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Species Diversity in *Colletotrichum* Causing Anthracnose of Aromatic and Ornamental Lamiaceae in Italy

Vladimiro Guarnaccia [1,2,*], Giovanna Gilardi [2], Ilaria Martino [2], Angelo Garibaldi [2] and Maria Lodovica Gullino [1,2]

[1] Department of Agricultural, Forest and Food Sciences (DISAFA), University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy; marialodovica.gullino@unito.it

[2] Centre for Innovation in the Agro-Environmental Sector, AGROINNOVA, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy; giovanna.gilardi@unito.it (G.G.); ilaria.martino88@edu.unito.it (I.M.); angelo.garibaldi@unito.it (A.G.)

* Correspondence: vladimiro.guarnaccia@unito.it; Tel.: +39-(0)-116708018

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Abstract: Species of *Colletotrichum* are considered important plant pathogens, saprobes, and endophytes on a wide range of host plants. In Italy, several *Colletotrichum* species have been reported in glasshouse environments. In this study, we have explored the occurrence, diversity, and pathogenicity of *Colletotrichum* spp. associated with aromatic and ornamental plants belonging to the Lamiaceae family. Surveys were carried out during the 2011–2018 period in Liguria and Piedmont, Italy. A total of 19 *Colletotrichum* isolates were collected from symptomatic leaves and seeds of *Ocimum basilicum* (basil), *Origanum vulgare* (oregano) and different *Salvia* spp. A multi-locus phylogeny was established based on the basis of four genomic loci (ITS, *GAPDH*, *ACT* and *TUB2*). The aggressiveness of selected, representative isolates were tested. *Colletotrichum* isolates were identified as being members of three major species complexes: *C. acutatum*, *C. destructivum*, and *C. gloeosporioides*. *Colletotrichum fioriniae*, *C. bryonicola*, and *C. fructicola* were found in association with leaf lesions on *Salvia leucantha*, *S. nemorosa*, and *S. greggii*, respectively. *Colletotrichum nigrum* was isolated from twig lesions of *S. greggii*. Moreover, *C. fioriniae* and *C. ocimi* were found to be responsible for causing leaf anthracnose of oregano and basil, respectively. All the tested isolates were pathogenic and reproduced identical symptoms to those observed in commercial glasshouses. The present study improves our understanding of *Colletotrichum* species associated with several hosts belonging to the Lamiaceae family, which are cultivated extensively throughout Italy for different purpose, and provides information that may be useful for an effective disease management program.

Keywords: Ornamentals; high-value crops; Anthracnose; multi-locus sequence typing; pathogenicity

1. Introduction

The genus *Colletotrichum* contains major plant pathogens, that cause diseases on a wide variety of woody and herbaceous plants. Although it has a primary tropical and subtropical distribution, it also affects temperate areas [1–3]. *Colletotrichum* spp. have been included in the 10 most important plant pathogenic fungi in the world, on the bases of their scientific and economic importance [4]. *Colletotrichum* species can infect more than 30 plant genera [5–9], causing anthracnose disease and postharvest decay over a wide range of tropical, subtropical and temperate fruit, grasses, vegetable crops, and ornamental plants [7–14]. Several *Colletotrichum* species are latent plant pathogens, endophytes or saprobes, which are able to switch to a pathogenic lifestyle when the host plants are subjected to different types of stress, or placed in postharvest storage [15].

With the increasing use of molecular data and multi-gene phylogenetic analysis, the combination of traditional morphology-based identification methods combined with these molecular tools has resulted in a major revision of the classification and species concepts of *Colletotrichum* [3,7,8,16,17]. Several systematic studies have led to the identification of 11 *Colletotrichum* species complexes, and more than 20 individual species [18]. The use of multi-locus phylogenetic analyses has shown that certain *Colletotrichum* spp. that were previously considered to be the causal agent of economically important plant diseases, have since been identified as different species. *Colletotrichum alienum* is the most important species in Proteaceae species cultivation [19], on the contrary *C. gloeosporioides* s. str. was previously assumed as the major *Colletotrichum* pathogen [20]. *Colletotrichum abscissum*, the causal agents of the post-bloom fruit drop of citrus fruit, was previously known as *C. acutatum* [21].

The species complex (SC) of *C. gloeosporioides* [17,22], *C. acutatum* [7,23,24], *C. boninense* [8], and *C. truncatum* [3,25] are considered the most important plant pathogens. Moreover, the *C. destructivum* SC includes important plant pathogens [16].

Ornamental plant production is an economically important sector of Italian agriculture with around 40,000 ha of cultivation [26]. The Lamiaceae family includes a number of plants cultivated because of their aromatic and medicinal properties, as well as for ornamental purposes. Several of these are economically important and cultivated extensively in the Mediterranean area. Oregano (*Origanum vulgare*) is a small shrub typical of the Mediterranean region, and which is also appreciated also as potted plants, because of its aromatic properties. Sweet basil (*Ocimum basilicum*) is a major herb crop in Mediterranean regions. Approximately 365 ha are grown annually in Italy for fresh consumption and processing [27]. *Salvia* spp., herbaceous perennials, are aromatic plants cultivated as ornamentals.

Aromatic plants are cultivated under protection in glasshouses or plastic tunnels and in open fields, while *Salvia* spp. are widely used in outdoor gardens and as potted plants in private or public gardens. A high level of humidity and a high plant density generally play major roles in the development and sporulation of *Colletotrichum* spp. [28].

Several *Colletotrichum* species have been reported on commercial farms and in private gardens or in public parks, in different environmental conditions in Northern and Southern Italy. Some of these species have been identified as pathogens on new hosts for the first time [29–32].

Several surveys were conducted in commercial farms as well as in private or public gardens in Liguria and Piedmont, Northern Italy, over an 8-year period. The aims of this study were (i) to identify the species of *Colletotrichum* isolated from aromatic plants on the basis of multi-locus phylogenetic analysis, and (ii) to compare the virulence of representative *Colletotrichum* isolates on the host species from which they were isolated.

2. Materials and Methods

2.1. Field Surveys and Fungal Isolation

During the 2010–2018 period, anthracnose symptoms were detected on *Or. vulgare*, *Oc. basilicum*, *S. greggii*, *S. leucantha,* and *S. nemorosa* plants. The samples from *Salvia* spp. and oregano were from public and private mountain gardens located near Biella (Northern Italy, 45.6121660 latitude and 8.0562970 longitude) at an altitude of 800 m, and from public parks in Torino, while the samples from basil were obtained from commercial farms in Liguria (one site) and Piedmont (three sites) over the years. Two *Colletotrichum* isolates were obtained from contaminated basil seeds using the protocol described by Mathur and Kongsdal [33]. Briefly, subsamples represented by 400 seeds were tested on Petri plates (10 seeds/plate) in two trials. Isolations were made from either non-disinfected or surface disinfected seeds for 1 min in 1% sodium hypochlorite, washed in sterile distilled water (SDW) for 5 min and dried under a sterile hood. The fungal colonies developing from the seeds, morphologically identified as *Colletotrichum* spp. [10], were transferred onto potato dextrose agar (PDA, Oxoid, Basingstoke, England) plates for monosporic cultures.

The disease incidence was recorded during the field survey for each host species on the basis of the number of symptomatic plants. Approximately 20 plants per species showing anthracnose symptoms were randomly collected for isolation. Small sections (0.2–0.5 cm long) from the edge of lesions were surface disinfected with 1% sodium hypochlorite for 1 min, rinsed once in SDW, dried on sterile absorbent paper and placed on PDA plates amended with 25 ppm streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated at 25 ± 1 °C under a 12 h photoperiod. Following 48 to 72 h of incubation, any hyphae from the margin of the colonies with characteristic features of *Colletotrichum* spp. were placed on PDA plates. After five days, single spores were selected and transferred into PDA plates to establish pure cultures.

A total of 19 isolates were obtained and used for molecular characterization (Table 1). Stock cultures are maintained at −80 °C in the Agroinnova (University of Torino) culture collection, Torino, Italy.

Table 1. Collection details of *Colletotrichum* isolates, virulence expressed after the pathogenicity test and GenBank accession numbers of other *Colletotrichum* isolates included in this study.

Species	Culture No. [1]	Host	Locality	Virulence [2]	GenBank No. [3]			
					ITS	*gapdh*	*act*	*tub2*
Colletotrichum abscissum	COAD 1877	*Citrus sinensis*	Brazil	-	KP843126	KP843129	KP843141	KP843135
C. acutatum	CBS 112996	*Carica papaya*	Australia	-	JQ005776	JQ948677	JQ005839	JQ005860
	CBS 979.69	*Coffea arabica*	Kenya	-	JQ948400	JQ948731	JQ949721	JQ950051
C. aenigma	ICMP 18608 *	*Persea americana*	Israel	-	JX010244	JX010044	JX009443	JX010389
C. alienum	ICMP 12071 *	*Malus domestica*	New Zealand	-	JX010251	JX010028	JX009572	JX010411
C. antirrhinicola	CBS 102189 *	*Antirrhinum majus*	New Zealand	-	KM105180	KM105531	KM105390	KM105460
C. asianum	ICMP 18580, CBS 130418	*Coffea arabica*	Thailand	-	FJ972612	JX010053	JX009584	JX010406
C. boninense	CBS 123755 *	*Crinum asiaticum* var. *sinicum*	Japan	-	JQ005153	JQ005240	JQ005501	JQ005588
C. bryoniicola	CBS 109849 *	*Bryonia dioica*	Netherlands	-	KM105181	KM105532	KM105391	KM105461
	CVG 256	*Salvia nemorosa*	Italy	M	*MN516549*	*MN535113*	*MN535132*	*MN535151*
	CVG 257	*Salvia nemorosa*	Italy	-	*MN516550*	*MN535114*	*MN535133*	*MN535152*
C. camelliae	CGMCC3.14925, LC1364 *	*Camellia sinensis*	China	-	KJ955081	KJ954782	KJ954363	KJ955230
C. coccodes	CBS 126378	*Solanum tuberosum*	South Africa	-	JX546835	JX546739	JX546643	JX546882
	CBS 150.33	*Anthurium* sp.	Germany	-	JX546826	JX546730	JX546634	JX546872
	CBS 369.75 *	*Solanum tuberosum*	Netherlands	-	HM171679	HM171673	HM171667	JX546873
C. conoides	CAUG17 *	*Capsicum annuum*	China	-	KP890168	KP890162	KP890144	KP890174
C. chrysanthemi	CBS 126519	*Chrysanthemum coronarium*	Netherland	-	JQ948272	JQ948602	JQ949593	JQ949923
C. destructivum	CBS 136228 *	*Crupina vulgaris*	Greece	-	KM105219	KM105574	KM105429	KM105499
C. fioriniae	ATCC 28992	*Malus domestica*	USA	-	JQ948297	JQ948627	JQ949618	JQ949948
	CBS 129916	*Vaccinium* sp.	USA	-	JQ948317	JQ948647	JQ949638	JQ949968
	CBS 293.67	*Persea americana*	Australia	-	JQ948310	JQ948640	JQ949631	JQ949961
	CVG 174	*Salvia leucantha*	Italy	M	*MN516538*	*MN535102*	*MN535121*	*MN535140*
	CVG 175	*Salvia leucantha*	Italy	-	*MN516539*	*MN535103*	*MN535122*	*MN535141*
	CVG 176	*Salvia leucantha*	Italy	-	*MN516540*	*MN535106*	*MN535123*	*MN535142*
	CVG 264	*Salvia leucantha*	Italy	-	*MN516551*	*MN535115*	*MN535134*	*MN535153*
	CVG 268	*Origanum vulgare*	Italy	H	*MN516552*	*MN535116*	*MN535135*	*MN535154*
	CVG 269	*Origanum vulgare*	Italy	-	*MN516553*	*MN535117*	*MN535136*	*MN535155*
C. fructicola	ICMP 18581, CBS 130416 *	*Coffea arabica*	Thailand	-	JX010165	JX010033	FJ907426	JX010405
	LC2923	*Camellia sinensis*	China	-	KJ955083	KJ954784	KJ954365	KJ955232
	CVG 170	*Salvia greggii*	Italy	L	*MN516535*	*MN535099*	*MN535118*	*MN535137*

Table 1. *Cont.*

Species	Culture No. [1]	Host	Locality	Virulence [2]	GenBank No. [3]			
					ITS	*gapdh*	*act*	*tub2*
C. gloeosporioides	ICMP 17821, CBS 112999 *	*Citrus sinensis*	Italy	-	JQ005152	JX010056	JX009531	JX010445
C. godetiae	CBS 133.44	*Clarkia hybrida*	Denmark	-	JQ948402	JQ948733	JQ949723	JQ950053
	CBS 796.72	*Aeschynomene virginica*	USA	-	JQ948407	JQ948738	JQ949728	JQ950058
C. kahawae subsp. *kahawae*	ICMP 17816 *	*Coffea arabica*	Kenya	-	JX010231	JX010012	JX009452	JX010444
C. lini	CBS 172.51 *	*Linum usitatissimum*	Netherlands	-	JQ005765	KM105581	JQ005828	JQ005849
C. nigrum	CBS 127562	*Cichorium intybus*	Chile	-	JX546842	JX546746	JX546650	JX546889
	CBS 169.49 *	*Capsicum* sp.	Argentina	-	JX546838	JX546742	JX546646	JX546885
	CVG 171	*Salvia greggii*	Italy	M	*MN516536*	*MN535100*	*MN535119*	*MN535138*
	CVG 173	*Salvia greggii*	Italy	-	*MN516537*	*MN535101*	*MN535120*	*MN535139*
C. nupharicola	CBS 470.96, ICMP 18187 *	*Nuphar lutea* subsp. *polysepala*	USA	-	JX010187	JX009972	JX009437	JX010398
C. nymphaeae	CBS 119294	*Leucaena* sp.	Mexico	-	JQ948205	JQ948535	JQ949526	JQ949856
	CBS 515.78	*Nymphaeae alba*	Netherlands	-	JQ948197	JQ948527	JQ949518	JQ949848
C. ocimi	CBS 298.94 *	*Ocimum basilicum*	Italy	-	KM105222	KM105577	KM105432	KM105502
	CVG 189	*Ocimum basilicum*	Italy	-	*MN516541*	*MN535105*	*MN535124*	*MN535143*
	CVG 190	*Ocimum basilicum*	Italy	H	*MN516542*	*MN535106*	*MN535125*	*MN535144*
	CVG 193	*Ocimum basilicum*	Italy	-	*MN516543*	*MN535107*	*MN535126*	*MN535145*
	CVG 200	*Ocimum basilicum*	Italy	-	*MN516545*	*MN535109*	*MN535128*	*MN535147*
	CVG 202	*Ocimum basilicum*	Italy	-	*MN516546*	*MN535110*	*MN535129*	*MN535148*
	CVG 203	*Ocimum basilicum*	Italy	-	*MN516544*	*MN535108*	*MN535127*	*MN535146*
	CVG 204	*Ocimum basilicum*	Italy	-	*MN516547*	*MN535111*	*MN535130*	*MN535149*
	CVG 205	*Ocimum basilicum*	Italy	-	*MN516548*	*MN535112*	*MN535131*	*MN535150*
C. siamense	ICMP 18578, CBS 130417 *	*Coffea arabica*	Thailand	-	JX010171	JX009924	FJ907423	JX010404
C. vignae	CBS 501.97	*Vigna unguiculata*	Nigeria	-	KM105183	KM105534	KM105393	KM105463
Moniolochaetes infuscans	CBS 869.96	*Ipomoea batatas*	South Africa	-	JQ005780	JX546612	JQ005843	JQ005864

[1] ATCC: American Type Culture Collection, Virginia, USA; CGMCC: The Microbiological Culture Collection, Beijing, China; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht; COAD: Coleção Octávio Almeida Drummond, Viçosa, Brazil; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; CVG: Agroinnova, Grugliasco, Torino, Italy. Ex-type and ex-epitype cultures are indicated with *. [2] Virulence: L, low virulence (10–30%); M, moderate virulence (31–60%); H, high virulence (61–100%). [3] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase gene; *act*: partial actin gene; *tub2*: partial beta-tubulin gene. Sequences generated in this study are indicated in *italics*.

2.2. DNA Extraction, PCR Amplification, and Sequencing

The total DNA was extracted for all *Colletotrichum* isolates with an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany) from 0.1 g of mycelium grown on PDA, according to the manufacturer's instructions. Portions of four loci were amplified. The primers ITS1 and ITS4 [34] were used to amplify the internal transcribed spacer region (ITS). The partial glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified using GDF1 and GDR1 primers [35]. The primers ACT-512F and ACT-783R [36] were used to amplify part of the actin gene (*ACT*). The partial beta-tubulin (*TUB2*) gene was amplified with T1 [37] and Bt-2b primers [38]. The PCR amplification mixtures and cycling conditions were adopted for all four loci were followed as described by Guarnaccia et al. [14]. Both strands of the PCR products were sequenced by Eurofins Genomics Service (Ebersberg, Germany). The generated DNA sequences were analyzed and consensus sequences were computed using the program Geneious v. 11.1.5 (Auckland, New Zealand).

2.3. Phylogenetic Analyses

The novel sequences generated in this study were compared using the NCBIs GenBank nucleotide database through the "BLAST" command, to determine the closest species for a taxonomic framework of the studied isolates. Different gene regions, including sequences obtained from this study and sequences downloaded from GenBank, were initially aligned by using the MAFFT v. 7 online servers (http://mafft.cbrc.jp/alignment/server/index.html) [39] and then manually adjusted in MEGA v. 7 [40]. Phylogenetic analyses were conducted individually for each locus (data not shown) and also as concatenated analyses of four loci with the aim of establishing the identity of the isolates at species level. Additional reference sequences were selected based on recent studies on *Colletotrichum* species [7,14,16,17,19]. Phylogenetic analyses were developed based on Maximum Parsimony (MP) for all individual loci, and based on both MP and Bayesian Inference (BI) for the combined multilocus analyses. For BI, the best evolutionary model for each partition was selected on the basis of MrModeltest v. 2.3 [41] and incorporated into the analyses. MrBayes v. 3.2.5 [42] was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set to 0.2 and trees were sampled every 1000 generations. The analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analyses were conducted using PAUP [43]. Phylogenetic relationships were estimated by heuristic searches with 100 random additional sequences. Tree bisection-reconnection was used, with the branch swapping option set at 'best trees' only with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated to establish the parsimony and the bootstrap analyses [44] were based on 1000 replications. Sequences generated in this study were deposited in GenBank (Table 1).

2.4. Pathogenicity

Five plant species, *Ocimum basilicum* (basil), *Origanum vulgare* (oregano), *Salvia leucantha*, *S. nemorosa*, and *S. greggii* were used for pathogenicity tests. Basil seeds were sown in 2-L plastic pots in triplicate (nine seeds per pot) in a sterilized mixture of peat and perlite. The basil plants were inoculated one month after seeding. Similarly, five-month oregano seedlings and cuttings of all the salvia species were cultivated in 2-L pots and inoculated. The plants were grown in a glasshouse at 23 to 25 °C before inoculation. One isolate for each of the *Colletotrichum* species (based on molecular identification) was used for each of the part hosts (Table 1). The isolates were grown on PDA with streptomycin sulfate (25 mg/L) and kept for a week with a 12 h photoperiod at 25 °C. Conidia suspensions with a final concentration of 10^6 conidia/ml were sprayed onto the leaves. One milliliter of suspension was used per single plant or pot with basil plants. Five pots of each host were inoculated using sterile water for control plants. The plants were covered with a transparent plastic film to keep a high level of relative humidity (RH) and transferred to a growth chamber and kept at 25 °C with a 12 h photoperiod. The plastic film was removed three days post-inoculation (dpi). A disease severity (DS) index was

adopted to rank the plants after 7 to 10 dpi, where 0 indicated healthy plants, 25, low virulent lesions and slight leaf chlorosis, 50, moderate presence of typical anthracnose on leaves, 100, the abundant presence of necrotic spots and dead plants. This trial was replicated once. Data of the replications of the repeated experiments were pooled and analyzed together. Therefore, the virulence was classified as low, moderate or high.

3. Results

3.1. Field Surveys and Fungal Isolation

Symptoms identified as those caused by *Colletotrichum* spp. were found at six sites (Table 2) with various disease incidence on five different species belonging to the Lamiaceae family (Figure 1). Disease incidence, considered as the percentage of affected leaves, varied from 10 to 80%, depending on the host species and environmental conditions (Table 1). The symptoms were observed on 15 to 30-day–old basil plants grown in open fields, or indoors in protected cultivations (plastic tunnels and glasshouse), and during different stages on established *Salvia* spp. plants (1–2 years old) as well as on oregano grown outdoors in public and private gardens. The observed symptoms consisted of brown to black, necrotic lesions on leaves of all the investigated species. The first symptoms on *S. leucantha* consisted of small necrotic spots, measuring 10 to 30 mm, which increased in number to cover a large percentage of the leaves. The symptoms generally started from the basal leaves on plants grown in the shade and at higher RH. Severely diseased plants were observed to be defoliated 30 to 35 days after the first symptoms appeared. Small irregular lesions of 3 to 40 mm developed on the leaves of *S. greggii* plants grown in the shade with high RH and, at the final stage, these plants were almost completely defoliated. Regarding *S. nemorosa*, initial symptoms appeared as small light brown, circular spots on the leaves, mainly at the leaf margin, surrounded by a chlorotic halo that subsequently enlarged in diameter and covered the entire leaf surface. Moreover, black and necrotic leaves were observed on *S. greggii*. At advanced stages of the disease, the canopies of the plants were partially or completely affected.

Figure 1. Anthracnose symptoms caused by *Colletotrichum* spp. on leaves of different Lamiaceae: (**a**) *Ocimum basilicum*, (**b**) *Origanum vulgare*, (**c**) *Salvia greggii*, (**d**) *Salvia nemorosa*, (**e**) *Salvia leucantha*.

Table 2. Hosts, sources, site of isolation, and disease incidence (%) of *Colletotrichum* spp.

Host Species	Common Name	ID of Collected Isolates	*Colletotrichum* Species	Source	Site (City, Region)	Year	Disease Incidence (%)
Salvia leucantha	Mexican bush sage	CVG174, CVG175, CVG176, CVG264	*C. fioriniae*	Leaves	Private garden (Biella, Piedmont)	2014	60–80
S. greggii	Autumn sage	CVG170	*C. fructicola*	Leaves	Private garden (Biella, Piedmont)	2015	30–40
S. greggii	Autumn sage	CVG171, CVG173	*C. nigrum*	Leaves	Private garden (Biella, Piedmont)	2015	30–40
S. nemorosa	Woodland sage	CVG256, CVG257	*C bryoniicola*	Leaves	Public garden (Torino, Piedmont)	2018	25–35
Ocimum basilicum	Basil cv. Aromatico della Riviera	CVG190	*C. ocimi*	Seeds	Unknown	2011	-
O. basilicum	Basil cv. Italiko	CVG189	*C. ocimi*	Seeds	Unknown	2011	-
O. basilicum	Basil	CVG193	*C. ocimi*	Leaves	Open field (Albenga, Savona, Liguria)	2016	25–35
O. basilicum	Basil	CVG200	*C. ocimi*	Leaves	Glasshouse (Torino, Piedmont)	2014	30–40
O. basilicum	Basil	CVG202	*C. ocimi*	Cotyledons	Open field (Castagnole, Torino, Piedmont)	2016	30–40
O. basilicum	Basil	CVG203, CVG204	*C. ocimi*	Leaves	Glasshouse (Torino, Piedmont)	2013	25–35
O. basilicum	Basil	CVG205	*C. ocimi*	Leaves	Plastic tunnel (Moncalieri, Torino, Piedmont)	2013	30–50
Origanum vulgare	Oregano	CVG268, CVG269	*C. fioriniae*	Leaves	Private garden (Biella, Piedmont)	2018	20–35

The *O. basilicum* plants showed black spot symptoms on leaves and stems as circular or irregular shaped necrotic spots. At the final stage, plants were almost completely defoliated. Moreover, necrotic spots on oregano with a purple margin were 5 to 30 mm in diameter, expanded and coalesced by interesting the entire leaf surface. The plants eventually died after total defoliation.

Pure fungal cultures resembling those of the *Colletotrichum* genus were obtained from symptomatic leaves collected during the surveys and from contaminated seeds used for isolation.

3.2. Phylogenetic Analyses

Five alignments were analyzed representing single gene analyses of Internal Transcribed Spacer (ITS), *act*, *gapdh*, *tub2,* and a combined alignment of the four genes, were analyzed. The alignments produced topologically similar trees. The combined species phylogeny of the *Colletotrichum* isolates consisted of 54 sequences, including the outgroup sequences of *Moniolochaetes infuscans* (CBS 896.96). A total of 1995 characters (ITS: 1–574, *act*: 581–873, *gapdh*: 880–1198, *tub2*: 1205–1995) were included in the phylogenetic analysis, 795 characters were parsimony-informative, 191 were variable and parsimony-uninformative, and 991 were constant. A maximum of 1000 equally most parsimonious trees were saved (Tree length = 2085, CI = 0.757, RI = 0.958 and RC = 0.725). Bootstrap support values from the parsimony analysis are plotted on the Bayesian phylogenies in Figure 2. For the Bayesian analyses, MrModeltest suggested that all partitions should be analyzed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: SYM + G for ITS, HKY + G for *act* and GTR + G for *gapdh* and *tub2*. In the Bayesian analysis, the ITS partition had 146 unique site patterns, the *act* partition had 163 unique site patterns, the *act* partition had 251 unique site patterns, the *tub2* partition had 354 unique site patterns and the analysis ran for 680,000 generations, resulting in 1362 trees of which 1022 trees were used to calculate the posterior probabilities.

In the combined analysis, six isolates (four from *S. leucantha* and two from *O. vulgare*) clustered with two reference strains and the ex-type of *C. fioriniae*, whilst eight isolates (from basil) clustered with the ex-type of *C. ocimi*. Two isolates from *S. nemorosa* were identified as *C. bryoniicola*. Furthermore, three isolates from *S. greggii* were identified as *C. nigrum* (two isolates) and as *C. fructicola*.

The individual alignments and trees of the four single loci used in the analyses were also compared with respect to their performance in species recognition.

3.3. Pathogenicity

All of the tested *Colletotrichum* species tested caused symptoms on the different, original inoculated hosts and were identical to those observed in naturally diseased plants. The DS caused by the *Colletotrichum* species inoculated on original hosts were ranged from 25 to 83.3% after 7 to 10 days at 25 °C. *Colletotrichum fioriniae* and *C. ocimi* caused the highest DS on oregano (83.3%) and basil (66.6%), respectively. Conversely, *C. fioriniae* produced a lower DS (33.3%) when inoculated on *S. leucantha*. Similarly, *C. bryoniicola* inoculation resulted in a moderate level of DS (33.3%) on *S. nemorosa*. The inoculated plants of *S. greggii* showed higher susceptibility to *C. nigrum* which led to a DS value of 41.6% than *C. fructicola* (25%). The pathogens were re-isolated from artificially inoculated plants and identified as previously described by means of blasting analysis of the *gapdh* locus, thus Koch's postulates were fulfilled. No symptoms were observed in the control plants.

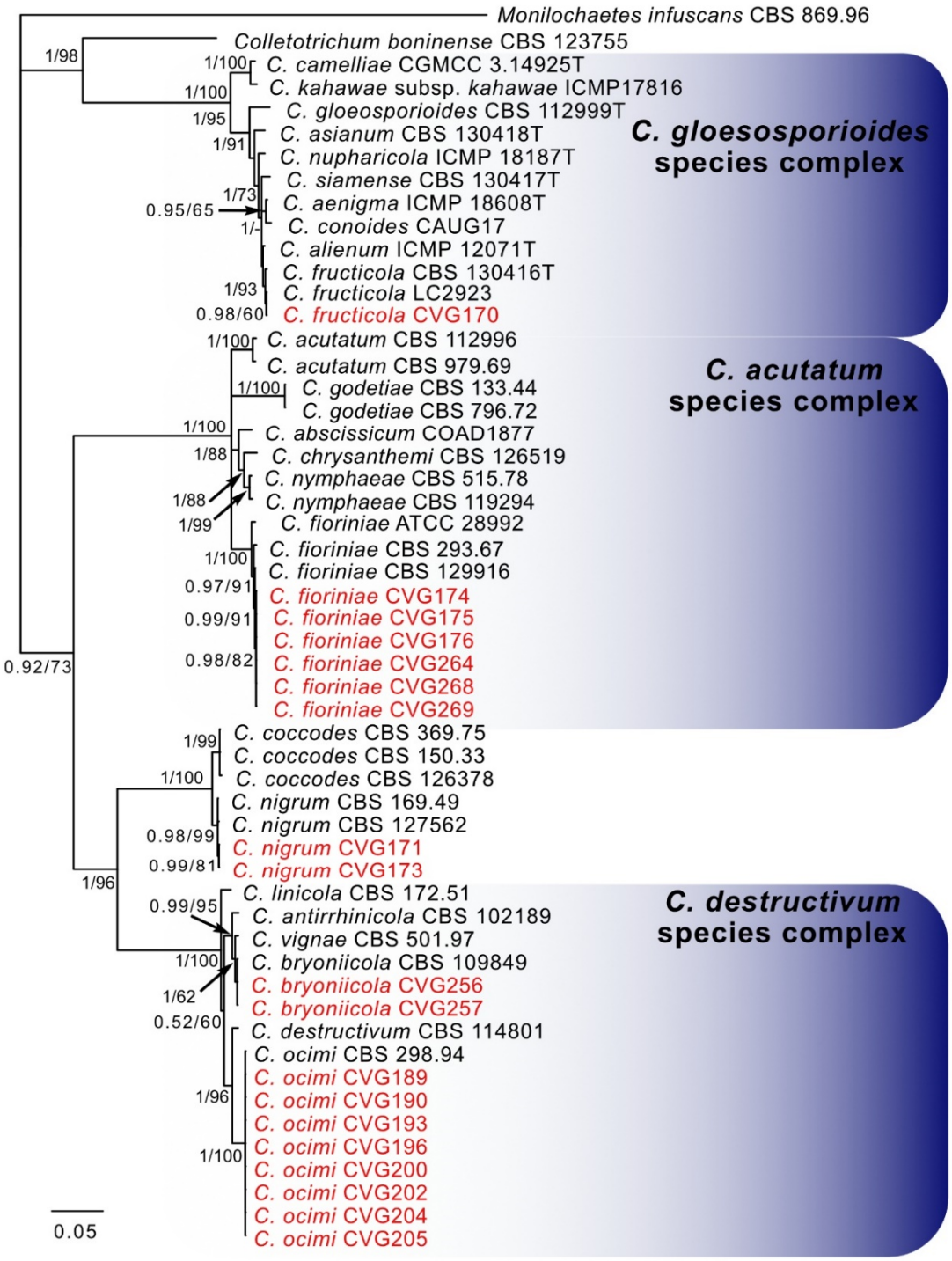

Figure 2. Consensus phylogram of the 1022 trees resulting from a Bayesian analysis of the combined ITS, *ACT*, *GAPDH*, and *TUB2* sequence of *Colletotrichum* spp. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. *Colletotrichum* species complexes are listed next to the isolate numbers. The isolates collected in this study are in red. The tree was rooted to *Moniolochaetes infuscans* (CBS 869.96).

4. Discussion

The present study provides the first overview of *Colletotrichum* diversity associated with the leaf anthracnose of several aromatic and ornamental host plants belonging to the Lamiaceae family and provides basic information on their aggressiveness.

The identification of *Colletotrichum* species, on the basis of the morphological characteristics, is no longer considered reliable anymore, because several species are not distinguishable from each other [14]. Multilocus sequence analyses, combined with a polyphasic approach is suggested for species differentiation for the *Colletotrichum* genus [3,45]. In this study, 19 *Colletotrichum* isolates were recovered from five aromatic and ornamental species belonging to the Lamiaceae family in Liguria and Piedmont (Northern Italy) over an 8-year period, and have been reported as the causal agents of anthracnose disease. This study has revealed a diversity in the composition of *Colletotrichum* species recovered from aromatic and ornamental Lamiaceae. The investigated isolates were characterized as five different species: *C. fioriniae* belonging to the *C. acutatum* SC, *C. bryoniicola* and *C. ocimi* belonging to the *C. destructivum* SC, *C. fructicola* into the *C. gloeosporioides* SC and the single-species *C. nigrum*. *Colletotrichum fioriniae* was found to be associated with diseased plants of oregano and *Salvia leucantha* plants. *Colletotrichum fructicola* and *C. nigrum* were isolated from lesions produced on *Salvia greggii*, and showed a mixed infection on the host. The lesions on the leaves of *Salvia nemorosa* were caused by isolates of *C. bryoniicola*. Moreover, all of the *Colletotrichum* isolates recovered from the seeds and affected plants were identified as *C. ocimi* and they developed typical anthracnose symptoms on *O. basilicum*. The virulence of the isolates obtained from the infected seeds was similar to that of the isolates from the basil plants. Circular to irregular and brown to black necrotic lesions appeared on all the *Salvia* spp., with severe defoliation in the case of high RH. Basil and oregano plants also showed necrotic spots which often became enlarged, thus affecting the entire leaf surface. Infections were generally observed in May to June during the early growing stages of basil grown under protection and in the open field, which was favored by the optimal conditions for the growth of the *Colletotrichum* species.

Colletotrichum ocimi is the unique *Colletotrichum* species that is known in association with basil plants [16]. Gullino et al. [29] reported a foliar disease of basil cultivated in a glasshouse in Northern Italy and consistently isolated a *Colletotrichum* species, which was initially considered as *C. gloeosporioides*. After the advent of the molecular era, those isolates from basil were moved into the *C. destructivum* SC and named as *C. ocimi* [16]. This species has also been reported in Australia on basil [23]. However, the ability of *C. ocimi* to colonize seeds as external contaminants has been demonstrated for the first time in this study. All the loci used in this study, except *gapdh*, can be used to distinguish this species. *Colletotrichum bryoniicola*, which is an also member of the *C. destructivum* SC, was first described by Damm et al. [16] and has only been reported on *Bryonia dioica* in the Netherlands. *Colletotrichum fructicola*, described by Prihastuti et al. [46], is mainly found in Asia but is present worldwide. It has recently been reported in Italy, where it has caused the postharvest decay of avocado [15] and is commonly associated with fruit diseases [17]. However, *C. fructicola* has never before been reported from infected *Salvia* spp. In this study, *C. fructicola* has been found in the presence of anthracnose symptoms on *S. greggii* in association with *C. nigrum* which is a single species closely related to *C. coccodes*. Previously reported in Canada, New Zealand, and the USA on affected plants of strawberry, sunflower and several Solanaceae [19], *C. nigrum* has never before been found in Europe. *Colletotrichum fructicola* may be distinguishable by sequencing each individual locus of those used, whilst *C. nigrum* has an identical *gapdh* sequence to *C. coccodes*.

Colletotrichum fioriniae has been implicated worldwide in the fruit rot of cranberry and blueberry, but also in lesions produced on broad numbers of fruit such as almond, apple, avocado, mango and nectarine [7,11]. Thus, *C. fioriniae* is considered as a lineage of the *C. acutatum* SC grouping isolates that are able to cross-infect fruit from multiple hosts. In accordance with this, this study reports the species associated with two plant hosts (*Oregano vulgare* and *Salvia leucantha*), that cause typical leaf anthracnose.

All isolates artificially inoculated on their original host developed symptoms, thereby fulfilling Koch's postulates. *Colletotrichum fioriniae* was more aggressive on oregano than on *Salvia leucantha*. *Colletotrichum ocimi* produced typical anthracnose symptoms on basil with high virulence. A medium level of aggressiveness was observed on *S. nemorosa* and *S. greggii* plants inoculated with *C. bryoniicola*

and *C. nigrum*, respectively. *Colletotrichum fructicola* was the only species that developed low levels of symptoms on *S. greggii*.

In recent years the production of medicinal and aromatic plants has been increasing with a current global value of approximately US$ 62 billion and expected annual growth of 15% [47,48].

Some of the ornamental hosts studied in our survey are also susceptible to other pathogens. For instance, *Phytophthora cryptogea* on *S. leuchanta* in southern Italy [49], *Puccinia ballotiflora* [50] and *Boeremia exigua* var. linicola on *S. greggii* [32]. *Salvia nemorosa* is affected by *Rhizoctonia solani* AG1 [51] and *Phoma herbarum* [52]. *Alternaria alternata*, *Botrytis cinerea* and *Corynespora cassiicola* cause the leaf spot on basil [53–55], while *Phoma multirostrata* and *R. solani* AG1 cause leaf blight of oregano [56,57]. Moreover, *C. fioriniae* was previously identified through *TUB2* sequencing after isolation from *S. leucantha* [58]. Aggressive *Colletotrichum* spp. could represent a serious threat to the Lamiaceae cultivation. Farming practices and several factors such as temperature, humidity, and irrigation systems, in addition to climatic changes, could induce suitable conditions for the development of *Colletotrichum* diseases. Thus, prevention is an important strategy to manage pathogenic *Colletotrichum* spp. An accurate identification of *Colletotrichum* species, based on multilocus analysis is important for correct disease diagnosis. The identification was based on a robust phylogeny analysis, performed by combining by four genomic loci, which provided tools that could be used to sequence target loci for rapid detection of the above mentioned *Colletotrichum* species. Artificial inoculations also demonstrated the ability of all these *Colletotrichum* spp. found in Northern Italy to cause disease on different salvia species, basil, and oregano. To our knowledge, this study represents the first report in Europe of *C. nigrum* and of *C. bryoniicola* in Italy. *Colletotrichum fioriniae* has been detected for the first time in Italy as pathogenic on oregano. Similarly, *C. fructicola* has been found for the first time on the *Salvia* genus in Italy. Moreover, this study highlights the ability of *C. ocimi* to colonize as contaminant microorganism, the seeds of basil.

5. Conclusions

Further studies are required to resolve the host range and cross pathogenicity of these *Colletotrichum* species. Moreover, further investigations are needed about other aromatic or ornamental plant species cultivated in Northern Italy as well as elsewhere.

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