Emergence of Leaf Spot Disease on Leafy Vegetable and Ornamental Crops Caused by *Paramyrothecium* and *Albifimbria* Species

Slavica Matić,^{1,†} Giovanna Gilardi,¹ Maria Lodovica Gullino,^{1,2} and Angelo Garibaldi¹

¹ Centre of Competence for the Innovation in the Agro-environmental Sector (AGROINNOVA), Università di Torino, 10095 Grugliasco, Torino, Italy

² Department of Agricultural, Forestry and Food Sciences, Università di Torino, 10095 Grugliasco, Torino, Italy

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ABSTRACT

The genera *Paramyrothecium* and *Albifimbria* have been established from the former genus *Myrothecium* and they generally comprise common soil-inhabiting and saprophytic fungi. Within these genera, only two fungi have been recognized as phytopathogenic thus far: *P. roridum* and *A. verrucaria*, both of which cause necrotic leaf spots and plant collapse. Severe leaf necrosis and plant decay have been observed in Northern and Southern Italy on leafy vegetable crops. Thirty-six strains of *Paramyrothecium*- and *Albifimbria*-like fungi were isolated from affected plants belonging to eight different species. Based on morphological characteristics, 19 strains were assigned to *A. verrucaria*, whereas the remaining strains, which mostly resembled *Paramyrothecium*-like fungi, could not be identified precisely. Molecular characterization of six loci (internal transcribed spacer [ITS], β -tubulin [*tub2*], calmodulin [*cmdA*], translation elongation

factor 1-alpha [*tef1*], large subunit ribosomal RNA [LSU], and mitochondrial ATP 6 synthase 6 [*ATP6*]) of the 36 new isolates and three previously ITS-characterized isolates assigned all strains to four species: *A. verrucaria*, *P. roridum*, *P. foliicola*, and *P. nigrum*. Single and concatenated phylogenetic analyses were conducted, and they clearly distinguished the isolated fungi into four different groups. *A. verrucaria*, *P. roridum*, *P. foliicola*, and *P. nigrum* were able to induce leaf necrosis singly, and they were confirmed to be the causal agents of the leaf spot disease through pathogenicity assays. The involvement of fungi previously considered saprophytic (i.e., *P. foliicola* and *P. nigrum*) in the development of plant disease for the first time deserves particular attention because of the possibility of their transmission by seeds and the limited knowledge of their management with chemicals.

Species of the genera *Paramyrothecium* and *Albifimbria* (formerly in the genus *Myrothecium*) (Lombard et al. 2016) are common soil inhabitants and saprophytes of decaying plant material worldwide (Domsch et al. 2007). They are efficient cellulose decomposers and have been used as biopesticides for the control of weeds, nematodes, and insects (Abbas et al. 2001; Perry et al. 2000; Zhao et al. 2011). The former genus, *Myrothecium*, was subjected to a profound revision (Lombard et al. 2016), which resulted in the recognition of 13 new genera on the basis of the polyphyletic origin of its species, and more than 15 species have been reported within two renamed genera, *Paramyrothecium* and *Albifimbria*. Despite the large number of species, only two of them (*P. roridum* and *A. verrucaria*) include important plant pathogens (Chen et al. 2016, 2018; Garibaldi et al. 2016a; Tulloch 1972). Both species cause similar types of leaf spot disease in various cultivated vegetable and ornamental hosts. *P. roridum* affects various economic crops, including tomato, cucurbits, pepper, lamb's lettuce, cotton, soybean, *Petunia* spp., Rieger begonia, *Anthurium andraeanum*, and others (Ben et al. 2015a, 2015b, 2017; Farr and Rossman 2018; Fish et al. 2012; Garibaldi et al. 2016a; Quezada Duval et al. 2010). *A. verrucaria* also infects important crops such as beet, spinach, sunflower, peanut, cotton, potato, wild rocket, and ornamental plants (Abbas et al. 2001; Farr and Rossman 2018; Garibaldi et al. 2016b, 2016c). *P. roridum* was previously reported in Italy on tomato and gardenia

(Cappelli 1996; Ciccarone 1953), whereas *A. verrucaria* was reported on rice (De Carolis 1972). However, the appearance of *P. roridum* and *A. verrucaria* on new hosts has increased, and these two species were observed for the first time in Italy in 2015 on leafy vegetable crops such as spinach, lamb's lettuce, and wild rocket (Gilardi et al. 2018).

Both *P. roridum* and *A. verrucaria* have been associated with the production of macrocyclic trichothecene (TRI) mycotoxins, such as verrucarins, roridin, epiroridin, mytoxin B, and epiroridin acid (Bosio et al. 2017; Liu et al. 2016; Siciliano et al. 2017; Weaver et al. 2009). These TRI compounds are phytotoxic and have cytotoxic mechanisms in humans (Chen et al. 2016). Their mode of action has allowed them to be used as bioherbicides for various weeds (Anderson and Hallett 2004; Lee et al. 2008; Weaver et al. 2016).

Species of the former genus *Myrothecium* were previously identified through the morphological characteristics of their asexual structures, particularly those of conidiophores and conidia. The difficulties involved in the clear differentiation between several *Paramyrothecium* spp., owing to their limited distinct morphological characteristics, have encouraged the development of molecular detection techniques to support their morphological characterization. These molecular techniques are based on the amplification and sequencing of the commonly used internal transcribed spacer (ITS) fungal barcoding marker and/or other genes (Chen et al. 2016; Krisai-Greilhuber et al. 2017; Lombard et al. 2016; Ruma et al. 2015; Trapp et al. 1998).

The objective of this study was to characterize the causal agents of leaf spot disease and collapse of eight different plant hosts on a molecular basis. Isolated fungal species were characterized by means of multilocus phylogenetic analyses. Pathogenicity tests and host range studies were carried out as well to evaluate disease severity and identify possible new hosts.

MATERIALS AND METHODS

Fungal isolates and morphological identification. Various leafy vegetable and ornamental crops (15 to 30 days after emergence) with necrotic leaf spot symptoms, which were grown

[†]Corresponding author: S. Matić; slavica.matic@unito.it

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in plastic houses and gardens, were collected between 2015 and 2018 in different areas in Northern and Southern Italy. Thirty-six fungal isolates obtained from leaf-spot samples of eight plant species (lettuce, lamb's lettuce, cultivated rocket, wild rocket, spinach, basil, orange coneflower, and *Echinacea* sp.) were used in this study (Table 1). Additionally, three fungal strains (PnITS4, AvITS6, and AvITS7), which were previously characterized on a morphological and molecular basis (ITS region) from lamb's lettuce, wild rocket, and spinach plants with leaf spot symptoms (Garibaldi et al. 2016a, 2016b, 2016c), were also used in this study (Table 1).

Isolations were performed from symptomatic leaves by dipping small sections of the leaves into 1% sodium hypochlorite, then rinsing them in sterile water and plating them in triplicate on 15 ml of potato dextrose agar (PDA; Merck, Darmstadt, Germany) amended with 25 mg per liter of streptomycin (Applichem, Darmstadt, Germany). Monoconidial cultures were prepared from each isolate and stored in 30% glycerol solution at -80°C . The 36 new isolates were identified morphologically as described by Lombard et al. (2016), based on microscopic observations of the conidial shape and size, and through an examination of the colonies.

DNA extraction and sequence amplification. An E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany) was used to extract the total DNA of 39 isolates from 100 mg of

mycelium grown on PDA dishes according to the manufacturer's instructions. Molecular identification was performed through amplification of the ITS (White et al. 1990), β -tubulin (*tub2*; Glass and Donaldson 1995), calmodulin (*cmdA*; Carbone and Kohn 1999; Groenewald et al. 2013), translation elongation factor 1- α (*tefl*; Carbone and Kohn 1999; O'Donnell et al. 1998), mitochondrial ATP synthase 6 (*ATP6*; Castlebury et al. 2004), and large subunit ribosomal RNA (LSU; Rehner and Samuels 1995; Vilgalys and Hester 1990). The primers used for the molecular identification are listed in Supplementary Table S1. The polymerase chain reaction (PCR) products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and sequenced in both directions (BMR Genomics Centre, Padua, Italy). All sequences were deposited in GenBank under the following accession numbers: ITS (MH707376 to MH707410, MH324475), *tub2* (MH824702 to MH824740), *cmdA* (MH807735 to MH807773), *tefl* (MH939202 to MH939240), *ATP6* (MH898688 to MH898726), and LSU (MH807502 to MH807540), with the exception of isolates PnITS4, AvITS6, and AvITS7, which were sequenced previously at the ITS region (Garibaldi et al. 2016a, b, c). The accession numbers of the reference isolates (Lombard et al. 2016) are also included in Supplementary Table S2.

TABLE 1. List of the *Albifimbria verrucaria*, *Paramyothecium foliicola*, *P. roridum*, and *P. nigrum* strains (1 to 39) and reference strains (40 to 45) used in this study

No.	Isolate	Host	Origin of isolation	Year of isolation	Location	Species
1	2-1	Lettuce	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
2	2-2	Lettuce	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
3	PnITS4	Lamb's lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
4	4 -1	Lamb's lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
5	4-2	Lamb's lettuce	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
6	4-3	Lamb's lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
7	5-1	Lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
8	5-2	Lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
9	5-3	Lettuce	Leaf	2015	Northern Italy	<i>P. nigrum</i>
10	AvITS6	Spinach	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
11	6-1	Spinach	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
12	6-2	Spinach	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
13	6-3	Spinach	Leaf	2016	Northern Italy	<i>A. verrucaria</i>
14	6-4	Spinach	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
15	6-5	Spinach	Leaf	2015	Northern Italy	<i>A. verrucaria</i>
16	AvITS7	Wild rocket	Stem	2015	Southern Italy	<i>A. verrucaria</i>
17	7-1	Wild rocket	Leaf	2015	Southern Italy	<i>A. verrucaria</i>
18	8	Lamb's lettuce	Stem	2016	Northern Italy	<i>A. verrucaria</i>
19	8-1	Lamb's lettuce	Leaf	2016	Northern Italy	<i>A. verrucaria</i>
20	8-2	Lamb's lettuce	Leaf	2016	Northern Italy	<i>A. verrucaria</i>
21	8-3	Lamb's lettuce	Leaf	2016	Northern Italy	<i>A. verrucaria</i>
22	9-1	Wild rocket	Leaf	2016	Southern Italy	<i>P. foliicola</i>
23	10-1	Lettuce	Leaf	2016	Northern Italy	<i>A. verrucaria</i>
24	11-1	Lettuce	Leaf	2017	Southern Italy	<i>A. verrucaria</i>
25	12-1	Basil	Seed	2017	Not available	<i>P. nigrum</i>
26	13-1	Basil	Leaf	2017	Northern Italy	<i>P. nigrum</i>
27	14-1	Cultivated rocket	Leaf	2015	Northern Italy	<i>P. nigrum</i>
28	14-2	Cultivated rocket	Leaf	2015	Northern Italy	<i>P. nigrum</i>
29	Rful	Orange coneflower	Leaf	2017	Northern Italy	<i>P. nigrum</i>
30	Bas5_18	Basil	Seed	2018	Not available	<i>A. verrucaria</i>
31	Bas4_1b	Basil	Seed	2018	Not available	<i>P. foliicola</i>
32	Bas3_m3	Basil	Seed	2018	Not available	<i>A. verrucaria</i>
33	Bas3_1a	Basil	Seed	2018	Not available	<i>A. verrucaria</i>
34	Bas4_m1	Basil	Seed	2018	Not available	<i>A. verrucaria</i>
35	Bas4_m2	Basil	Seed	2018	Not available	<i>P. foliicola</i>
36	AGR1r1	Orange coneflower	Leaf	2017	Northern Italy	<i>P. roridum</i>
37	AGR1r2	Orange coneflower	Leaf	2017	Northern Italy	<i>P. nigrum</i>
38	AGR2r2	Orange coneflower	Leaf	2017	Northern Italy	<i>P. nigrum</i>
39	Echi_orig	<i>Echinacea</i>	Leaf	2017	Northern Italy	<i>P. nigrum</i>
40	CBS 189.46	Potato	Leaf	— ^a	Cyprus	<i>A. verrucaria</i>
41	CBS 372.50	<i>Coffea</i> sp.	Twig	—	Colombia	<i>P. roridum</i>
42	CBS 357.89	<i>Gardenia</i> sp.	Unknown	—	Italy	<i>P. roridum</i>
43	CBS 419.93	Air	Air	—	Cuba	<i>P. foliicola</i>
44	CBS 113121	Decaying leaf	Leaf	—	Brazil	<i>P. foliicola</i>
45	CBS 116537	Soil	Soil	—	Spain	<i>P. nigrum</i>

^a Dashes indicate undefined year of isolation.

Sequence analyses. A sequence comparison was performed with sequences available in GenBank by means of the Basic Local Alignment Search Tool (BLAST) software package (<https://www.ncbi.nlm.nih.gov/>). Phylogenetic analyses were conducted on the basis of maximum likelihood and Bayesian inference. The following reference strains were included for each species: CBS 189.46 (*A. verrucaria*), CBS 372.50 and CBS 357.89 (*P. roridum*), CBS 419.93 and CBS 113121 (*P. foliicola*), and CBS 116537 (*P. nigrum*). MEGA 7 software was used for the maximum likelihood analysis (Kumar et al. 2016). A 2,728-bp concatenated data set was obtained with the ITS, *tub2*, *cmdA*, *tef1*, and LSU sequences, whereas the phylogenetic analysis was performed separately for the *ATP6* gene because of a lack of sequences for those reference strains. FindModel was used to select the best-fit nucleotide model of each region (<https://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) as follows: Tamura-Nei (TrN) for LSU, TrN plus gamma for ITS and *tub2*, Hasegawa-Kishino-Yano plus gamma for *tef1*, and general time reversible (GTR) plus gamma for *cmdA*, *ATP6*, and the concatenated tree. Maximum-likelihood trees were then constructed with bootstrap values based on 1,000 replications. The best-fit model of each data set was determined for the Bayesian analysis (Huelsenbeck and Ronquist 2001) using TOPALI version 2.5 (Milne et al. 2004); GTR plus gamma for ITS, *tub2*, *cmdA*, *tef1*, and *ATP6*; Felsenstein (1981) for LSU; and GTR plus invariable sites plus gamma for the concatenated tree. The Bayesian analysis was performed by discarding the first 25% of the saved trees as burn-in.

PCR-based detection. The portion of the *cmdA* gene showing the highest polymorphism between four characterized fungal species was identified by sequence analyses. This region was used to design forward and reverse primers for differentiation of four species. Thus, four sets of primers were designed: VERRCAL1×VERRCAL2 (*A. verrucaria*), FOLCAL1×FOLCAL2 (*P. foliicola*), NIGRCAL1×NIGRCAL2 (*P. nigrum*), and RORCAL1×RORCAL2 (*P. roridum*) amplifying 343, 354, 349, and 337 bp, respectively. Primer quality and specificity were checked by means of Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA) and BLAST sequence alignment (National Center for Biotechnology Information [NCBI]), respectively. The final volume of the PCR reaction (25 µl) contained 10 ng of DNA, 1× PCR buffer, 1.5 mM of MgCl₂, 0.2 µM of each primer, 0.2 mM of dNTPs, and 1 U of Taq DNA polymerase (Qiagen). The PCR cycling protocol consisted of 5 min at 94°C of initial denaturation; followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. The PCR products (5 µl) were analyzed by electrophoresis on a 1.2% agarose gel prestained with RedGel (Biotium, Hayward, CA), and the bands were visualized under ultraviolet light.

Pathogenicity tests. Six plant species were used for pathogenicity tests: lettuce (cultivar Gentilina; Maraldi Sementi, Cesena, Italy), lamb's lettuce (cultivar Palace; Meilland Richardier, Diemoz, France), cultivated rocket (cultivar Rucola Coltivata; La Semiorto, Sarno, Italy), wild rocket (cultivar Frastagliata Mazzocchi; Casalpusterlengo, Italy), spinach (cultivar Crocodile; Rijk Zwaan, The Netherlands), and basil (cultivar Genovese Gigante Italiko; Furia Sementi, Parma, Italy). Seeds were sown in 2-liter plastic pots in triplicate (nine seeds per pot) in a sterilized mixture of peat and perlite (80:20). The plants were grown in a greenhouse at 22 to 24°C before inoculation.

Following molecular identification, six strains that included all four fungal species were selected randomly for the pathogenicity tests (*P. nigrum* 13-1, *P. nigrum* Echi_orig, *P. foliicola* Bas4_1b, *P. roridum* AGR1r1, *A. verrucaria* AvITS6, and *A. verrucaria* Bas5_18). Isolates were grown on PDA amended with streptomycin sulfate for 10 days with a 12-h photoperiod in a growth chamber at 20 to 22°C. Spore suspensions with a final spore concentration of 10⁶/ml were prepared and used for the spray inoculation of above-ground organs of the plants (1 ml of suspension/pot). The plants

were artificially inoculated at 20 to 25 days after emergence, enclosed in a clear polyethylene moist chamber, covered by a transparent polyethylene film (to reach 100% relative humidity), and then moved to a growth chamber. They were maintained at temperatures of 24°C (day) and 22°C (night) at a photoperiod of 12 h and were watered twice a day. The moist chamber was removed 5 days postinoculation (dpi).

Disease severity was evaluated 7 to 10 dpi using a diagrammatic scale for percentage of infected leaf area as follows (Gullino et al. 2017): 0 indicated symptomless plants, 1 indicated up to 5%, 2 indicated 6 to 10%, 3 indicated 11 to 25%, 4 indicated 26 to 50%, and 5 indicated 51 to 100%. Symptoms were observed on 50 leaves per pot, searching for round, sunken, dark gray to brown spots. Disease severity was calculated as follows: Disease severity = $\Sigma(\text{number of leaves} \times X_{0-5}) / (\text{number of leaves})$, where X_{0-5} refers to an approximate class midpoint ($X_0 = 0\%$, $X_1 = 3\%$, $X_2 = 8\%$, $X_3 = 18\%$, $X_4 = 38\%$, and $X_5 = 76\%$).

RESULTS

Disease symptoms and fungal isolation. The observed symptoms of affected leafy vegetable and ornamental crops initially were circular, small 1- to 2-mm gray to brown spots with well-defined borders. Spots gradually expanded and coalesced to form necrotic lesions (around 30 mm) with concentric rings, and the affected leaf tissue had a water-soaked consistency. The fungal isolates were obtained from symptomatic plant tissue by plating on PDA plates, and a single strain was isolated from each individual plant. Fungal isolates were subjected to morphological and molecular identification.

Morphological identification. Fungal isolates showed abundant white aerial mycelium and dark sporodochia distributed in concentric rings resembling the mycelium of the former *Myrothecium* genus. The shape of the conidia permitted the separation of the isolates in two groups belonging to two genera: *Albifimbria* (more subglobose conidia) and *Paramyrothecium* (more cylindrical conidia). Furthermore, the first group of isolates was identified morphologically as *A. verrucaria* showing nonseptate and subglobose to ellipsoidal conidia without appendages (average $7 \times 3 \mu\text{m}$). They had white aerial mycelium with sporodochia, which had formed on the surface of the growth medium that contained dark to olive-green conidial masses.

Another group of isolates was *Paramyrothecium*-like and had almost identical morphological features. The isolates of this group were most similar to *P. roridum*, *P. foliicola*, and *P. nigrum*. These isolates showed nonseptate conidia with a cylindrical to ellipsoidal shape, hyaline to pale green color, and an approximate size of $6 \times 2 \mu\text{m}$. Additional structures consisted of abundant and white aerial mycelium and sporodochia that had developed in the form of concentric rings that contained dark green to black conidial masses. These species were primarily isolated from necrotic plant tissues. *P. foliicola* was distinguished from other *Paramyrothecium*-like fungi by the production of orange exudates, which diffused in the PDA medium, and by shorter conidiophore stipes (approximately 0.6 times the length of those of *P. nigrum* and *P. roridum*).

Molecular identification and phylogenetic analyses. When the ITS sequences of the 36 new strains were compared with those available at NCBI nucleotide database, 19 gave the highest identity (100%) with *A. verrucaria*, whereas 17 other strains showed 99 to 100% identity with different *Paramyrothecium* spp. Thirteen of the 17 strains were 99 to 100% similar to *P. roridum*, *P. nigrum*, *P. viridisporum*, *P. acadiense*, and a few others, whereas four strains (9-1, Bas4_1b, Bas4_m2, and AGR1r1) were 98 to 99% similar to *P. foliicola* and *P. roridum*. Since the ITS region was not conclusive for many of the strains, the molecular characterization was continued with another five loci (*tub2*, *cmdA*, *tef1*, LSU, and *ATP6*). Four loci (*tub2*, *cmdA*, *tef1*, and LSU) of 13 unidentified strains gave the highest identity with *P. nigrum* (reference strain

CBS 116537): 100% for *tub2* and LSU, 99% for *cmdA*, and 97% for *tef1*. Another four strains were similar (99%) to both *P. roridum* and *P. foliicola* for LSU, but three of them were more similar to *P. foliicola* for *tub2*, *cmdA*, and *tef1* (reference strains CBS 419.93 and CBS 113121), whereas one strain (AGR1r1) was more similar to *P. roridum* (reference strains CBS 372.50 and CBS 357.89). Furthermore, 17 unidentified strains were most similar to *P. roridum* (97 to 98%) regarding the *ATP6* gene, for which no *P. nigrum* and *P. foliicola* sequences were available at NCBI nucleotide database.

Phylogenetic analyses were then performed on gene portions of around 400 to 550 bp for ITS, *tub2*, *cmdA*, and *tef1* and portions of 600 to 800 bp for *ATP6* and LSU. The concatenated tree obtained on the basis of five partial sequences (ITS, *tub2*, *cmdA*, *tef1*, and LSU) was used to evaluate the genetic relatedness of all 39 studied fungal strains. Two different clusters were observed (Fig. 1); the first grouped all strains of *A. verrucaria*, including the reference CBS 189.46 strain, whereas the second was divided into two subclusters. One subcluster included the *P. nigrum*, *P. viridisporum*, *P. foeniculicola*, and *P. cupuliforme* strains with a 77% bootstrap value, and the other contained the *P. roridum* and *P. foliicola* strains with a 99% bootstrap value (together with their respective CBS reference strains). Furthermore, the *P. nigrum* subgroup (99% bootstrap value) contained all of the studied *P. nigrum* strains, together with the reference CBS 116537 strain, which was clearly separated from the phylogenetically close *Paramyrothecium* spp. (*P. viridisporum*, *P. foeniculicola*, and *P. cupuliforme*) (Lombard et al. 2016). The *P. foliicola* subgroup (66% bootstrap value) contained three of the studied strains (9-1, Bas4_1b, and Bas4_m2) together with the *P. foliicola* reference strains, which were separated from the *P. roridum* subgroup (73% bootstrap value) that contained the AGR1r1 strain and the CBS reference strain. The Bayesian consensus tree for five loci was concordant with the tree topologies obtained from the maximum likelihood analyses (data not shown). A single *ATP6* phylogenetic tree was also able to differentiate four species, although the reference strain sequences of *P. nigrum* and *P. foliicola* were not available (Supplementary Fig. S1).

PCR-based detection. Variable regions that were able to discriminate *P. foliicola*, *P. nigrum*, *P. roridum*, and *A. verrucaria* were searched for in a multiple sequence alignment of the six loci. The highest diversity was identified in the *cmdA* region of the studied strains, which was used to design the primers for differentiation of four identified species (Supplementary Fig. S2). Estimated amplicons using these primers were obtained for *P. foliicola*, *P. nigrum*, *P. roridum*, and *A. verrucaria* by conventional PCR. The amplicons were only observed in the species for which specific primers had been designed and not in those of the other three fungal species.

Pathogenicity assays. The first symptoms appeared as small necrotic spots on the leaf surface of four artificially inoculated plant hosts (lamb's lettuce, cultivated rocket, spinach, and basil), whereas necrosis was more distributed in patches and at the leaf margins on lettuce and wild rocket at 7 dpi (Fig. 2). The necrotic lesions expanded and coalesced (3 to 4 cm of length) at 15 dpi, and this was followed by a general plant collapse. All studied strains were able to reproduce the leaf spot symptoms on their original hosts of isolation. Furthermore, the strains isolated from one host in natural conditions were able to infect other hosts under experimental conditions (Supplementary Table S3).

No important difference in disease severity was observed between the four fungal species on the cultivated and wild rocket at 1 week after inoculation, where disease severity ranged from 39 to 59%, depending on the fungal species and strain (Fig. 3). On the other hand, *P. foliicola* (Bas4_1b) on lettuce, *A. verrucaria* (AvITS6) on lamb's lettuce, and *P. nigrum* (Echi_orig) on basil and spinach were the most severe (39, 42, 26, and 54% disease severity, respectively). Interestingly, pathogenicity of *P. nigrum* and *P. foliicola* (either naturally or following artificial inoculation) has not been reported previously.

The highest percentage of plant mortality (83%) was observed in lamb's lettuce infected with *A. verrucaria* AvITS6 and spinach infected with *P. nigrum* Echi_orig, followed by wild rocket, cultivated rocket, and lettuce infected with *P. foliicola* Bas4_1b (82, 67, and 50%, respectively). In general, these strains appeared to be more detrimental to plant vitality on plant hosts that were different from the original plant hosts of isolation.

DISCUSSION

A phylogenetic inference study of the family *Stachybotriaceae* has revealed a polyphyletic origin of the *Myrothecium* genus, which has been revised and divided into 13 new genera (Lombard et al. 2016), including genera *Paramyrothecium* and *Albifimbria*. Of these genera, only *P. roridum* and *A. verrucaria* are presently considered prominent plant pathogens (Belisario et al. 1999; Bharath et al. 2006; Garibaldi et al. 2016b, c; Saira et al. 2017; Tulloch, 1972). Furthermore, there have been increasing reports of diseases caused by *P. roridum*; leaf spots have been reported in Italy, the United States, Brazil, China, Pakistan, and Ghana on crops such as lamb's lettuce, pepper, watermelon, and soybean, as well as on many ornamentals (Alam et al. 2017; Ben et al. 2015a, b, 2016, 2017; Farr and Rossman 2018; Fujinawa et al. 2016; Haudenschild et al. 2018; Jordan et al. 2018; Li et al. 2015). There have also been reports concerning *A. verrucaria*, and these have been prevalent on spinach and wild rocket in Italy (Garibaldi et al. 2016b, c) and on maize in Pakistan (Saira et al. 2017). On the other hand, to the best of the authors' knowledge, there have been no reports of *P. nigrum* and *P. foliicola* as plant pathogenic fungi. Only one CBS strain, isolated from soil, has been characterized on a morphological and molecular basis as *P. nigrum* (CBS 116537; Lombard et al. 2016), whereas a few gene sequences of other *P. nigrum* strains, originating from *Catharanthus roseus*, *Vinca minor*, and *V. herbacea*, are available at NCBI nucleotide database but without any phytopathological data or additional details. Furthermore, two *P. foliicola* strains from air and decaying leaf tissue (CBS 419.93 and CBS 113121), which were characterized in the study by Lombard et al. (2016), are the only available strains that have been described thus far.

A total of 36 new strains from six leafy vegetable species and two ornamental crops and three previously ITS-identified strains of *P. roridum* and *A. verrucaria* (Garibaldi et al. 2016a, b, c) were documented in this study as the causal agents of necrotic leaf spot and plant collapse. The morphological characteristics of the isolated fungi were similar, and they were considered members of the former genus *Myrothecium*. Microscopic observations of sporodochia and conidia allowed 19 *A. verrucaria* isolates to be identified, whereas the other 17 isolates could not be identified precisely as a result of the presence of morphologically identical species within the genus *Paramyrothecium* (Krisai-Greilhuber et al. 2017; Lombard et al. 2016). These *Paramyrothecium*-like isolates were most similar to *P. roridum*, *P. nigrum*, and *P. foliicola* (Krisai-Greilhuber et al. 2017).

The lack of distinct morphological characteristics suggested the use of a multilocus phylogenetic approach, since ITS-based identification alone was not conclusive to identify these species, as already reported previously (Chen et al. 2016). The ITS sequences confirmed the highest identity with *A. verrucaria* in those strains that were morphologically identified as that species. On the other hand, *Paramyrothecium*-like strains gave the highest identity to several *Paramyrothecium* spp., which could not be distinguished by means of ITS. Multilocus combined phylogenetic analysis, based on ITS, *tub2*, *cmdA*, *tef1*, and LSU, permitted the studied strains to be clearly identified as *A. verrucaria* and *Paramyrothecium* spp. (*P. nigrum*, *P. foliicola*, and *P. roridum*), which were further grouped into different subclusters and subgroups. All of the studied strains were grouped together with their corresponding reference strains. This study was in accordance with the multigene phylogenetic approach performed by Chen et al.

(2016), which allowed the separation of *A. verrucaria* and *P. roridum* into different clades using five loci (SSU, LSU, *ATP6*, *EF1-α*, and *RPB1*). Furthermore, our study confirmed the results of the combined phylogenetic analysis of Lombard et al. (2016) and allowed a precise separation of *P. nigrum*, *P. roridum*, and *P. foliicola*, together with the differentiation of *A. verrucaria*.

The molecular characterization of the 39 strains in this study revealed, for the first time, the natural presence of additional fungal species (besides *A. verrucaria* and *P. roridum*) involved in the leaf spot disease reported in Northern Italy (Garibaldi et al. 2016a, b, c), and increased the number of reported hosts as well. Thus, apart from *A. verrucaria*, *P. foliicola* has also been found to be involved in

Fig. 1. Phylogenetic relationships among *Paramyrothecium nigrum*, *P. foliicola*, *P. roridum*, and *Albifimbria verrucaria*, based on internal transcribed spacer (ITS), β -tubulin (*tub2*), calmodulin (*cmdA*), translation elongation factor (*tef1*), and large subunit ribosomal RNA (LSU) sequences. The concatenated phylogenetic tree was obtained by maximum likelihood analysis using the general time reversible plus gamma model to differentiate the four fungal species. The strain designation, host affiliation, and fungal species are indicated for each strain. The reference isolates of the four fungal species and phylogenetically close species (Lombard et al. 2016) are shown in italics.

this disease on wild rocket (Garibaldi et al. 2016c). *A. verrucaria* has been identified from infected lamb's lettuce plants along with the previously reported, ITS-characterized *P. roridum* (Garibaldi et al. 2016a). Furthermore, the multilocus molecular identification in this study showed that what was thought to be *P. roridum* from lamb's lettuce is actually *P. nigrum*. The presence of either *A. verrucaria* or *P. nigrum* has been observed on infected lettuce. Three species (i.e., *A. verrucaria*, *P. foliicola*, and *P. nigrum*) have

been identified in leaf spot-diseased basil. Furthermore, their presence has been observed on ornamentals: *P. roridum* and *P. nigrum* on orange coneflower and *P. nigrum* on *Echinacea* sp. When artificial inoculation was carried out, these four fungal species had a wider host range compared with that in natural conditions.

The sudden outbreak of the previously unknown leaf spot disease in Northern and Southern Italy, the involvement of four fungal species of the former genus *Myrothecium*, and the expansion of its

Fig. 2. Symptomatology observed 1 week postinoculation with *Paramyrothecium nigrum*, *P. foliicola*, *P. roridum*, and *Albifimbria verrucaria* on 4-week-old leafy vegetable hosts. The strains used for pathogenicity tests are indicated at the top of the figure.

natural host range could be related to climatic changes. Siciliano et al. (2017) reported a significant increase in leaf spot on spinach caused by *A. verrucaria* in increased temperature and CO₂ conditions. Furthermore, the concentrations of the verrucarins A and roridin E mycotoxins were almost two times higher in increased temperature or CO₂ conditions. Similar accounts of new necrotic leaf spot diseases have been reported on leafy vegetable crops in Northern Italy, such as those caused by *Pleospora bjoerlingii* and *Fusarium equiseti* (Garibaldi et al. 2007; Gullino et al. 2014).

Simulated increases in global air temperatures and CO₂ levels have evidenced a higher leaf spot incidence on leaf beet by *Pleospora bjoerlingii* as a result of increasing CO₂, whereas *F. equiseti* has been found to cause severe losses on lettuce as a result of temperature increases (25 to 30°C) as well as on wild rocket (30 to 35°C) (Garibaldi et al. 2016d; Gilardi et al. 2017; Gullino et al. 2018). Furthermore, increased temperature and CO₂ levels might be responsible for the emergence of the new pathogen races, as a result of the action of evolutionary forces on boosting pathogen

Fig. 3. Disease severity (percentage of affected leaf area) 1 week postinoculation with *Paramyothecium nigrum*, *P. foliicola*, *P. roridum*, and *Albifimbria verrucaria* on 4-week-old artificially inoculated leafy vegetable hosts. Values are means and standard deviations of nine replicates.

populations through raised fecundity and the number of infection cycles (Chakraborty 2013).

Apart from a changing environment, the wide host range of the four studied fungal species should also be taken into consideration. We found an expanded host range of vegetable and ornamental crops affected by leaf spot disease, compared with 2015 when the disease was observed for the first time. It would be interesting to evaluate whether only host range expansion occurred or whether it was supported by a host shift, in which the fungus lost its capacity to infect the primary hosts such as tomato, gardenia, and others. *Myrothecium*-like fungi are common soil inhabitants and saprophytes on a range of plant residues but could possibly become phytopathogenic on various plant hosts as a result of their polyphagous nature. There have been examples of increased pathogen virulence on new hosts. Two new *Diaporthe* species (*D. limonicola* and *D. melitensis*), which represent saprobes, endophytes, or plant pathogens, were found to be associated with an emergence of a new destructive dieback disease of lemon plants in Europe, and they affected all tested *Citrus* species (Guarnaccia and Crous 2017). *Ramularia collo-cygni*, a European barley pathogen that has emerged in South Africa and Russia, has become pathogenic on oats and wheat (Havis et al. 2015; Huss et al. 2005; Kaczmarek et al. 2017). In China, a new species that affects stored asparagus has emerged (*Fusarium asiaticum*) (Zhu et al. 2013). Often the emergence of new diseases is associated with the introduction of pathogens into new geographic areas (Gladioux et al. 2015; Pautasso et al. 2015; Summerell 2017), but this appears unlikely for the four identified species in this study because they show moderately high intraspecific genetic variation, at least in the case of *A. verrucaria* and *P. nigrum*, for which a larger number of strains was available for diversity studies.

The risks posed by new disease outbreaks may also be associated with seed transmission of the causal agent, the globalization of the seed market, and changes in agricultural practices. Both *A. verrucaria* and *P. roridum* are seed-transmitted pathogens (Belisario et al. 1999; Nguyen et al. 1973).

A. verrucaria, *P. roridum*, *P. foliicola*, and *P. nigrum* have been found as single isolates obtained from individual plants. Furthermore, fungal isolates originated from different hosts, different locations, and different years of isolation, and all have shown the ability to cause leaf spot disease as single isolates. Further work involving field isolations and artificial inoculations is needed to establish whether mixed infections of these four species could be associated with the leaf spot disease in Italy 4 years after disease establishment.

In conclusion, this study is the first overview of the diversity of *Myrothecium*-like fungi involved in newly emerged leaf spot diseases of leafy vegetables and ornamental crops in Italy. The presence of four species (*A. verrucaria*, *P. roridum*, *P. foliicola*, and *P. nigrum*) as the causal agents of this disease was documented, and data about their symptomatology and pathogenicity were provided. Multilocus phylogenetic analyses allowed precise species differentiation and identified, for the first time, *P. nigrum* and *P. foliicola* as the causal agents of a plant disease. Studies using more loci and more reference strain sequences are needed to address inter- and intraspecific evolutionary questions pertaining to members of this important polyphyletic *Stachybotriaceae* family.

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