus Sinensis in Italy. *J. Phytopathol.* **2015**, *163*, 168–177. [CrossRef]
29. Ismail, A.M.; Cirvilleri, G.; Yaseen, T.; Epifani, F.; Perrone, G.; Polizzi, G. Characterisation of *Colletotrichum* Species Causing Anthracnose Disease of Mango in Italy. *J. Plant Pathol.* **2015**, *97*, 167–171.
30. Yang, Z.J.; Yin, Y.; Wang, Z.Q.; Yang, T.; Chen, D.J. Cytotoxic Metabolites of Endophytic Fungus *Colletotrichum* Sp. from *Aristolochia* spp. *Nat. Prod. Res. Dev.* **2012**, *24*, 329–332.
31. Tekade, A.; Mohod, Y.N. Occurrence of Diseases on Medicinal Plants in Vidarbha Region and Correlation of Weather Factors with Leaf Blight of Coleus. *Med. Plant Res.* **2015**, *5*, 1–4.