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Paraphoma garibaldii sp. nov. causing leaf spot disease of *Campanula rapunculoides* in Italy

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pathogenicity
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Abstract: Leaf and stem spots are among the most important diseases compromising ornamental plants worldwide. In this study, *Paraphoma garibaldii sp. nov*. is described from leaf lesions on *Campanula rapunculoides* in Piedmont, Northern Italy. The new species was characterised using a polyphasic approach including morphological characterisation and a multilocus molecular phylogenetic analysis based on partial nucleotide sequences of the translation elongation factor 1-α (*tef1*), the internal transcribed spacers (ITS) region and the β-tubulin (*tub2*) markers. Pathogenicity tests and the fulfilment of Koch's postulates confirm *P. garibaldii* as a novel foliar pathogen of *Campanula rapunculoides*. Presently, the fungal infection due to *Paraphoma garibaldii* is known from a single location in Italy, and further surveys are required to determine its distribution and relative importance.

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INTRODUCTION

The genus *Phoma* was introduced by Saccardo (1880), but the generic concept was significantly revised by Boerema & Bollen (1975). Boerema *et al.* (2004) divided this genus in nine sections based on morphological features. The section *Paraphoma* was distinguished based on the presence of setose pycnidia and muriform chlamydospores. However, this classification system revealed several difficulties in understanding species boundaries and in reflecting the evolutionary relationships among species. Furthermore, molecular analyses revealed that *Paraphoma* is polyphyletic, and related to genera affiliated with the families *Phaeosphaeriaceae* (de Gruyter *et al.* 2010), *Cucurbitariaceae* and *Coniothyriaceae* (Chen *et al.* 2015). *Paraphoma* is based on *P. radicina*, which was isolated from roots of *Prunus cerasus* in Australia and from rootstocks of *Malus sylvestris* in the Netherlands (de Gruyter *et al.* 2010). Currently, 14 species are included within the genus: *P. chlamydocopiosa*, *P. chrysantemicola*, *P. convolvuli*, *P. dioscoreae*, *P. fimeti*, *P. ledniceana*, *P. melnickii*, *P. pye*, *P. radicina*, *P. rhaphiolepidis*, *P. salicis, P. variabilis* and *P. vinacea* (Crous *et al*. 2021). Species within *Paraphoma* are generally regarded as soil-borne pathogens. They usually cause root and crown rot disease, but they have been isolated from necrotic leaf spots on *Tanacetum cinerariifolium* (Moslemi *et al.* 2016, 2018). Several *Paraphoma* spp. have been reported in association with ornamental and herbaceous plant hosts. For instance, *P. chrysanthemicola* was isolated from leaf spots on *Atractylodes japonica* in China (Ge *et al.* 2016). Three *Paraphoma* species were found in association with *T. cinerariifolium* in Australia: *P. vinacea* (Moslemi *et al.* 2016), *P. chlamydocopiosa* and *P. pye* (Moslemi *et al.* 2018), *Paraphoma radicina* was isolated from crown rot on *Medicago sativa* in China (Cao *et al.* 2020), while *P. convolvuli* and *P. melnikiae* were identified in association with leaf spots of *Convolvulus arvensis* in Russia (Gomzhina *et al.* 2020).

Ornamental plants represent an economically important sector of agriculture worldwide. Presently, Europe is leading in ornamental plant production, with The Netherlands ranking first, followed by Italy (DG-AGRI-G2 2020). In particular, bedding plants represent a major group in the ornamental sector with a continuous increasing commercial value and relevance. However, seeds, propagation materials and growing media could consistently influence bedding plants cultivation, as there are several diseases affecting them (Guarnaccia *et al.* 2021a). *Campanula* spp. are popular bedding plants, and these are planted on the borders of parks and gardens (Garibaldi *et al.* 2017a). Several fungal pathogens have been found in association with *Campanula* spp. in Italy including *Sclerotinia sclerotium* on *Ca. carpatica* (Garibaldi *et al.* 2002), *Coleosporium campanulae* on *Ca. rapunculoides* and *Ca. trachelium* (Garibaldi *et al.* 2017b, 2021), and *Golovinomyces orontii* on *Ca. glomerata* and *Ca. rapunculoides* (Garibaldi *et al.* 2012, 2018). *Campanula* spp. are also severely affected by leaf anthracnose caused by *Colletotrichum lineola* and *C. nymphaeae* (Guarnaccia *et al.* 2021b) and Alternaria leaf spot caused by *Alternaria alternata* which can cause severe defoliation (Garibaldi *et al.* 2017a). Moreover, different *Campanula* spp. were reported as susceptible to *Rhizoctonia solani* and as hosts of phoma-like taxa, such as *Stagonosporopsis trachelii* (Garibaldi *et al.* 2015, Guarnaccia *et al.* 2021a).

In this study a new *Paraphoma* sp. associated with leaf spots on *Campanula rapunculoides* was identified and characterised on the basis of morphological features and multi-locus DNA phylogeny. Pathogenicity and Koch's postulates were tested.

MATERIALS AND METHODS

Field surveys and fungal isolation

The surveys were conducted in a garden in Piedmont, Northern Italy (45°36'43.8"N 8°03'22.7"E), a site constantly monitored as a representative area exposed to the introduction of new plant pests since the historical data known for this site and its geographical isolation.

At the end of June 2020, leaf spots and stem necrosis were observed on 6-mo-old plants of *Ca. rapunculoides*. The disease index was recorded as the number of symptomatic plants. Small sections (0.2–0.5 cm long) from the margin of lesions were surface disinfected with 1 % sodium hypochlorite for 1 min, rinsed once in sterile distilled water, dried on sterile filter paper and placed on 2 % potato dextrose agar (PDA) plates amended with 25 ppm streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated at 25 ± 1 °C under a 12 h photoperiod. After 48–72 h of incubation, mycelial plugs were taken from the margin of the resulting colonies and transferred to fresh PDA plates. After 5 d, pure cultures were established from single hyphal tip transfers. Stock cultures were maintained at -80 °C in the Agroinnova (University of Torino) culture collection, Torino, Italy. Reference strains and specimens are maintained in the CBS culture collection of the Westerdijk Fungal Biodiversity Institute (WI), Utrecht, the Netherlands.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted with an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany), according to the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using ITS1 and ITS4 primers (White *et al.* 1990). The primers TUB2Rd and TUB4Fd (Aveskamp *et al.* 2009) were used to amplify part of the β-tubulin (*tub2*) gene. The partial translation elongation factor 1-α (*tef1*) gene was amplified with EF1-728F (Carbone & Kohn 1999) and EF2 (O'Donnell *et al.* 1998) primers. The amplification mixtures and cycling conditions for all three loci were followed as described in each of the cited references. Both strands of the PCR products were sequenced by Eurofins Genomics Service (Ebersberg, Germany). The sequences generated were analysed using Geneious v. 11.1.5 (Kearse *et al*. 2012, Auckland, New Zealand) and consensus sequences were processed.

Phylogenetic analyses

The newly generated sequences were analysed using BLAST search on the NCBIs GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database to achieve a taxonomic framework by determining the closest relatives. The MAFFT v. 7 online program (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh & Standley 2013) was used to align each gene region of the sequences obtained from this study and sequences downloaded from GenBank. Alignments were then manually adjusted by MEGA v. 7 (Kumar *et al.* 2016). The analyses were conducted individually for each locus (data not shown) and as multi-locus analysis, with the aim of identifying the isolates at species level. Reference sequences were selected based on recent studies on *Paraphoma* species (Moslemi *et al.* 2016, Cao *et al.* 2020, Gomzhina *et al.* 2020, Magaña-Dueñas *et al*. 2021). The phylogeny was developed based on Maximum Parsimony (MP) approach for all individual loci, and on both MP and Bayesian Inference (BI) methods for the concatenated multilocus analyses. For BI, the best evolutionary model for each partition was selected with MrModeltest v. 2.3 (Nylander 2004) and incorporated into the analyses. MrBayes v. 3.2.5 (Ronquist *et al.* 2012) 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 established at 0.2 and trees were sampled every 1 000 generations. The analyses were considered done when the average standard deviation of split frequencies was less than 0.01. The MP analyses were conducted using PAUP (Swofford 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random additional sequences. Tree bisection-reconnection was adopted, 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 re-scaled consistency index (RC) were calculated, and the parsimony and the bootstrap analyses (Hillis & Bull 1993) were based on 1 000 replications. Sequences generated in this study were deposited in GenBank (Table 1), and the alignments in TreeBASE (www.treebase.org; study number S29045).

Morphology

Slide preparations were mounted in lactic acid from colonies sporulating on sterilised pine needles placed on 2 % tap water agar (PNA) (Smith *et al.* 1996). Observations were performed under a Nikon SMZ25 dissection-microscope, and a Zeiss Axio Imager 2 bright field microscope using differential interference contrast (DIC) illumination, and images recorded on a Nikon DS-Ri2 camera with associated software. Colony features and pigment production were described on 2 % malt extract agar (MEA), PDA and oatmeal agar (OA; Crous *et al*. 2019) after 2 wk at 25 °C. Colony colours were scored using the colour charts of Rayner (1970). The taxonomic novelty was registered in MycoBank (www.MycoBank.org; Crous *et al*. 2004).

Pathogenicity

Pathogenicity tests were performed on healthy *Ca. rapunculoides* plants grown in 2 L pots. The virulence of a representative isolate (CBS 148459) grown for 15 d on PDA at 25 °C, was tested. Leaves of three 5-mo-old plants of *Ca. rapunculoides* were sprayed with a conidial suspension (1×10^5 conidia/mL). Sterile water was sprayed on three plants used as negative control. All inoculated and non-inoculated plants were covered with a transparent plastic film to retain a high level of relative humidity (RH) and kept in a growth chamber at 23 °C with a 12 h photoperiod. The plastic film was removed after 7 d. The experiment was repeated once. All plants were irrigated 2–3 times per week and examined daily for disease symptom development. Disease incidence (DI) was recorded as described above. The inoculated fungi were re-isolated and identified by sequencing the *tub2* and *tef1* loci, thus fulfilling Koch's postulates.

Table 1. GenBank accession numbers of *Paraphoma* spp. and closely related taxa included in this study.

		GenBank accession no.[2]		
Species	**Culture No.[1]**	**ITS**	***tub2***	***tef1***
Juncaceicola alpina	CBS 456.84	KF251181	KF252285	KF253139
J. typharum	CBS 296.54	KF251192	KF252686	KF253148
Neosetophoma samarorum	CBS 138.96	KF251160	KF252655	KF253119
Neostagonospora caricis	CBS 135092	KF251163	KF252658	–
Paraphoma aquatica	FMR 16956^{T}	OU612361	OU612355	–
P. chlamydocopiosa	UMPc01	KU999072	KU999084	KU999080
P. chrysanthemicola	CBS 172.70	KF251165	KF252660	KF253123
	CBS 522.66^{T}	KF251166	KF252661	KF253124
P. convolvuli	MF 9.222	MG764055	–	–
	MF 9.265	MG764062	MG779457	–
	MF 9.301	MG764060	MG779461	–
P. dioscoreae	CBS 135100^{T}	KF251167	KF252662	KF253125
	CPC 11355	KF251168	KF252663	KF253126
	CPC 11361	KF251169	KF252664	KF253127
P. fimeti	CBS 170.70^{T}	KF251170	KF252665	KF253128
	CBS 368.91	KF251171	KF252666	KF253129
P. garibaldii	**CBS 148459**	**OL435708**	**OL449254**	**OL449256**
	CBS 148460	**OL435709**	**OL449255**	**OL449257**
P. ledniceana	CBS 146533	MT371091	MT372661	MT372654
P. melnikiae	MF 9.182	MG764058	MG779454	–
	MF 9.294^{T}	MG764059	MG779455	–
	MF 9.88	MG764063	MG779456	–
P. pye	UMPp02	KU999073	KU999087	KU999081
P. radicina	CBS 102875^{T}	KF251173	KF252668	KF253131
	CBS 111.79	KF251172	KF252667	KF253130
P. rhaphiolepidis	CBS 142524^{T}	KY979758	KY979924	KY979896
P. salicis	CBS 146797	MW883437	MW890140	–
P. vinacea	UMPV002	KU176885	KU176893	KU176897
Setophoma terrestris	CBS 335.29	KF251246	KF252729	KF253196
Xenoseptoria neosaccardoi	CBS 120.43	KF251280	KF252761	KF253227

[1] CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CPC: Culture collection of Pedro Crous housed at Westerdijk Fungal Biodiversity Institute; FMR: Faculty of Medicine and Health Sciences, Reus, Spain; MF: All-Russian Institute of Plant Protection; UMP: University of Melbourne, Ex-type and ex-epitype cultures are indicated with superscript T.

[2] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *tub2*: beta-tubulin gene; *tef1*: translation elongation factor 1-α gene. Sequences newly generated in this study are indicated in **bold**.

RESULTS

Field survey and fungal isolation

Leaf symptoms identified as those caused by *Paraphoma* spp. were found in the investigated site with a disease incidence value of 50 %, considered as the percentage of affected leaves. The symptoms were observed on 6-mo-old *Campanula rapunculoides* plants grown in open fields in a private garden. The observed symptoms consisted of grey to brown, necrotic, circular, converging lesions on leaves, chlorotic yellowing and, in some case, defoliation of the investigated host. Moreover, necrosis on stems and wilting of the apical part of the plant were observed. Several colonies resembling *Paraphoma* sp. appeared following isolation, and two monohyphal strains (CBS 148459, CBS 148460) were used for morphological and molecular characterisation.

Taxonomy

Paraphoma garibaldii Guarnaccia, M.L. Gullino & Crous, ***sp. nov.*** MycoBank MB 842029. Fig. 1.

Etymology: Named after Prof. Angelo Garibaldi, in recognition of his contribution to research on ornamental plant diseases.

Conidiomata pycnidial, erumpent to superficial on PNA, wall of 3-4 layers of brown, thin-walled *textura angularis*, globose, 200–300 µm diam, covered by brown, septate, thick-walled, subcylindrical

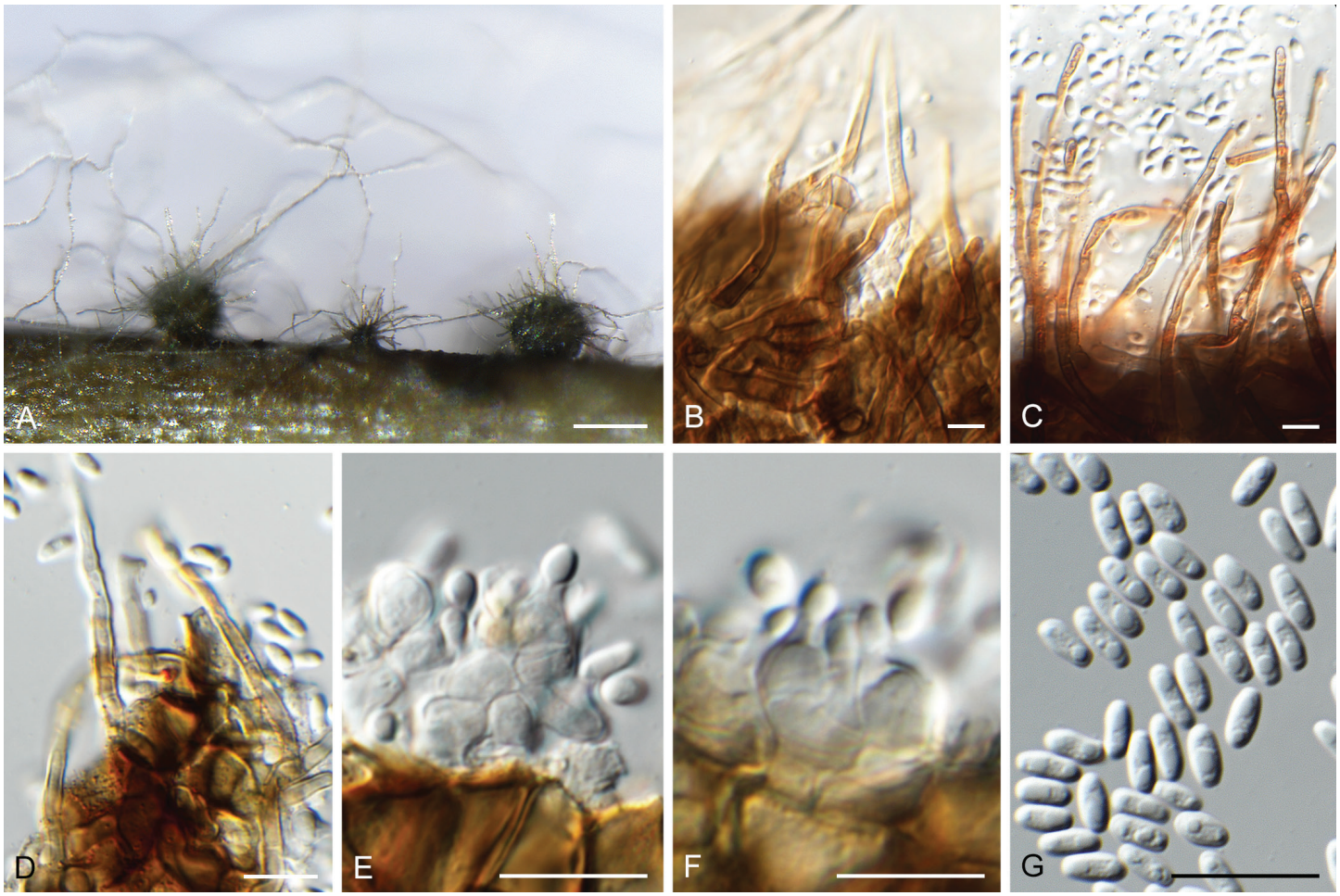

Fig. 1. *Paraphoma garibaldii* (CBS 148459). **A.** Pycnidia with setae forming on PNA. **B–D.** Brown setae arising from outer pycnidial wall. **E, F.** Conidiogenous cells giving rise to conidia. **G.** Aseptate, guttulate conidia. Scale bars: A = 300 µm; All others = 10 µm.

setae, 30–70 × 3–4 µm, with obtuse ends. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* lining the inner cavity, phialidic, hyaline, smooth-walled, ampulliform to doliiform, 5–8 × 4–6 µm, with prominent periclinal thickening. *Conidia* solitary, aseptate, hyaline, smooth-walled, guttulate, subcylindrical, obtuse at the apex and truncate at the base, (4–)5–6(–7) × (2–)2.5(–3) µm.

Culture characteristics: On MEA, PDA and OA, colonies erumpent, spreading with moderate aerial mycelium and even lobate margins, up to 70 mm diam after 2 wk, surface and reverse red.

Typus: **Italy**, Piedmont, Biella, on leaf spots of *Campanula rapunculoides* (*Campanulaceae*), May 2021, *A. Garibaldi* (**holotype** CBS H-24894, culture ex-type CBS 148459).

Additional material examined: **Italy**, Piedmont, Biella, on leaf spots of *Ca. rapunculoides*, May 2021, *A. Garibaldi,* CBS 148460.

Notes: *Paraphoma garibaldii* is phylogenetically distinct from all 14 species of the genus. Morphologically, its conidia are similar to those of *P. variabilis* (4–8 × 2–3 µm, from dung, Spain; Crous *et al*. 2021), but distinct in that the latter has greyish colonies and shorter (7–25 × 2.5–3 µm), subhyaline setae.

Phylogenetic analyses

Based on the results by BLAST search, all the sequences obtained in this study showed high similarity (around 96 %) with species included in the *Paraphoma* genus, however they were identical with no particular species. Three alignments representing single locus analyses of ITS, *tub2*, *tef1* (data not shown), and a combined alignment of the three loci were analysed. The single phylogenetic analysis generated by each locus produced a similar tree topology. The strains of *Paraphoma garibaldii* formed a well-supported monophyletic clade in the ITS, *tub2* and *tef1* single-locus trees, with maximum bootstrap values, respectively. The multi-locus phylogeny consisted of 30 sequences, including *Setophoma terrestris* (CBS 335.29, Gomzhina *et al.* 2020) as outgroup. A total of 1 172 characters (ITS: 1–502, *tub2*: 509–777, *tef1*: 784–1 172) were included in the phylogenetic analysis, 456 characters were parsimony-informative, 239 were variable and parsimony-uninformative, and 464 were constant. A maximum of 1 000 equally MP trees were saved (Tree length = 1 899, CI = 0.656, RI = 0.737 and RC = 0.483). Bootstrap support values from the MP analysis are included on the Bayesian tree in Fig. 2. For the BI, MrModeltest suggested that all partitions should be analysed with dirichlet state frequency distributions. The following models were recommended by MrModeltest and used: GTR+I+G for ITS, K80+G for *tub2* and HKY+G for *tef1*. In the BI, the ITS partition had 219 unique site patterns, the *tub2* partition had 161 unique site patterns, the *tef1* partition had 245 unique site patterns and the analysis ran for 675 000 generations, resulting in 1 352 trees of which 534 trees were used to calculate the posterior probabilities.

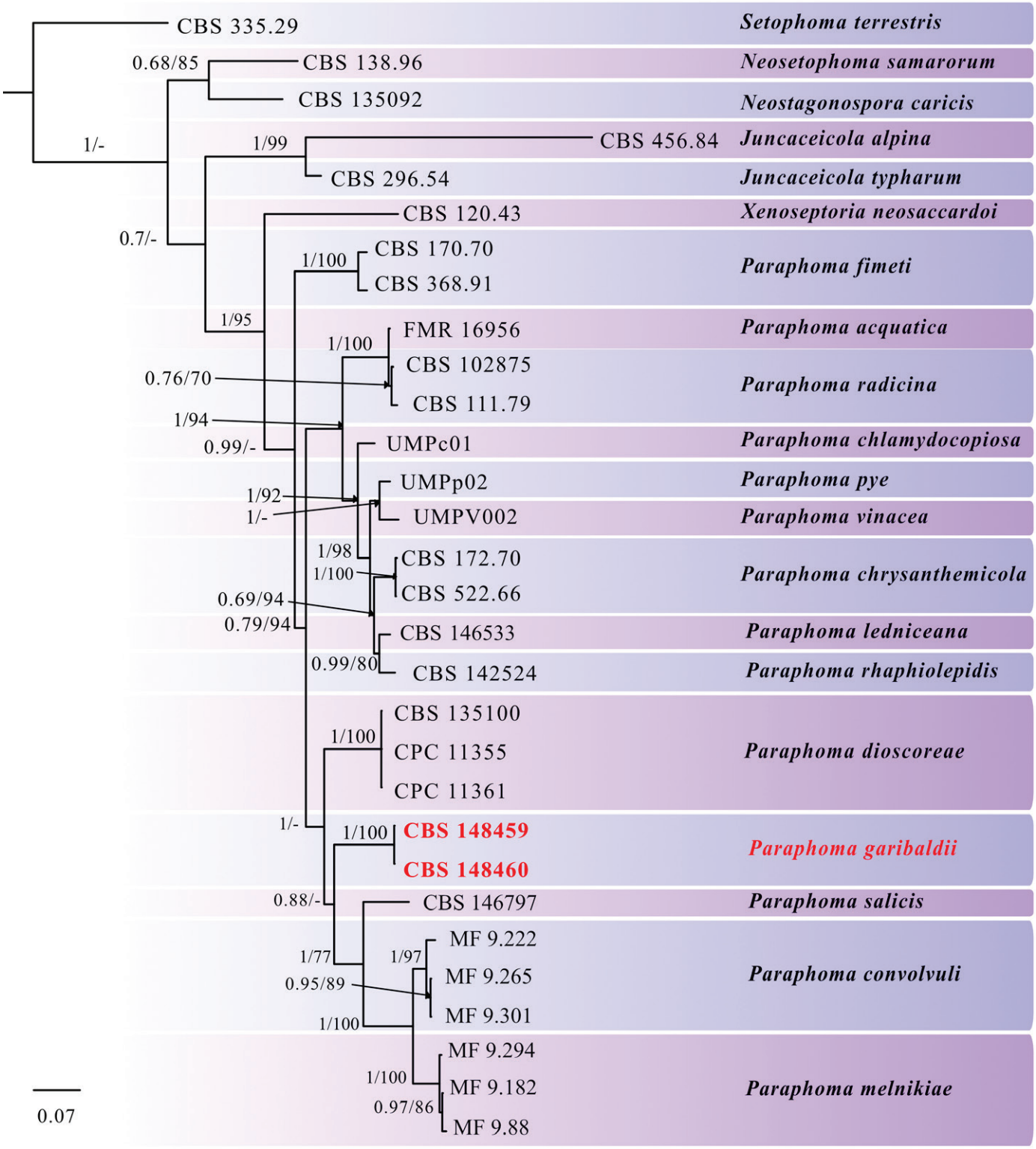

Fig. 2. Consensus phylogram of 1352 trees resulting from BI of the combined ITS, *tub2* and *tef1* datasets. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted with *Setophoma terestris* (CBS 335.29).

Pathogenicity

Isolate CBS 148459 was pathogenic for 100 % of the inoculated *Ca. rapunculoides* plants causing similar symptoms observed for the first time on the cultivated plants grown in the garden. Dark brown leaf spots appeared 7 d after inoculation, and leaves wilted 5 d after the appearance of large chlorotic areas and the expansion of necrotic tissues (Fig. 3). No symptoms appeared on control plants. The pathogen was consistently re-isolated from the inoculated plants and identified with molecular analysis as described above.

DISCUSSION

In this study two *Paraphoma* isolates were recovered from *Ca. rapunculoides* plants showing leaf spot symptoms in Piedmont, Northern Italy during 2021, and identified based on single and multi-locus (ITS, *tub2* and *tef1*) phylogenetic analyses, as well as morphological characters. These analyses revealed the two isolates to represent a novel species erected here as *Paraphoma garibaldii*.

The robust three-locus based analysis distinguished *P. garibaldii* from other *Paraphoma* species, and other genera

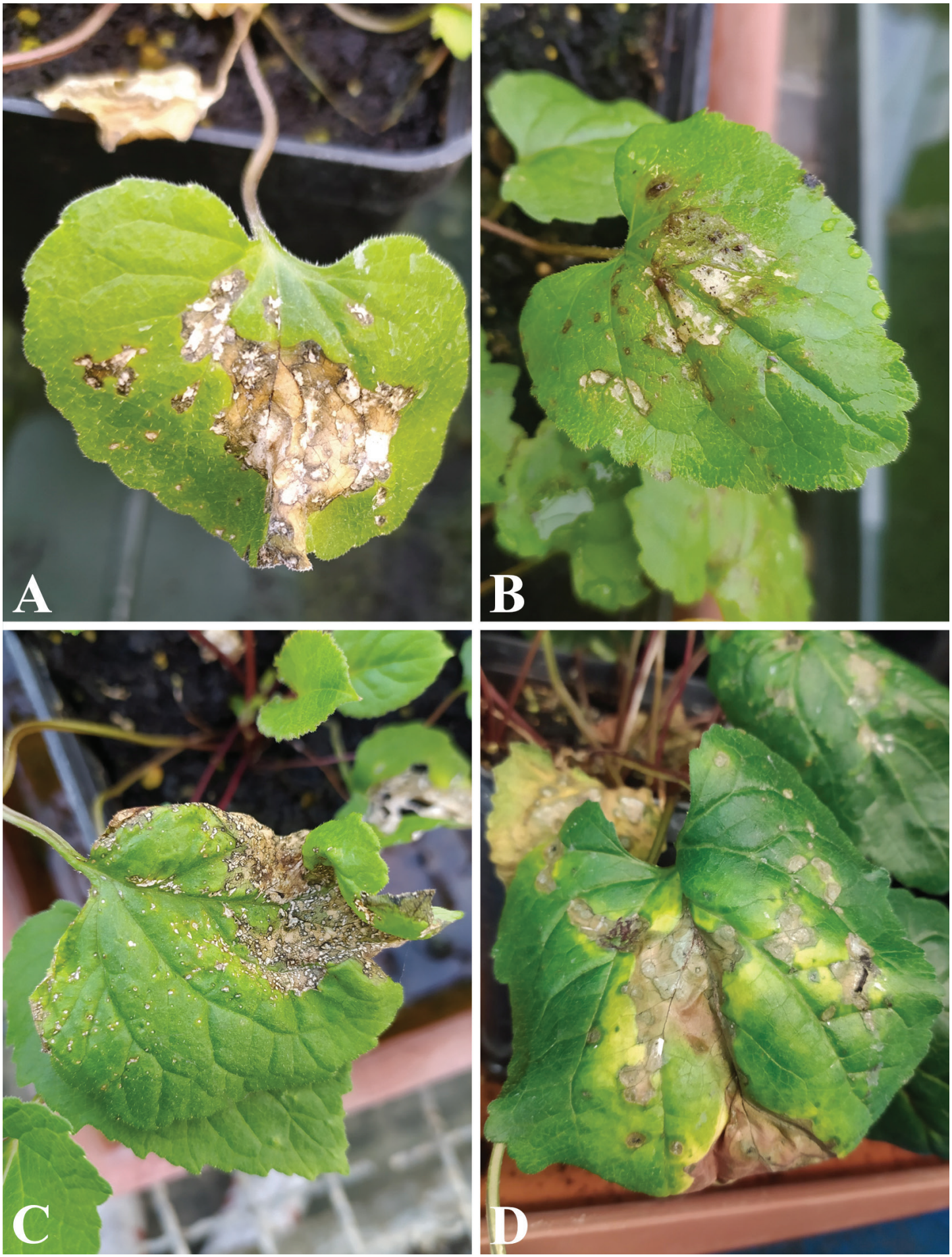

Fig. 3. Symptoms caused after artificial inoculation of *Paraphoma garibaldii* on *Campanula rapunculoides*. **A.** Necrotic leaf area. **B, C.** Leaf spots. **D.** Necrosis surrounded by a chlorotic area.

causing foliar diseases on this crop, such as *Alternaria*, *Coleosporium*, *Colletotrichum* and *Stagonosporopsis*. In spite on the recent detection of similar leaf diseases caused by other fungal species in the same geographic area (Guarnaccia *et al*. 2021b), *P. garibaldii* was the only fungus associated with leaf spot disease in this survey, demonstrating it was able to cause leaf spot disease independently. Furthermore, pathogenicity tests confirmed that *P. garibaldii* causes the disease on *Ca. rapunculoides,* thereby fulfilling Koch's postulates.

This study has revealed and characterised a novel pathogenic fungal species, *P. garibaldii,* associated with leaf spot on *Campanula rapunculoides*, which is one of the most common ornamental bedding plants in Italy. As no epidemiological data are yet available, it is not possible to suggest any control strategies to control *P. garibaldii* infections. Several previous studies in the same geographical area have revealed a wide diversity of soil- and air-borne fungal species (Garibaldi *et al*. 2017a), including more taxa pathogenic to *Campanula* spp. (Guarnaccia *et al*. 2021b). Further surveys are required to determine the distribution of *P. garibaldii*, as it might represent a limiting factor for future cultivation of *Ca. rapunculoides*.

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Conflict of interest: The authors declare that there is no conflict of interest.

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