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# Characterization and pathogenicity assessment of *Plectosphaerella* species associated with stunting disease on tomato and pepper crops in Italy

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This study follows a survey carried out in 2012 and 2013 on tomato and pepper crops in the Foggia province (southern Italy), for morphological, molecular and pathogenicity analyses of *Plectosphaerella* fungi. The *Plectosphaerella* genus includes several species that are pathogens of horticultural plants. The survey identified tomato and pepper crops that showed abundant wilt, leaf yellowing, and discolouration and necrosis of roots, plus collar and stem symptoms. Different fungi including *Plectosphaerella* spp. were isolated from tissues with and without symptoms. Subsequent molecular and morphological studies identified first records of *P. citrulli* infecting tomato and pepper, and *P. pauciseptata* and *P. ramiseptata* infecting pepper. Pathogenicity testing confirmed that most isolated species of *Plectosphaerella* caused symptoms on tomato and pepper, with *P. ramiseptata* the most aggressive. On the basis of these data, it is considered that *Plectosphaerella* species may cause stunting disease in tomato and pepper.

*Keywords: Capsicum annum*, MSP-PCR, pathogenic behaviour, phylogenetic analysis, Plectosphaerellaceae, *Solanum lycopersicum*

## Introduction

The Foggia province of southern Italy is one of the main areas for tomato and pepper production, although the volume of pepper production is less than for tomato. It is estimated that in the Apulia region about 20 570 ha are used for tomato (*Solanum lycopersicum*), of which 16 500 ha are in Foggia, compared to 2200 ha for pepper (*Capsicum annuum*), of which 1000 ha are in Foggia (ISTAT, 2014). Several diseases are responsible for serious losses in these crops. The most important pathogens for tomato and pepper are considered to be *Fusarium* spp., *Verticillium dahliae*, *Sclerotinia sclerotiorum*, *Phytophthora infestans*, *Rhizoctonia solani* and *Pyrenochaeta lycopersici* (Purdy & Bardin, 1953; Grogan *et al.*, 1979; Knapova & Gisi, 2002; Kuramae *et al.*, 2003; Lops *et al.*, 2011). In particular, some species of *Fusarium*, such as *F. oxysporum*, *F. solani* and *F. proliferatum* (Namiki *et al.*, 1994), and *V. dahliae* (Karagiannidis *et al.*, 2002), have spread rapidly and are aggressive, causing vascular wilt disease in Solanaceae in various areas of the world (Mao *et al.*, 1998).

Recently, Carlucci *et al.* (2012) described *Plectosphaerella* spp. as a new group of pathogens of several horticultural crops in Italy, including four novel species, *Plectosphaerella citrullae* (renamed according to Article 60 of the International Code of Nomenclature for Fungi as *Plectosphaerella citrulli*, based on the host from which it was isolated), *Plectosphaerella pauciseptata*, *Plectosphaerella plurivora* and *Plectosphaerella ramiseptata*; and another four renamed as *Plectosphaerella alismatis*, *Plectosphaerella delsorboi*, *Plectosphaerella oratosquillae* and *Plectosphaerella melonis*. However, no specific description of the symptoms was reported, as the main focus was the taxonomic reassessment of the genus. Moreover, *Plectosphaerella oligotrophica* from soil and *Plectosphaerella populi* from poplar are the other two new species described by Liu *et al.* (2013) and Crous *et al.* (2015), respectively. To date, based on previous and recent reports, the *Plectosphaerella* genus is composed of 11 species, which includes *Plectosphaerella cucumerina*.

Over the last 30 years, the *Plectosphaerella* genus has been isolated from different hosts throughout the world, with the most common species being *Plectosporium tabacinum*, synonymous with *P. cucumerina*.

Host plant infections by *Plectosphaerella* spp. are generally not well defined, as they are sometimes reported as either causal agents of wilt disease (Xu *et al.*, 2014), or root rot disease (Carrieri *et al.*, 2014). The aim of the present study was to identify and characterize a collection of fungal isolates obtained from tomato and pepper plants in Apulia (southern Italy). In particular, a detailed study was performed using morphological and molecular tools on all of the *Plectosphaerella* isolates retrieved from tomato and pepper plants both with and without symptoms, to understand which species are involved in wilt or root rot in these crops, and to establish the isolation frequencies of each species of tomato and pepper plants with and without symptoms. In addition, through

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the use of pathogenicity tests carried out under *in vitro* and *in vivo* conditions, a direct correlation is made between disease symptoms and *Plectosphaerella* species occurrence.

## Materials and methods

### Sampling of plants, isolates and isolations

From May to September 2012 and 2013, a total of 308 tomato and pepper plants that were symptomless or were showing various degrees of wilt severity were collected from several fields in the Foggia province of southern Italy. The symptoms observed consisted of yellowing and/or wilting foliage, yellow-brown discolouration of roots, collars and basal stems, corky brown areas on main roots, and/or discolouration and/or necrosis of rootlets.

The plants were uprooted at different growth stages: post-transplantation, blossom, green fruit and ripened fruit. They were transferred to the laboratory and examined either immediately or within 24 h of storage at 4 °C. Sampled plants consisted of 135 tomato plants with symptoms and 98 without, and 42 pepper plants with symptoms and 33 without (Table 1). The plant samples were washed and surface sterilized according to Fisher *et al.* (1992), and five tissue portions (5 × 5 mm) were cut from the primary and/or secondary roots, collars and basal stems. These pieces of tissue were placed on potato dextrose agar (PDA; 3.9% potato dextrose agar; Oxoid Ltd) supplemented with 400 ppm streptomycin sulphate (Sigma-Aldrich), and incubated in the dark at 23 ± 2 °C. All of the fungal colonies isolated were grown until sporulation, and then a conidial suspension was spread onto agar plates. After 24–36 h incubation, single germinated conidia were transferred to fresh PDA plates.

Morphological and culture features (i.e. type and colour of colonies, presence, shape and size of conidia, ascomata or conidiomata formation) were initially used to distinguish all of the fungal genera and species isolated from tissues with and without symptoms, which were grown on PDA medium for 10–21 days, at 23 ± 2 °C in the dark. A total of 942 isolates of *Plectosphaerella* spp. were collected, with the reference strains maintained in the culture collection of the Department of Sciences of Agriculture, Food and the Environment, of the University of Foggia, Italy. A detailed morphological study was carried out on this group based on structure and size, and conidiophore, phialide and conidia shape and size, according to Carlucci *et al.* (2012). The isolation frequency per host was calculated as the number of tissue portions infected by a given fungus divided by the total number of tissue segments incubated, expressed as percentages (Table 1).

To define any correlation between symptom expression and isolation frequency, factorial analysis of variance (ANOVA) was performed, after the datasets were evaluated in terms of whether they followed normal distributions (using Shapiro–Wilk tests; *W* tests), and had homogeneity of variance (using Levene tests). Statistical analyses were performed using STATISTICA v. 6 (StatSoft). Factorial ANOVA was performed separately on both datasets (i.e. tomato and pepper) for each fungal species isolated, on the basis of presence/absence of symptoms and vegetation stages, and interactions between these factors.

### DNA extraction and microsatellite PCR profiles

Genomic DNA of all isolates was extracted from fresh mycelia grown on PDA plates in the dark at 23 ± 2 °C for 10 days, according to Carlucci *et al.* (2013).

*Alternaria*, *Botrytis*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Pyrenochaeta*, *Rhizoctonia*, *Trichoderma* and *Verticillium* isolates were identified according to keys available in the related literature. As there was a large number of *Plectosphaerella* isolates (942), a preliminary screening was carried out based on the M13 minisatellite primers (5′-GAGGGTGGCGGTTCT-3′) (Meyer *et al.*, 1993), and microsatellite (MSP)-PCR profiles were generated according to Santos & Phillips (2009). The DNA banding patterns were analysed using the BIONUMERICS v. 5.1 software (Applied Maths), with calculation of Pearson's correlation coefficients and the unweighted pair group method with arithmetic means. The reproducibility levels were calculated by comparing the banding profiles obtained for the M13 primer. For this purpose, from any cluster, 10% of the strains were chosen at random, and their profiles were analysed again.

### Sequence analysis

The MSP dendrogram of isolates of the 942 *Plectosphaerella* spp. generated eight clades (Fig. 1), from which three isolates from both tomato and pepper samples were chosen as representative for sequencing of the internal transcribed spacer (ITS) region, giving a total of 24 and 21 isolates, respectively (Table 2). The ITS1 and ITS2 regions flanking the 5.8S ribosomal DNA were amplified with the ITS1 and ITS4 primers (White *et al.*, 1990). PCR amplifications were performed according to Carlucci *et al.* (2012). The amplified PCR fragments were purified before DNA sequencing, using NucleoSpin extract II purification kits (Macherey-Nagel). Both strands of the PCR products were sequenced by Eurofins Genomics Service (Milan, Italy).

### Phylogenetic analysis

The nucleotide sequences obtained were manually edited using BIOEDIT v. 7.0.19 (http://www.mbio.ncsu.edu/BioEdit). All of the sequences were aligned using CLUSTALX v. 1.83 (Thompson *et al.*, 1997), with additional *Plectosphaerella* sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov) that showed 98–100% similarity. Alignment gaps were treated as missing, and all of the characters were unordered and of equal weight. Maximum parsimony analysis was performed with PAUP v. 4.0b10 (Swofford, 2003), using the heuristic search option with 1000 random taxa additions, and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed, and all multiple equally parsimonious trees were saved. Bootstrap support values were calculated from 1000 heuristic search replicates and 100 random taxon additions. The tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI), and rescaled consistency index (RC) were calculated, and the resulting trees were visualized with TREEVIEW v. 1.6.6 (Page, 1996). Maximum likelihood analyses were carried out using RAxML (Stamatakis, 2006) on the webserver (http://phylobench.vital-it.ch/raxml-bb/index.php; Stamatakis *et al.*, 2008), with the gamma model of rate heterogeneity in effect and using the maximum likelihood search. The phylogenetic information contained in the indels was incorporated into the Bayesian phylogenetic analysis using simple indel coding, as implemented by GAPCODER (Young & Healy, 2003). Bayesian analyses were carried out with MRBAYES v. 3.0b (Ronquist & Huelsenbeck, 2003) using the Markov chain Monte Carlo method. The general time-reversible model of evolution was used (Rodriguez *et al.*, 1990), which included estimation of invariable sites and assumption of a discrete gamma distribution with six rate categories. Four Markov chain Monte Carlo chains were run simultaneously, starting from

Table 1 Isolation frequencies of fungal species from tomato and pepper plants with and without symptoms on the basis of plant growth stages.

| | Isolation frequency (%)[a] according to plant growth stage | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tomato plants | | | | | | | | | | Pepper plants | | | | | | | | | |
| | With symptoms | | | | | Symptomless | | | | | With symptoms | | | | | Symptomless | | | | |
| Fungal species | PTS | BS | GFS | RFS | Total | PTS | BS | GFS | RFS | Total | PTS | BS | GFS | RFS | Total | PTS | BS | GFS | RFS | Total |
| *Alternaria alternata* | – | 0.9 | 1.3 | 1.2 | 1.1 | – | 0.3 | 2.4 | 2.1 | 1.4 | – | 2.7 | 3.3 | 2.1 | 2.2 | – | 1.3 | – | 0.6 | 0.4 |
| *Botrytis cinerea* | – | 0.3 | 0.3 | 1.1 | 0.6 | – | – | 0.5 | 1.8 | 0.7 | – | – | – | 1.6 | 0.6 | – | – | – | 0.6 | 0.2 |
| *Rhizoctonia solani* | – | – | 0.3 | 0.1 | 0.1 | – | – | 0.5 | 0.2 | 0.2 | – | 0.7 | – | 0.4 | 0.3 | – | – | – | 0.6 | 0.2 |
| *Phytophthora infestans* | – | – | 0.1 | 0.3 | 0.2 | – | – | 0.3 | 0.6 | 0.3 | – | – | – | 0.8 | 0.3 | – | – | – | – | – |
| *Fusarium* spp. | 14.0 | 20.1 | 18.8 | 13.1 | 16.2 | 4.4 | 7.7 | 5.6 | 7.5 | 6.5 | 12.2 | 14.6 | 16.0 | 30.9 | 20.8 | 4.4 | 2.7 | 0.7 | 2.8 | 2.4 |
| *Penicilllium* spp. | 1.4 | 0.9 | 2.0 | 2.4 | 2.0 | 0.4 | 0.7 | 3.7 | 0.6 | 1.4 | 2.2 | 2.0 | 2.7 | 3.4 | 2.7 | – | – | 0.7 | 0.6 | 0.4 |
| *Plectosphaerella* spp. | 20.7 | 22.4 | 24.5 | 14.7 | 19.7 | 15.6 | 15.3 | 13.1 | 12.8 | 13.9 | 24.4 | 22.0 | 40.0 | 39.6 | 33.3 | 21.2 | 33.3 | 27.4 | 25.0 | 26.3 |
| *Pyrenochaeta lycopersici* | – | – | 0.9 | 1.5 | 0.9 | – | – | – | 0.4 | 0.1 | – | – | 2.7 | 0.8 | 1.0 | – | – | – | 0.6 | 0.2 |
| *Trichoderma* spp. | 1.3 | 0.9 | 0.3 | 0.8 | 0.7 | – | – | 0.3 | 0.8 | 0.3 | – | – | 2.0 | 0.4 | 0.6 | – | – | – | – | – |
| *Macrophomina phaseolina* | 2.7 | 1.5 | 0.7 | 1.4 | 1.3 | 2.2 | 2.4 | 1.3 | 1.7 | 1.8 | – | 6.0 | 4.0 | 3.8 | 3.8 | 2.2 | 4.0 | 2.0 | 2.3 | 12.4 |
| *Verticillium dahliae* | – | – | 0.4 | 0.7 | 0.4 | – | – | 0.3 | 0.2 | 0.1 | – | 0.7 | 0.7 | 0.4 | 0.5 | – | – | – | – | 0.0 |
| Other fungal species | 14.0 | 27.9 | 27.8 | 34.2 | 29.5 | 50.4 | 44.6 | 44.5 | 42.3 | 44.8 | 14.0 | 27.9 | 27.8 | 34.2 | 20.8 | 46.7 | 37.4 | 28.7 | 26.7 | 32.5 |
| Bacteria or no growth | 46.1 | 25.2 | 22.2 | 28.5 | 27.2 | 27.1 | 29.0 | 27.5 | 29.4 | 28.4 | 46.1 | 25.2 | 22.2 | 28.5 | 13.0 | 25.6 | 21.3 | 40.7 | 40.5 | 34.9 |
| Total tissue portions examined | 150 | 330 | 675 | 870 | 100.0 | 270 | 300 | 375 | 525 | 100.0 | 90 | 150 | 150 | 240 | 100.0 | 90 | 75 | 150 | 180 | 100.0 |
| Total[b] | 10 | 22 | 45 | 58 | 135 | 18 | 20 | 25 | 35 | 98 | 6 | 10 | 10 | 16 | 42 | 6 | 5 | 10 | 12 | 33 |

PTS, post-transplantation stage; BS, blossom stage; GFS, green fruit stage; RFS, ripened fruit stage. –, no species detected.

[a]Isolation frequency per host calculated as number of segments infected by a given fungus divided by total number of segments incubated.

[b]Total plants examined.

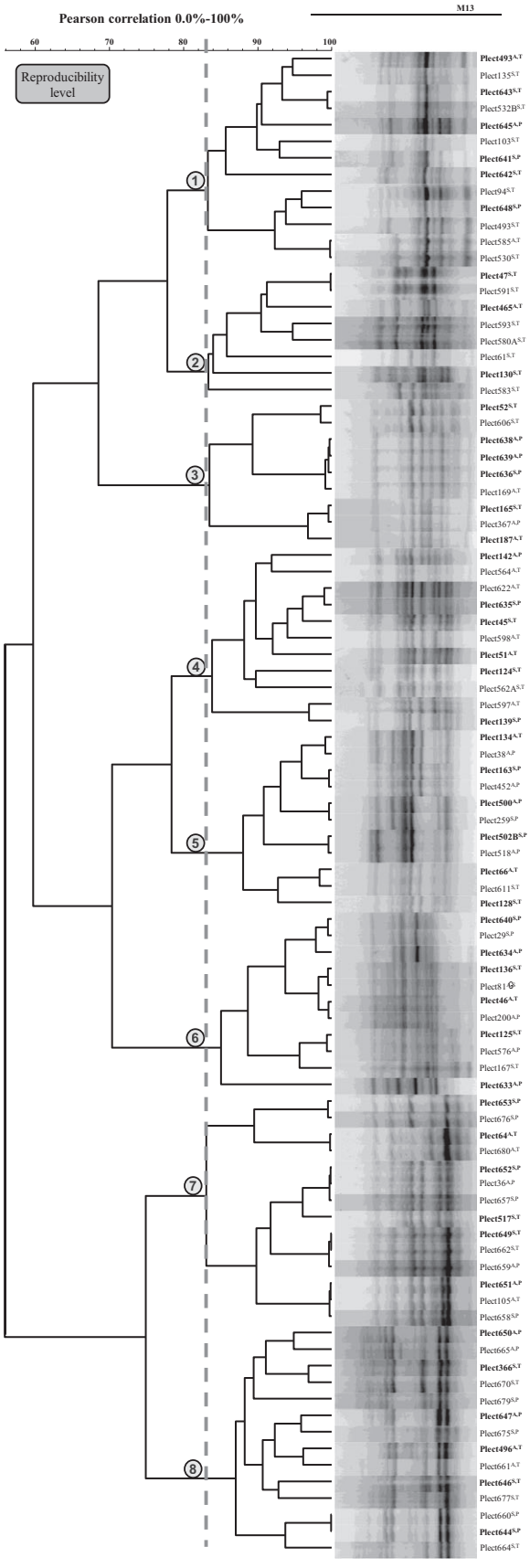


Figure 1 Consensus cladogram from the MSP-PCR profiles obtained with primer M13. Vertical dashed line, reproducibility level (83%) from which eight groups of isolates were inferred (indicated by numbered circles). On the right of the cladogram, electrophoretic profile of each strain is showed. A, symptomless; S, with symptoms; T, tomato plant; P, pepper plant.

random trees, for $10^6$ generations. The trees were sampled every 100th generation for a total of $10^4$ trees. The first $10^3$ trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang, 1996) were determined from a 50% majority-rule consensus tree generated from the remaining 9000 trees. The analysis was repeated three times starting from different random trees, to ensure that trees from the same tree space were being sampled during each analysis. Newly generated sequences were lodged in GenBank (Table 2), and the alignments and trees in TreeBASE (www.treebase.org). The sequences of *Gibellulopsis nigrescens* (CBS 387.35; GenBank: ITS, EF543854) and *Musicillium theobromae* (CBS 458.51; GenBank: ITS, EF543858) were used as out-groups in the phylogenetic analysis. To confirm the molecular identifications, all 45 of the isolates were subjected to microscopic observations, according to Carlucci *et al.* (2012).

On the basis of phylogenetic resolution of the *Plectosphaerella* clade, to determine whether the main factors of symptom presence/absence and vegetation stage were associated with the species of *Plectosphaerella*, factorial ANOVA was performed after evaluation of whether the datasets followed normal distributions, using Shapiro–Wilk tests (*W* tests), with homogeneity of the variance determined using Levene tests. Statistical analyses were performed using STATISTICA v. 6. Factorial ANOVA was performed separately on both datasets (i.e. tomato and pepper) for each fungal species isolated, on the basis of presence/absence of symptoms, vegetation stage, and the interactions between these factors.

## Pathogenicity tests

### In vitro *conditions*

To perform the pathogenicity tests, five fungal isolates for each *Plectosphaerella* spp. were used where possible, one as reference strain and two isolated from tomato and pepper plants with and without symptoms. Three fungal isolates of *P. melonis* and reference strains of *P. alismatis*, *P. delsorboi* and *P. oratosquillae* were also included in the pathogenicity tests.

Leaves from 35-day-old tomato cv. Talent and pepper cv. Pompeo seedlings grown from seed in a greenhouse were surface sterilized by immersion in 20% ethanol for 30 s. The disinfected leaves were dried with sterile paper and placed in Petri dishes containing 0.3% water agar (Difco). From each fungal isolate, three to five 20 μL drops of conidial suspensions adjusted to $10^4$ conidia $mL^{-1}$ with distilled water with Tween 20 (Sigma Aldrich) were placed on the leaf surfaces. The conidial suspensions were obtained from 10-day-old colonies of *P. alismatis* (CBS113362), *P. citrulli* (CBS131741, Plect64, Plect517, Plect651, Plect653), *P. cucumerina* (CBS131739, Plect128, Plect163, Plect167, Plect633), *P. delsorboi* (CBS116708), *P. melonis* (CBS131859, CBS131858, Plect148), *P. oratosquillae* (NJM0662), *P. pauciseptata* (CBS131745, Plect493, Plect641, Plect642, Plect645), *P. plurivora* (CBS131742, Plect130, Plect465) and *P. ramiseptata* (CBS131861, Plect496, Plect644, Plect646, Plect650). Control leaves were treated in the same manner, using sterile distilled water instead of inoculum suspension. The experimental design was performed as two independent batches. Each host × isolate combination was replicated

Table 2 Sources of fungal species and ITS GenBank accession numbers used in the phylogenetic analysis

| Fungal species | Isolate number | Host | Locality | Collector | GenBank |
|---|---|---|---|---|---|
| *Gibellulopsis nigrescens* | CBS 387.35 | *Amaranthus tricolor* | Italy | — | EF543854 |
| *Musicillium theobromae* | CBS 458.51 | — | Japan | K. Kominami | EF543858 |
| *Plectosphaerella alismatis* | CBS 113362[a] | *Alismata plantago-aquatica* | Pijnenburg, Netherlands | W. Gams | JF780523 |
| | RH62 | *A. plantago-aquatica* | Khancoban, Australia | — | AY258150 |
| *Plectosphaerella citrulli* | Plect 157; CBS 131741[a] | Watermelon root | Foggia, Italy | A. Carlucci | HQ238962 |
| | **Plect 64** | Symptomless tomato collar | Rignano Garganico, Foggia, Italy | M. L. Raimondo | KF648358 |
| | **Plect 517** | Tomato collar | Foggia, Italy | A. Carlucci | KF648359 |
| | **Plect 649** | Tomato root | San Severo, Foggia, Italy | A. Carlucci | — |
| | **Plect 651** | Symptomless pepper collar | San Severo, Foggia, Italy | A. Carlucci | — |
| | **Plect 652** | Pepper root | Foggia, Italy | A. Carlucci | KF648378 |
| | **Plect 653** | Pepper collar | Torremaggiore, Foggia, Italy | M. L. Raimondo | KF648379 |
| *Plectosphaerella cucumerina* | Plect 4 | Melon collar | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238977 |
| | Plect 7 | Melon collar | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238978 |
| | Plect 10 | Melon collar | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238979 |
| | Plect 11; CBS 131739 | Melon collar | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238980 |
| | **Plect 45** | Tomato collar | Rignano Garganico, Foggia, Italy | M. L. Raimondo | KF648367 |
| | **Plect 46** | Symptomless tomato collar | Cerignola, Foggia, Italy | A. Carlucci | KF648365 |
| | **Plect 51** | Symptomless tomato collar | San Severo, Foggia, Italy | M. L. Raimondo | — |
| | **Plect 52** | Tomato collar | San Severo, Foggia, Italy | M. L. Raimondo | KF648366 |
| | **Plect 66** | Symptomless tomato collar | Orta Nova, Foggia,Italy | A. Carlucci | — |
| | **Plect 124** | Tomato stem | Manfredonia, Foggia, Italy | A. Carlucci | — |
| | **Plect 125** | Tomato root | Foggia, Italy | A. Carlucci | — |
| | **Plect 128** | Tomato root | Foggia, Italy | M. L. Raimondo | KF648364 |
| | **Plect 134** | Symptomless tomato collar | Foggia, Italy | A. Carlucci | — |
| | **Plect 136** | Tomato stem | San Severo, Foggia, Italy | M. L. Raimondo | — |
| | **Plect 139** | Pepper collar | Cerignola, Foggia, Italy | A. Carlucci | KF648363 |
| | **Plect 142** | Symptomless pepper collar | Manfredonia, Foggia, Italy | A. Carlucci | — |
| | Plect 143 | Pepper collar | Rignano Garganico, Foggia, Italy | A. Carlucci | HQ238987 |
| | Plect 144 | Pepper collar | Rignano Garganico, Foggia, Italy | A. Carlucci | HQ238988 |
| | **Plect 163** | Pepper root | Foggia, Italy | A. Carlucci | KF648360 |
| | **Plect 165** | Tomato collar | Rignano Garganico, Foggia, Italy | M. L. Raimondo | — |
| | **Plect 187** | Symptomless tomato root | Foggia, Italy | A. Carlucci | — |
| | Plect 373 | Tomato collar | Potenza, Italy | A. Carlucci | HQ239006 |
| | **Plect 500** | Symptomless pepper stem | San Giovanni Rotondo, Foggia, Italy | M. L. Raimondo | — |
| | **Plect 502B** | Pepper collar | Cerignola, Foggia, Italy | A. Carlucci | — |
| | **Plect 633** | Symptomless pepper root | Cerignola, Foggia, Italy | A. Carlucci | KF648361 |
| | **Plect 634** | Symptomless pepper collar | Cerignola, Foggia, Italy | A. Carlucci | — |

(continued)

Table 2 (continued)

| Fungal species | Isolate number | Host | Locality | Collector | GenBank |
|---|---|---|---|---|---|
| | **Plect 635** | Pepper root | Foggia, Italy | A. Carlucci | — |
| | **Plect 636** | Pepper stem | Cerignola, Foggia, Italy | A. Carlucci | KF648362 |
| | **Plect 638** | Symptomless pepper root | Cerignola, Foggia, Italy | A. Carlucci | — |
| | **Plect 639** | Symptomless pepper collar | Cerignola, Foggia, Italy | A. Carlucci | — |
| | **Plect 640** | Pepper collar | Orta Nova, Foggia, Italy | A. Carlucci | — |
| *Plectosphaerella delsorboi* | CBS 116708 | *Curcuma alismatifolia* | Portici, Napoli, Italy | V. Antignani | EF543847 |
| *Plectosphaerella melonis* | CA-1103 | *Cucumis melo* | California, USA | P. V. Martinez Culebras | AJ621767 |
| | CA-1509 | *Cucumis melo* | California, USA | P. V. Martinez Culebras | AJ621768 |
| | CBS 488.96 | *Cucumis melo* | Japan | P. V. Martinez Culebras | AJ621769 |
| | CBS 489.96 | *Cucumis melo* | Japan | P. V. Martinez Culebras | AJ621770 |
| | Plect 148 | Melon root | Nardò, Lecce, Italy | A. Carlucci | HQ238968 |
| | Plect 211; CBS 131858 | Melon collar | Lecce, Italy | A. Carlucci | HQ238965 |
| | Plect 228; CBS 131859 | Melon root | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238967 |
| *Plectosphaerella oligotrophica* | LC1990[a] | Soil | China | — | JX508810 |
| | LC1991 | Soil | China | — | JX508811 |
| *Plectosphaerella oratosquillae* | NJM 0662[a] | Mantis shrimp (*Oratosquilla oratoria*) | Yamaguchi, Japan | — | AB425974 |
| | NJM 0678 | *O. oratoria* | Aichi, Japan | — | AB425977 |
| | RM1-12 | Marine sponge (*Suberites zeteki*) | Hawaii, USA | — | DQ993622 |
| *Plectosphaerella pauciseptata* | Plect 186; CBS 131745[a] | Tomato root | Rignano Garganico, Foggia, Italy | A. Carlucci | HQ238971 |
| | **Plect 493** | Symptomless tomato collar | Cerignola, Foggia, Italy | A. Carlucci | KF648370 |
| | **Plect 642** | Tomato root | Stornara, Foggia, Italy | A. Carlucci | KF648371 |
| | **Plect 643** | Tomato collar | Stornara, Foggia, Italy | A. Carlucci | — |
| | **Plect 641** | Pepper collar | Stornara, Foggia, Italy | A. Carlucci | — |
| | **Plect 645** | Symptomless pepper root | San Severo, Foggia, Italy | M. L. Raimondo | KF648368 |
| | **Plect 648** | Pepper stem | San Severo, Foggia, Italy | M. L. Raimondo | KF648369 |
| *Plectosphaerella plurivora* | **Plect 47** | Tomato collar | Foggia, Italy | A. Carlucci | KF648373 |
| | **Plect 130** | Tomato root | Orta Nova, Foggia, Italy | A. Carlucci | KF648372 |
| | Plect 365; CBS 131742[a] | Asparagus apex turion | Borgo Cervaro, Foggia, Italy | A. Carlucci | HQ238975 |
| | **Plect 465** | Symptomless tomato root | Foggia, Italy | A. Carlucci | — |
| *Plectosphaerella populi* | CBS 139623 | Branch of *Populus nigra* | Brandenburg, Germany | C. Ullah | KR476750 |
| | CBS 139624 | Branch of *P. nigra* | Brandenburg, Germany | C. Ullah | KR476751 |
| *Plectosphaerella ramiseptata* | **Plect 366** | Tomato stem | Torremaggiore, Foggia, Italy | M. L. Raimondo | KF648376 |
| | Plect 403; CBS 131861[a] | Symptomless tomato root | Borgo Cervaro, Foggia, Italy | A. Carlucci | JQ246953 |
| | **Plect 496** | Symptomless tomato collar | San Severo, Foggia, Italy | M. L. Raimondo | KF648377 |
| | **Plect 646** | Tomato root | Foggia, Italy | A. Carlucci | — |
| | **Plect 644** | Pepper root | Foggia, Italy | A. Carlucci | KF648374 |
| | **Plect 647** | Symptomless pepper collar | Cerignola, Foggia, Italy | A. Carlucci | KF648375 |
| | **Plect 650** | Symptomless pepper collar | Foggia, Italy | A. Carlucci | — |
| *Plectosphaerella* sp. | MAFF 238629 | Petiole of *Ranunculus asiaticus* | Kagawa, Japan | T. Sato | AB264781 |
| | Ppf4 | *Paris polyphylla* var. *yunnanensis* | China | — | EF495236 |

[a]Ex-type isolates. Isolates collected in the present study are in bold.

10 times. The same experiment was repeated after 1 month, and the data from each host × isolate combination were averaged before statistical analysis. The individual disease severities were assessed after 15 days' incubation at 23 ± 2 °C, using a scale of 0–5, where 0 = no symptoms observed; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 = 81–100% of leaf surface showing necrotic symptoms. The overall disease severities (DS) were calculated according to Eqn 1:

$$DS = \frac{\sum(\text{No. of observations} \times \text{values of scores})}{\text{Total no. of cases}} \quad (1)$$

To determine whether these data followed normal distributions, Shapiro–Wilk tests (*W* tests) were used. The homogeneity of the variance of the datasets was assessed on the basis of two plant hosts and fungal species inoculated, using Levene tests. Factorial ANOVA was performed separately on both datasets (i.e. leaves, seedlings) for each fungal species, on the basis of two plant hosts, to determine the significance of the isolates for the same fungal species of the plant hosts inoculated, and to detect any interactions between these factors (plant host × fungal species), when possible. One-way ANOVA was performed to determine the significant differences in disease severities caused by each fungal species inoculated, and any differences due to plant species (i.e. tomato, pepper). Fisher's tests were used for the comparison of the treatment means, at $P < 0.05$.

In vivo *conditions*

A mixture containing soil and peat (3:1) was sterilized twice at 121 °C for 30 min, and kept for 20 days in a greenhouse at 25 ± 3 °C, 70% relative humidity, and under natural light, before transplanting the seedlings. Young, 35-day-old seedlings of tomato cv. Talent and pepper cv. Pompeo plants were transplanted into small pots (180 mL) containing the above-mentioned mixture. Seven days after transplanting the seedlings, the soil of each pot was inoculated by applying 5 mL conidial suspension adjusted to $10^6$ conidia $mL^{-1}$ with distilled water, obtained from 10-day-old colonies of each of the above-mentioned fungal species. Control pots were treated in the same manner, using sterile distilled water instead of inoculum. The experimental design was performed as two independent batches. Each host × isolate combination was replicated 10 times. The same experiment was repeated after 1 month, and the data from each host × isolate combination were averaged before statistical analysis. The pots were then kept in a greenhouse at 25 ± 3 °C, 70% relative humidity, and under natural light, for up to 35 days. Subsequently, each young plant was removed from the pots, the roots and collar were carefully washed, and the presence/absence of intensity of browning symptoms observed on root and collar was evaluated and described using a scale of 0 to 5, where 0 = no symptoms observed; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 = 81–100%, and the disease severities on the roots and collar were determined according to Eqn 1. All of the fungi underwent reisolation from the root, collar and stem of the inoculated plants, to fulfil Koch's postulates. Statistical analyses were performed as described above.

## Results

### Sampling of plants and isolates, and isolations

The isolation data combining all of the tissues analysed (i.e. roots, collar, basal stems) for tomato and pepper plants both with and without symptoms, and the mycobiota composition obtained, were grouped on the basis of the vegetation stages, as given in Table 1. Known causal agents of important diseases, such as *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Phytophthora infestans*, *Pyrenochaeta lycopersici*, *Macrophomina phaseolina* and *Verticillium dahliae*, were isolated from tomato and pepper plants showing symptoms with variable isolation frequencies from 0.1% to 3.8%, and from symptomless tomato and pepper plants with variable isolation frequencies from 0.1% to 12.4% (Table 1). Fungi belonging to the *Fusarium* and *Plectosphaerella* genera were isolated at higher frequencies than other fungi. *Fusarium* spp. were isolated from 16.2% and 20.8% of tomato and pepper plants with symptoms, and from 6.5% and 2.4% without symptoms, respectively, whilst *Plectosphaerella* spp. were isolated from 19.7% and 33.3% of tomato and pepper plants with symptoms, and from 13.9% and 26.3% without symptoms, respectively (Table 1).

Shapiro–Wilk and Levene testing showed the isolation frequency data from both tomato and pepper plants followed normal distributions, and the homogeneity of the variance was significant. Factorial ANOVA performed on the tomato plants demonstrated that there were no significant differences between fungal isolation frequencies from plants with and without symptoms, except for *Fusarium* spp. ($F = 28.48$; $P < 0.01$) and *Plectosphaerella* spp. ($F = 9.52$; $P < 0.01$). Factorial ANOVA performed on the pepper plants demonstrated that there were no significant differences between fungal isolation frequencies from plants with and without symptoms, except for *Fusarium* spp. ($F = 40.59$; $P < 0.01$). No significant differences were observed when factorial ANOVA was performed for 'vegetation stages' and 'interaction' factors (presence/absence of symptoms × vegetation stages) for either host.

### Morphological analysis

All of the *Plectosphaerella* isolates produced colonies on PDA within 2 weeks that were appressed, slimy, buff to salmon pink, with sparse or absent aerial mycelium. No ascomata were seen in culture. Microscopic observations revealed that the isolates produced conidia both septate or aseptate, with phialides or coils. Based on the description provided by Carlucci *et al.* (2012), the isolates were attributed to Plectosphaerellaceae.

### Molecular identification and phylogenetic analysis

ITS sequences were generated for 45 isolates selected from the MSP-PCR profiles, and these were aligned with 35 sequences retrieved from GenBank (Table 2). The dataset consisted of 80 taxa, which included the out-group taxa (i.e. *Gibellulopsis nigrescens*, *Musicillium theobromae*). After alignment and exclusion of incomplete portions at either end, the dataset consisted of 482 characters (including alignment gaps). Of these

482 characters, 385 were constant, while 36 were variable and parsimony uninformative. Maximum parsimony analysis of the remaining 61 parsimony-informative characters resulted in 28 most-parsimonious trees (TL = 161; CI = 0.727; RI = 0.928; RC = 0.674; HI = 0.273). Maximum likelihood, maximum parsimony and Bayesian analyses resulted in trees with similar topologies (TreeBASE S18320). The isolates sequenced in this study clustered into five clades (Fig. 2). Of the 45 isolates, 24 clustered with *P. cucumerina*, six with the ex-type of *P. ramiseptata*, another six with the ex-type of *P. pauciseptata*, three with the ex-type of *P. plurivora*, and six with the ex-type of *P. citrulli*. According to Carlucci *et al.* (2012), the microscopic features of each species identified confirmed the molecular results.

On the basis of these phylogenetic data, the numbers and isolation frequencies of isolates of *Plectosphaerella* spp. from tomato and pepper plants both with and without symptoms were determined and are reported in Table 3. The results obtained show that tomato plants with symptoms were more abundantly infected with *P. cucumerina*, with an isolation frequency of 44.6%, than with *P. citrulli*, *P. pauciseptata*, *P. plurivora* and *P. ramiseptata* (isolation frequencies of 18.3%, 4.0%, 14.0% and 19.1%, respectively; Table 3). The symptomless tomato plants were also abundantly infected with *P. cucumerina*, with an isolation frequency of 60.8%, which was higher than that for the tomato plants with symptoms. The isolation frequencies for the other *Plectosphaerella* spp. for these symptomless tomato plants were similar to those of the plants with symptoms, except for the lower isolation frequency of *P. ramiseptata* (4.9%). In addition, these data indicated that 24.4% and 18.4% of the examined tomato plants with and without symptoms, respectively, were affected by *Plectosphaerella* spp. (Table 3).

For the pepper plants with symptoms, the isolation frequency of *P. cucumerina* was 52.9%, which was higher than the isolation frequencies for the other *Plectosphaerella* spp. (Table 3). The isolation frequencies of *P. citrulli*, *P. pauciseptata* and *P. ramiseptata* were 19.5%, 5.7% and 21.9%, respectively. For the symptomless pepper plants, the isolation frequencies were close to those of plants with symptoms, although the isolation frequency of *P. cucumerina* was higher (64.6%). No *P. plurivora* isolate was detected from either set of pepper plants. In addition, these data show that 50.4% and 42.0% of the examined pepper plants with and without symptoms, respectively, were affected by *Plectosphaerella* spp. (Table 3).

Shapiro–Wilk and Levene testing showed the data for the isolation frequencies of *Plectosphaerella* spp. from tomato and pepper plants followed normal distributions, and the homogeneity of the variance was significant. Factorial ANOVA demonstrated that the symptom expression was not significantly correlated with presence/absence of *Plectosphaerella* isolates, apart for *P. cucumerina* ($F = 52.40$; $P < 0.001$) and *P. ramiseptata* ($F = 11.55$; $P < 0.001$) detected for tomato plants.

No significant correlations on the basis of the factors were observed in either tomato or pepper plants, such as for vegetation stage and interaction between presence/absence of symptoms per vegetation stage.

## Pathogenicity tests

### In vitro *conditions*

Shapiro–Wilk and Levene testing showed that the data from the *in vitro* pathogenicity tests carried out on the tomato and pepper leaves followed normal distributions, and the homogeneity of the variance was significant. Factorial ANOVA demonstrated that there were significant differences between the plant hosts inoculated with different *Plectosphaerella* spp., while there were no significant differences among the isolates related to the same fungal species inoculated, nor significant correlations on the basis of the interactions between host per fungal species (Table 4). This means that all of the *Plectosphaerella* isolates belonging to the same fungal species produced similar symptoms.

One-way ANOVA carried out on the tomato and pepper leaves revealed that the *Plectosphaerella* spp. used in this study were not consistently pathogenic (Table 5). The symptoms on tomato leaves 15 days after inoculation with the nine *Plectosphaerella* spp. were determined, with variable disease severities ranging from 1.2 (*P. cucumerina*) to 4.8 (*P. ramiseptata*). No symptoms were observed when *P. melonis* and *P. oratosquillae* were inoculated. *Plectosphaerella citrulli* and *P. delsorboi* produced parenchymatous necrotic spots with light discoloured rings, while *P. alismatis*, *P. cucumerina* and *P. ramiseptata* produced parenchymatous necrotic patches with hydropic rings. Finally, *P. plurivora* and *P. pauciseptata* caused vascular necrosis (Fig. 3).

Of the nine *Plectosphaerella* spp. inoculated on pepper leaves, only *P. delsorboi*, *P. melonis*, *P. ramiseptata* and *P. pauciseptata* produced symptoms 15 days after inoculation, with variable disease severities from 0.9 (*P. delsorboi*) to 4.2 (*P. ramiseptata*). *Plectosphaerella delsorboi* produced parenchymatous necrotic spots with light discoloured rings, while *P. melonis* and *P. ramiseptata* produced parenchymatous necrotic patches with hydropic rings. *Plectosphaerella pauciseptata* caused vascular necrosis that developed as hydropic patches around the main veins (Fig. 4).

### In vivo *conditions*

Shapiro–Wilk and Levene testing showed the data from the *in vivo* pathogenicity tests carried out on the tomato and pepper seedlings followed normal distributions, and the homogeneity of variance was significant. Factorial ANOVA demonstrated that there were significant differences between the plant hosts inoculated with different *Plectosphaerella* spp., except for *P. citrulli* and *P. cucumerina*, while there were neither significant differences among the isolates related to the same fungal species

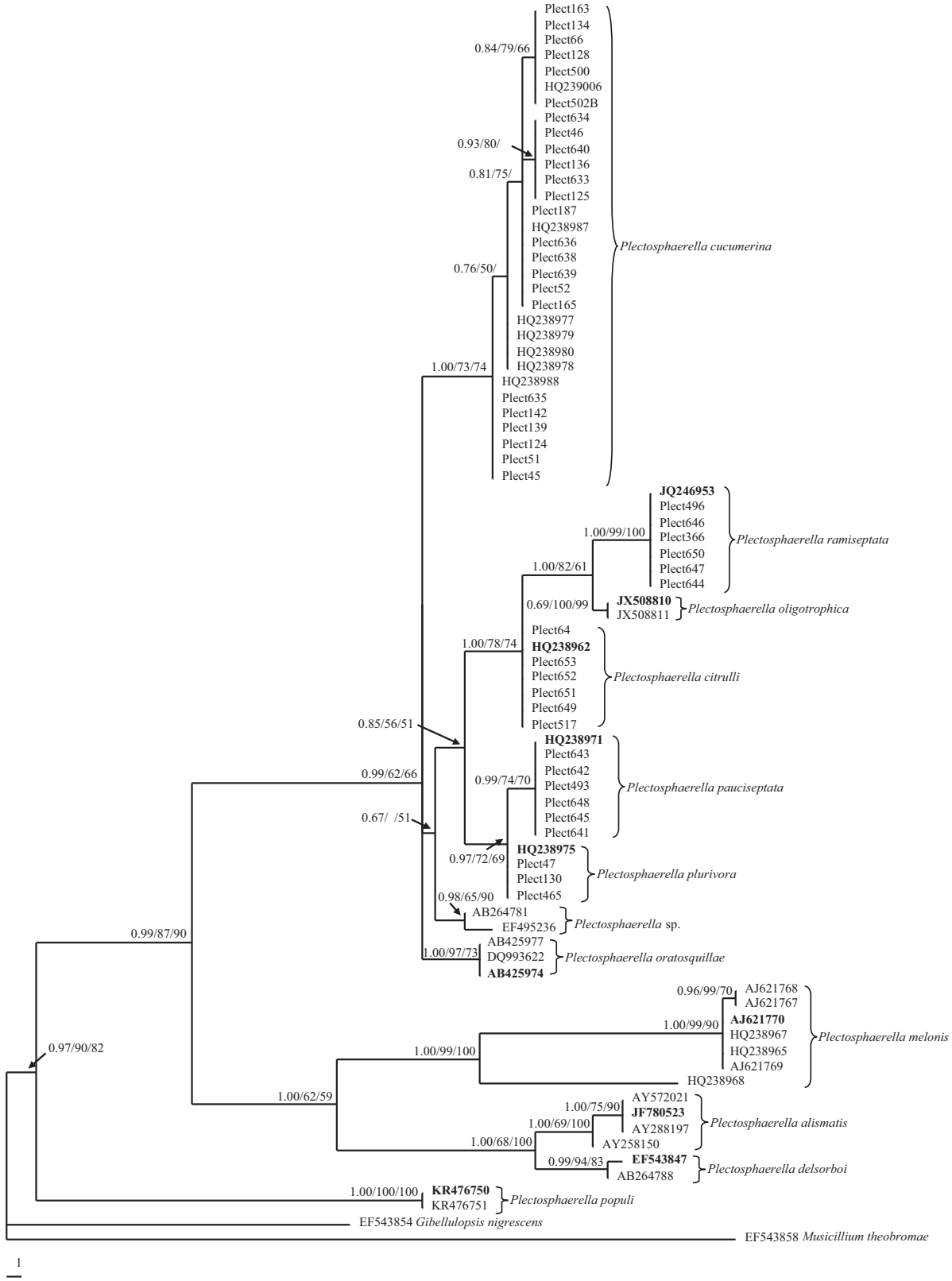


Figure 2 One of the 28 most-parsimonious trees obtained from the alignment of the ITS sequence data with bootstrap support values from Bayesian posterior probability/maximum likelihood/maximum parsimony. Ex-type isolates are in bold. *Gibellulopsis nigrescens* and *Musicillium theobromae* are included as out-groups.

Table 3 Numbers and isolation frequencies of *Plectosphaerella* spp. from tomato and pepper plants with and without symptoms, according to plant growth stage.

| | Number (isolation frequency) [*n* (%)][a] according to plant growth stage | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Plants with symptoms | | | | | Plants without symptoms | | | | |
| Plant/fungal species | PTS | BS | GFS | RFS | Total [*N* (%)] | PTS | BS | GFS | RFS | Total [*N* (%)] |
| Tomato | | | | | | | | | | |
| *Plectosphaerella citrulli* | 6 (19.3) | 15 (20.2) | 36 (21.6) | 16 (12.5) | 73 (18.3) | 6 (14.4) | 8 (17.3) | 6 (12.2) | 13 (19.5) | 33 (16.2) |
| *Plectosphaerella cucumerina* | 13 (42.0) | 31 (43.2) | 68 (41.2) | 65 (50.7) | 177 (44.6) | 25 (59.4) | 29 (63.1) | 32 (65.4) | 38 (56.5) | 124 (60.8) |
| *Plectosphaerella pauciseptata* | 1 (3.2) | 3 (4.0) | 6 (3.6) | 6 (4.8) | 16 (4.0) | 2 (4.8) | 5 (10.8) | 2 (4.1) | 1 (1.5) | 10 (4.9) |
| *Plectosphaerella plurivora* | 3 (9.7) | 10 (13.6) | 27 (16.2) | 16 (12.5) | 56 (14.0) | 8 (19.0) | 2 (4.4) | 6 (12.2) | 11 (16.5) | 27 (13.2) |
| *Plectosphaerella ramiseptata* | 8 (25.8) | 14 (19.0) | 29 (17.4) | 25 (19.5) | 76 (19.1) | 1 (2.4) | 2 (4.4) | 3 (6.1) | 4 (6.0) | 10 (4.9) |
| Total (*N*) *Plectosphaerella* spp. | 31 | 73 | 166 | 128 | 398 | 42 | 46 | 49 | 67 | 204 |
| Total [*N* (%)] fungi by plant stage | 31 (7.8) | 73 (18.5) | 166 (41.6) | 128 (32.1) | 398 (100) | 42 (20.6) | 46 (22.5) | 49 (24.0) | 67 (32.9) | 204 (100) |
| Total plants affected by *Plectosphaerella* spp. [*N* (%)] | 3 (30.0) | 7 (31.8) | 13 (28.9) | 10 (17.2) | 33 (24.4) | 4 (22.2) | 4 (20.0) | 4 (16.0) | 6 (17.1) | 18 (18.4) |
| Total plants examined | 10 | 22 | 45 | 58 | 135 | 18 | 20 | 25 | 35 | 98 |
| Pepper | | | | | | | | | | |
| *P. citrulli* | 5 (22.7) | 6 (18.2) | 9 (15) | 21 (22.1) | 41 (19.5) | 5 (26.3) | 3 (12) | 5 (12.2) | 6 (13.3) | 19 (14.6) |
| *P. cucumerina* | 9 (41.0) | 16 (48.4) | 38 (63.3) | 48 (50.4) | 111 (52.9) | 8 (42.2) | 15 (60) | 30 (73.2) | 31 (69.0) | 84 (64.6) |
| *P. pauciseptata* | 2 (9.1) | 2 (6.1) | 3 (5.0) | 5 (5.4) | 12 (5.7) | – | 1 (4.0) | 1 (2.4) | 1 (2.2) | 3 (2.3) |
| *P. ramiseptata* | 6 (27.2) | 9 (27.3) | 10 (16.7) | 21 (22.1) | 46 (21.9) | 6 (31.5) | 6 (24) | 5 (12.2) | 7 (15.5) | 24 (18.5) |
| Total (*N*) *Plectosphaerella* spp. | 22 | 33 | 60 | 95 | 210 | 19 | 25 | 41 | 45 | 130 |
| Total [*N* (%)] fungi by plant stage | 22 (10.5) | 33 (15.7) | 60 (28.6) | 95 (45.2) | 210 | 19 (14.6) | 25 (19.3) | 41 (31.5) | 45 (34.6) | 130 |
| Total plants affected by *Plectosphaerella* spp. [*N* (%)] | 2 (33.3) | 4 (40.0) | 7 (70.0) | 8 (50.0) | 21 (50.4) | 2 (33.3) | 3 (60.0) | 4 (40.0) | 5 (41.5) | 14 (42.0) |
| Total plants examined | 6 | 6 | 10 | 16 | 42 | 6 | 5 | 10 | 12 | 33 |

PTS, post-transplantation stage; BS, blossom stage; GFS, green fruit stage; RFS, ripened fruit stage; –, no species detected.

[a]Isolation frequency per host calculated as the number of segments infected by a given fungus divided by the total number of segments incubated.

Table 4 Factorial analysis of variance (ANOVA) among the main factors (i.e. plant host, isolate) and their interactions in the pathogenicity tests.

| | | ANOVA[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Leaves | | | Seedlings | | |
| *Plectosphaerella* species | Factor | d.f. | *F* | *P* | d.f. | *F* | *P* |
| *P. alismatis* | Host[b] | 1 | 129.71 | 0.00 | 1 | 52.10 | 0.00 |
| | CBS113362 | — | — | — | — | — | — |
| | Host × CBS113362 | — | — | — | — | — | — |
| *P. citrulli* | Host | 1.00 | 138.80 | 0.001 | 1.00 | 0.00 | 1.00 |
| | CBS131741/Plect64/Plect517/Plect651/Plect653[c] | 4.00 | 0.08 | 0.92 | 2.00 | 0.17 | 0.85 |
| | Host × CBS131741/Plect64/Plect517/Plect651/Plect653 | 2.00 | 0.08 | 0.92 | 2.00 | 0.07 | 0.93 |
| *P. cucumerina* | Host | 1.00 | 84.52 | 0.001 | 1.00 | 0.34 | 0.56 |
| | CBS131739/Plect128//Plect163/Plect187/Plect633 | 4.00 | 0.85 | 0.43 | 2.00 | 0.50 | 0.61 |
| | Host × CBS131739/Plect128//Plect163/Plect187/Plect633 | 2.00 | 0.20 | 0.82 | 2.00 | 0.11 | 0.89 |
| *P. delsorboi* | Host | 1.00 | 65.22 | 0.001 | 1.00 | 72.43 | 0.001 |
| | CBS116708 | — | — | — | — | — | — |
| | Host × CBS116708 | — | — | — | — | — | — |
| *P. melonis* | Host | 1.00 | 194.54 | 0.001 | 1.00 | 9.89 | 0.001 |
| | CBS131859/CBS131858/Plect148 | 2.00 | 1.79 | 0.18 | 2.00 | 0.10 | 0.90 |
| | Host × CBS131859/CBS131858/Plect148 | 2.00 | 1.79 | 0.18 | 2.00 | 0.03 | 0.97 |
| *P. oratosquillae* | Host | 0.00 | 0.00 | 0.001 | 0.00 | 0.00 | 0.001 |
| | NJM 0662 | — | — | — | — | — | — |
| | Host × NJM 0662 | — | — | — | — | — | — |
| *P. pauciseptata* | Host | 1.00 | 4.96 | 0.03 | 1.00 | 97.60 | 0.001 |
| | CBS131745/Plect493/Plect641/Plect642/Plect645 | 4.00 | 0.08 | 0.93 | 2.00 | 0.25 | 0.78 |
| | Host × CBS131745/Plect493/Plect641/Plect642/Plect645 | 2.00 | 1.24 | 0.30 | 2.00 | 0.36 | 0.70 |
| *P. plurivora* | Host | 1.00 | 366.76 | 0.001 | 1.00 | 299.64 | 0.001 |
| | CBS131742/Plect130/Plect465 | 2.00 | 0.17 | 0.85 | 2.00 | 2.71 | 0.08 |
| | Host × CBS131742/Plect130/Plect465 | 2.00 | 0.17 | 0.85 | 2.00 | 3.59 | 0.03 |
| *P. ramiseptata* | Host | 1.00 | 13.34 | 0.001 | 1.00 | 24.69 | 0.001 |
| | CBS131861/Plect496/Plect644/Plect646/Plect650 | 4.00 | 0.18 | 0.83 | 2.00 | 1.07 | 0.35 |
| | Host × CBS131861/Plect496/Plect644/Plect646/Plect650 | 2.00 | 0.74 | 0.48 | 2.00 | 1.67 | 0.20 |

—, no data due to one isolate available.

[a]d.f., degrees of freedom; *F*, *F*-value; *P*, *P*-value ($P < 0.01$).

[b]Tomato × Pepper.

[c]Isolate × Isolate × Isolate × Isolate × Isolate.

inoculated, nor significant correlations on the basis of the interactions between host per fungal species (Table 4).

The results from one-way ANOVA carried out on the tomato and pepper seedlings are reported in Table 5. The symptoms observed for the tomato seedlings 15 days after inoculation with the nine *Plectosphaerella* spp. were determined, with variable disease severities from 1.0 (*P. melonis*) to 4.8 (*P. ramiseptata*). No symptoms were observed when *P. delsorboi* and *P. oratosquillae* were inoculated. *Plectosphaerella alismatis*, *P. melonis*, *P. pauciseptata* and *P. plurivora* produced root browning, although with different disease severities (Fig. 3). Moreover, *P. plurivora* also produced collar browning. Tomato seedlings inoculated with *P. citrulli* showed collar browning and reduced root growth, while those inoculated with *P. cucumerina* showed light leaf yellowing, and browning of the basal stem. Finally, *P. ramiseptata* induced reduced growth of tomato seedlings and collar browning (Fig. 3).

The symptoms observed for the pepper seedlings 15 days after inoculation with the nine *Plectosphaerella* spp. were also determined, with variable disease severities from 1.2 (*P. cucumerina*) to 4.0 (*P. ramiseptata*). No symptoms were observed when *P. alismatis*, *P. oratosquillae* and *P. plurivora* were inoculated. *Plectosphaerella delsorboi* and *P. melonis* produced light root browning (Fig. 4), *P. citrulli* produced reduced root growth and collar browning, *P. cucumerina* produced light leaf yellowing and browning of the basal stem, and *P. pauciseptata* produced reduced growth of the pepper seedlings, and collar and root browning. Finally, *P. ramiseptata* caused light leaf yellowing and extended collar and root browning (Fig. 4). All of these *Plectosphaerella* spp. were reisolated from the tomato and pepper seedlings (Table 5).

## Discussion

This study confirmed the presence of known fungal pathogens including *Alternaria alternata*, *Pyrenochaeta lycopersici*, *Phytophthora infestans*, *Verticillium dahliae* and *Fusarium* spp., with particular focus on the high frequency of fungi attributed to the *Plectosphaerella* genus, as reported by Carlucci *et al.* (2012) in a study carried out on several horticultural crops affected by general collapse symptoms.

Table 5 Pathogenicity assays carried out with the different species of *Plectosphaerella* on the tomato and pepper leaves and seedlings (one-way ANOVA).

| Plant/fungal species | Leaves | | | | Seedlings | | | | Reisolation (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Disease severity | | | | Disease severity | | | | |
| | Mean[a] | SD | Min.–max.[b] | Symptoms | Mean | SD | Min.–max. | Symptoms | |
| Tomato | | | | | | | | | |
| Control | 0.0 A | — | — | No symptoms observed | 0.0 A | — | — | No symptoms observed | 0 |
| *P. alismatis* | 3.5 D | 1.0 | 2.0–5.0 | Necrotic patches (PPB[c]) | 1.8 CD | 0.8 | 1.0–3.0 | Root browning | 90 |
| *P. citrulli* | 1.4 B | 0.6 | 1.0–3.0 | Necrotic spots (PPB) | 3.0 E | 0.9 | 2.0–5.0 | Collar browning, reduced root growth | 100 |
| *P. cucumerina* | 1.2 B | 0.7 | 0.0–3.0 | Necrotic patches (PPB) | 1.3 BC | 0.7 | 0.0–3.0 | Leaf yellowing and browning of basal stem | 90 |
| *P. delsorboi* | 1.4 B | 0.5 | 1.0–2.0 | Necrotic spots (PPB) | 0.0 A | — | — | No symptoms observed | 90 |
| *P. melonis* | 0.0 A | — | — | No symptoms observed | 1.0 B | 0.6 | 0.0–2.0 | Root browning | 90 |
| *P. oratosquillae* | 0.0 A | — | — | No symptoms observed | 0.0 A | — | — | No symptoms observed | 90 |
| *P. pauciseptata* | 3.5 D | 0.8 | 2.0–5.0 | Necrosis (PTB[d]) | 4.0 F | 0.8 | 3.0–5.0 | Root browning | 90 |
| *P. plurivora* | 2.7 C | 0.7 | 1.0–4.0 | Necrosis (PTB) | 2.3 D | 0.8 | 1.0–4.0 | Collar and root browning | 90 |
| *P. ramiseptata* | 4.8 E | 0.5 | 3.0–5.0 | Necrotic patches (PPB) | 4.8 G | 0.4 | 4.0–5.0 | Reduced growth, collar browning | 90 |
| Pepper | | | | | | | | | |
| Control | 0.0 A | — | — | No symptoms observed | 0.0 A | — | — | No symptoms observed | 0 |
| *P. alismatis* | 0.0 A | — | — | No symptoms observed | 0.0 A | — | — | No symptoms observed | 90 |
| *P. citrulli* | 0.0 A | — | — | No symptoms observed | 3.0 D | 0.7 | 2.0–4.0 | Collar browning, reduced root growth | 100 |
| *P. cucumerina* | 0.0 A | 0.2 | 0.0–1.0 | No symptoms observed | 1.2 B | 0.6 | 0.0–3.0 | Leaf yellowing and browning of basal stem | 90 |
| *P. delsorboi* | 0.9 B | 0.7 | 0.0–2.0 | Necrotic spots (PPB) | 1.3 B | 0.5 | 1.0–2.0 | Root browning | 90 |
| *P. melonis* | 1.8 C | 0.7 | 0.0–3.0 | Necrotic patches (PPB) | 1.5 B | 0.7 | 0.0–3.0 | Root browning | 90 |
| *P. oratosquillae* | 0.0 A | — | — | No symptoms observed | 0.0 A | — | — | No symptoms observed | 90 |
| *P. pauciseptata* | 3.1 D | 0.8 | 1.0–4.0 | Necrosis (PTB) | 2.1 C | 0.7 | 1.0–3.0 | Reduced growth, collar, root browning | 100 |
| *P. plurivora* | 0.0 A | — | — | No symptoms observed | 0.0 A | 0.2 | 0.0–1.0 | No symptoms observed | 90 |
| *P. ramiseptata* | 4.2 E | 0.7 | 3.0–5.0 | Necrotic patches (PPB) | 4.0 E | 0.8 | 3.0–5.0 | Leaf yellowing, collar and root browning | 100 |

—, datum not applicable.

[a]Means, different capital letters within columns indicate significant differences ($P < 0.01$; Duncan tests).

[b]Minimum and maximum values on the basis of 10 observations.

[c]PPB, putative parenchymatous behaviour.

[d]PTB, putative tracheomycotic behaviour.

The factorial analysis carried out on the isolation frequencies of all of the fungi identified in the present study demonstrated that *Fusarium* spp. and *Plectosphaerella* spp. were the most isolated species. Although specific studies on *Fusarium* spp. were not carried out here, a comparative study was undertaken to determine the isolation frequencies between the *Plectosphaerella* and *Fusarium* genera. In particular, the composition of the *Fusarium* population mainly consisted of *F. oxysporum* and *F. solani* (data not shown), which are known fungal pathogens of tomato and pepper plants. On the basis of these results, it is interesting that the isolation frequencies of *Fusarium* isolates were always lower than those of *Plectosphaerella* isolates from plants of both crops. *Plectosphaerella* isolation frequencies from tomato plants with symptoms were significantly different to those of symptomless tomato plants, while no significant difference was detected in pepper plants. That may be due to differences in disease susceptibility of pepper and tomato, or it may be explained on the basis that this fungal group can induce different symptom severity, probably influenced by different variables such as pedoclimatic conditions, telluric mycobiota close to the roots of the plants, soil texture, and plant host susceptibility. For the putative differences in host susceptibility, further studies are needed on different cultivars of tomato and pepper.

These data led to further studies being carried out to identify and define which *Plectosphaerella* spp. occurred on these tomato and pepper plants. The use of MSP-PCR was successful for screening the large number of strains isolated from the tomato and pepper plants. This grouped them in eight main clades, which allowed the further study to focus on a smaller number of isolates. Based on the phylogenetic and morphological analysis, four groups contained four specific *Plectosphaerella* species (i.e. *P. citrulli*, *P. pauciseptata*, *P. plurivora*, *P. ramiseptata*), while the other four included all of the *P. cucumerina* strains. These same


Figure 3 Representative samples in the pathogenicity assays for disease severity carried out on tomato leaves (a–j) and seedlings (k–p) under artificial conditions. Inoculations were: sterile distilled water (a); conidial suspensions from fungal colonies of *Plectosphaerella alismatis* (b), *P. citrulli* (c), *P. cucumerina* (d), *P. delsorboi* (e), *P. melonis* (f), *P. oratosquillae* (g), *P. pauciseptata* (h), *P. plurivora* (i) and *P. ramiseptata* (j); browning and rot root caused by *P. delsorboi* (k); different disease severities on root caused by *P. pauciseptata* (l), *P. plurivora* (m), and *P. alismatis* (n); cortical browning and collar and root destruction caused by *P. citrulli* (o); leaf chlorosis and yellowing caused by *P. cucumerina* (p). [Colour figure can be viewed at wileyonlinelibrary.com].

species were also isolated from the pepper plants, except for *P. plurivora*. To the best of the authors' knowledge, this is the first time that isolation of *P. citrulli* has been reported from tomato and pepper plants, and *P. pauciseptata* and *P. ramiseptata* from pepper plants. The subsequent factorial analysis showed that *P. cucumerina* was the most frequently isolated species from both hosts with and without symptoms. This high frequency of *P. cucumerina* in the present study is in agreement with that reported in the literature (Garibaldi *et al.*, 2012, 2013; Carrieri *et al.*, 2014). Moreover, the high frequency of *P. cucumerina* detected from symptomless hosts is an indication of putative latent infections.

Through the use of nine different species of *Plectosphaerella* in pathogenicity assays, it was possible to determine if they were able to infect, and which symptoms they produced on the tomato and pepper hosts.

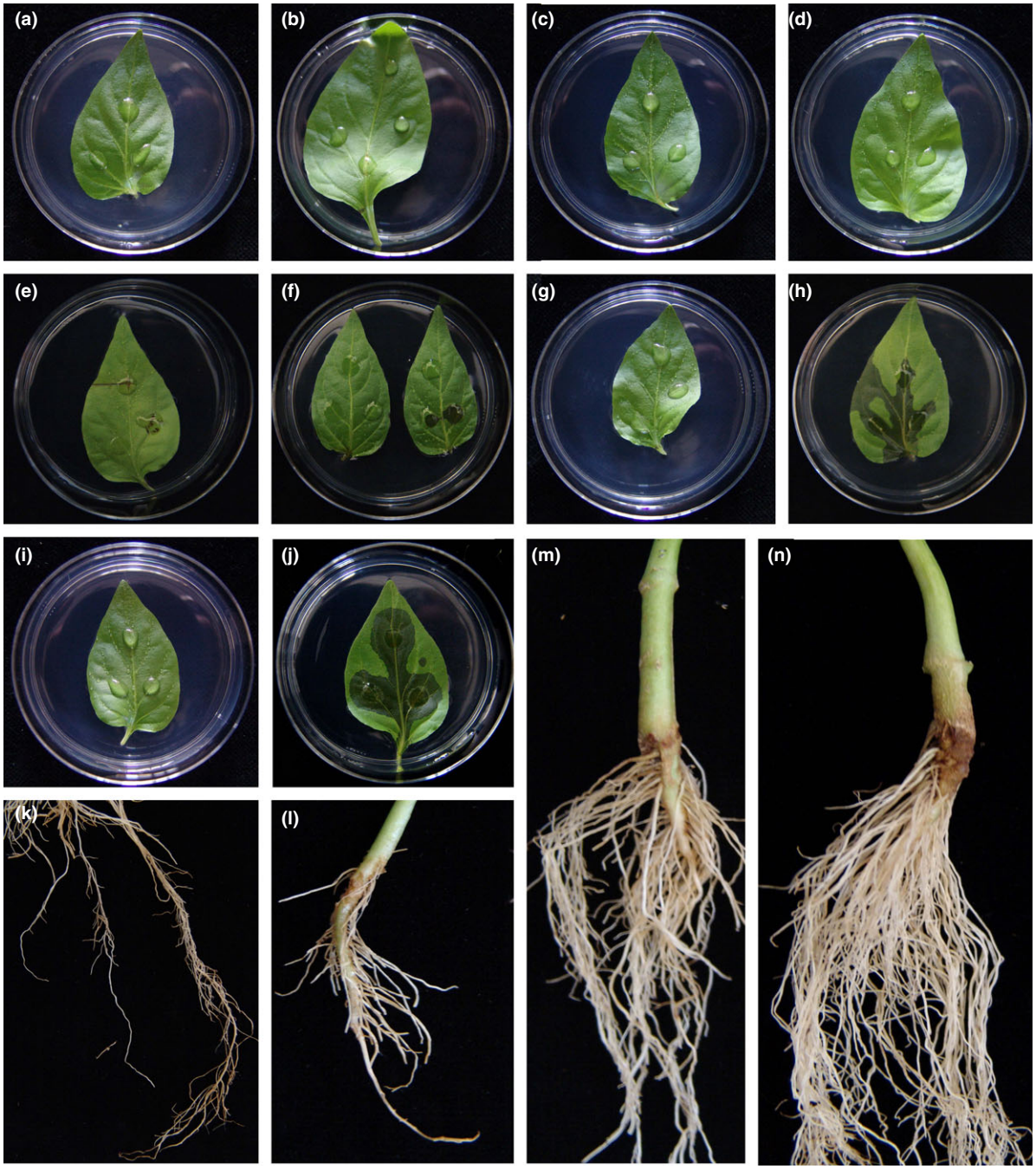


Figure 4 Representative samples in the pathogenicity assays for disease severity carried out on pepper leaves (a–j) and seedlings (k–n) under artificial conditions. Inoculations were: sterile distilled water (a); conidial suspensions from fungal colonies of *Plectosphaerella alismatis* (b), *P. citrulli* (c), *P. cucumerina* (d), *P. delsorboi* (e), *P. melonis* (f), *P. oratosquillae* (g), *P. pauciseptata* (h), *P. plurivora* (i) and *P. ramiseptata* (j); browning and rot root caused by *P. delsorboi* (k); reduction of radicular hairs caused by *P. melonis* (l); light browning of collar caused by *P. pauciseptata* (m); collar browning caused by *P. ramiseptata* (n). [Colour figure can be viewed at wileyonlinelibrary.com].

The pathogenicity trials revealed that except for *P. oratosquillae*, all of these *Plectosphaerella* spp. were pathogenic, although they showed different degrees of aggressiveness.

Results from *in vitro* inoculation of detached leaves should be regarded as a preliminary confirmation of pathogenicity. However, the symptoms described supported those observed on inoculation of young intact plants. The most aggressive species was *P. ramiseptata*, as it showed higher disease severities for both tomato and pepper young plants. The symptoms observed indicate that *P. ramiseptata* has a pathogenic role based on

cell wall degradation by pectinolytic enzymes, inducing reduced growth, root and collar browning, and then death of the plants. Other *Plectosphaerella* species, such as *P. alismatis* and *P. cucumerina*, induced root and basal stem browning and general decline of the tomato and pepper seedlings. *Plectosphaerella melonis* produced similar symptoms consisting of root browning on tomato and pepper seedlings. Similar symptoms produced by *P. alismatis* on some Cucurbitaceae and Solanaceae hosts were reported by Cother (1999), and on Alismataceae by Pitt & Gams (2005). For *P. cucumerina*, the literature describes various pathogenic behaviours that are strongly linked to its complexity. For instance, Egel *et al.* (2010), El-Gindy (1991) and Garibaldi *et al.* (2012, 2013) associated various symptoms with *P. cucumerina* that resembled rot, leaf chlorosis, leaf necrotic enlarging spots, and browning of the basal stem, with this seen as a necrotrophic pathogen according to the present study. Moreover, Vitale *et al.* (2007), Xu *et al.* (2014) and Zhang *et al.* (2015) associated *P. cucumerina* with wilting symptoms that occurred on zucchini (courgette), tomato and sunflower hosts, respectively. In addition, Li *et al.* (2008) reported *P. cucumerina* (Ppf4; acc. no. EF495236) as an endophyte of *Paris poliphylla* var. *yunnannensis*, although through taxonomic revision based on molecular and morphological descriptions, it is a different species of the *Plectosphaerella* genus (Carlucci *et al.*, 2012).

*Plectosphaerella citrulli* and *P. delsorboi* caused collar browning and reduced root growth, and root browning, respectively, for both tomato and pepper seedlings, in agreement with Antignani *et al.* (2008), who reported similar symptoms on curcuma.

Vascular behaviour can be attributed to *P. pauciseptata* and *P. plurivora*, as they produced extending necrosis of leaf veins when they were inoculated on the leaf surface. This symptom was confirmed when these fungi were inoculated in pots, causing collar and root browning on tomato seedlings, which evolved into wilt. Similar symptoms were associated with *P. pauciseptata* by Usami *et al.* (2012) on lettuce, coriander and chervil, although they considered *P. pauciseptata* as the causal agent of plectosphaerella rot. Satou *et al.* (2010) associated black discolouration and decay of chrysanthemum cuttings with *P. tabacinum* (syn. *P. cucumerina*), although on the basis of the recent taxonomic revision (Carlucci *et al.*, 2012), their strain of MAFF 712335 (acc. no. AB537556) was *P. plurivora*.

Although the results obtained from pathogenicity tests carried out under *in vitro* conditions provided clear indications about the putative pathogenic and/or endophytic role of *Plectosphaerella* spp., they are not enough to confirm *Plectosphaerella* pathogenicity, but support the results from tests carried out under *in vivo* conditions. The high isolation frequency from symptomless pepper and tomato plants suggests possible early infections prior to disease expression. Therefore, the *Plectosphaerella* species investigated in the present study are not considered endophytic fungi, but pathogenic fungi that are mainly necrotrophic, although they showed different degrees of disease severity. *Plectosphaerella oratosquillae* was not pathogenic on tomato or pepper plants. This species has previously been isolated from mantis shrimp (Duc *et al.*, 2009), and may not infect plant hosts.

Previous different symptoms associated with *Plectosphaerella* spp. infection have been described as 'plectosporium blight' on pumpkin and *Ranunculus* (Sato *et al.*, 2005) and cucurbits (Bost & Mullins, 1992), 'plectosphaerella root rot' on tomato (El-Gindy, 1991), *Diplotaxis* (Garibaldi *et al.*, 2012), lettuce, coriander and chervil (Usami *et al.*, 2012) and white lupin (Youssef *et al.*, 2001), 'plectosphaerella wilt' on tomato (Xu *et al.*, 2014), 'black leg' on basil (Egel *et al.*, 2010; Mersha *et al.*, 2012), 'cutting rot' on *Chrysanthemum* (Satou *et al.*, 2010), and 'collapse' on cucurbits (García-Jiménez *et al.*, 2000). The present study describes 'stunting disease' of tomato and pepper resulting from *Plectosphaerella* spp. infection which includes root and collar rot, plus vascular and leaf symptoms.

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