Calonectria spp. causing leaf spot, crown and root rot of ornamental plants in Tunisia

L. Lombard¹, G. Polizzi², V. Guarnaccia², A. Vitale², P.W. Crous¹

INTRODUCTION

Species of Calonectria are common pathogens of a wide range of plant hosts in nurseries cultivated through seedings or vegetative propagation (Crous 2002, Lombard et al. 2010a). Nursery disease symptoms associated with these fungi include crown, collar and root rot, leaf spots and cutting rot (Crous 2002, Vitale & Polizzi 2008, Polizzi et al. 2009, Lombard et al. 2010a). Calonectria spp. have been reported worldwide from agricultural and forestry nurseries (Crous et al. 1991, Crous 2002, Lombard et al. 2009, 2010a, b, d), whereas in Europe, they have only been reported from commercial ornamental nurseries (Polizzi & Crous 1999, Polizzi 2000, Crous 2002, Henricot & Culham 2002, Polizzi et al. 2007a, b, Vitale & Polizzi 2008, Polizzi et al. 2009, Lombard et al. 2010a).

Past reports have shown that C. morganii and C. pauciramosa are the most common Calonectria spp. found in ornamental nurseries in the Northern Hemisphere (Polizzi & Crous 1999, Polizzi 2000, Polizzi & Catara 2001, Polizzi et al. 2006a, b, 2007a, b). Based on phylogenetic studies, C. morganii appears to be restricted to Brazil, Europe and the USA (Crous et al. 1993, Overmeyer et al. 1996, Schoch et al. 2000), whereas C. pauciramosa has a more global distribution and has been shown to better adapt to different environmental conditions (Crous et al. 2002, Lombard et al. 2010b, Chen et al. 2011). Calonectria pauciramosa was also regarded as the dominant pathogen in nurseries in Australia and South Africa (Crous 2002, Schoch et al. 2001, Lombard et al. 2010b).

In November 2010, a survey was conducted in an ornamental nursery in Carthage, Tunis, Tunisia. Various plant species were collected showing symptoms of leaf spots, crown and root rot. Isolations consistently yielded Calonectria spp. and the aim of this study was to identify these species, and confirm their pathogenicity.

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MATERIALS AND METHODS

Disease survey and isolates

During November 2010, an ornamental nursery located in Carthage, Tunis, Tunisia was surveyed for diseased plants. Several samples of Callistemon spp., Dodonaea viscosa, Myrtus communis and Metrodieros spp. showing leaf spots, crown and root rot were randomly collected for analysis (Fig. 1, Table 1). Infected tissues collected from symptomatic plants were superficially disinfected with 1.0 % sodium hypochlorite for 2 min, rinsed with sterile water, placed on potato-dextrose agar (PDA, Oxoid) and incubated in the dark at 24 °C. Representative isolates of Calonectria from each ornamental species were obtained from single-spore colonies made from 14 d old cultures grown on PDA. Representative isolates have been deposited at the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (Table 1).

DNA sequence comparisons

Total genomic DNA was extracted from single-conidial isolates grown on 2 % malt extract agar (MEA) for 7 d, using the Ultra-Clean™ Microbial DNA isolation kits (Mo Bio Laboratories, Inc., California, USA) according to the manufacturer’s protocol. Partial gene sequences were determined for β-tubulin (BT), histone H3 (HIS3) and translation elongation factor-1α (TEF-1α) using the primers and protocols described by Lombard et al. (2010c).

To ensure the integrity of the sequences, the amplicons were sequenced in both directions using the same primer pairs used for amplification. Sequence data from Lombard et al. (2010b, d) were used as reference data and subsequent alignments were generated using MAFFT v. 6 (Katoh & Toh 2010) and manually corrected where necessary. Congruency of the sequence datasets for the separate loci was determined using tree topologies of 70 % reciprocal Neighbour-Joining bootstrap trees with Maximum Likelihood distances that were compared visually to identify conflicts between partitions (Gueidan et al. 2007). Molecular evolution models for the separate gene regions were determined in Modeltest v. 3.7.
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(Morphological characterisation of the Calonectria isolates was done using single conidial cultures prepared on MEA and synthetic nutrient-poor agar (SNA; Nirenburg 1981, Lombard et al. 2009). Inoculated plates were incubated at room temperature and examined after 7 d. Gross morphological characteristics of the anamorph state were determined by mounting fungal structures in clear lactic acid and 30 measurements at \( \times 1000 \) magnification were made for each isolate using a Zeiss Axioscope 2 microscope with interference contrast (DIC) illumination. The 95 % confidence levels were determined and extremes of conidial measurements are given in parentheses. For other structures, only extremes are presented. Colony characteristics were noted after 7 d of growth on MEA at 24 °C and colony colours determined using the colour charts of Rayner (1970). Descriptions, nomenclature and illustrations were deposited in MycoBank (Crous et al. 2004).

**Pathogenicity**

In order to test the pathogenicity of the Calonectria spp. collected in this study, seven isolates representing different Calonectria species identified by morphology and DNA sequence comparisons were selected for inoculation trials (Table 1). A conidial suspension (\( 1.0 \times 10^5 \) conidia/mL) was prepared for each isolate by adding sterile water to plates of carnation leaf agar (CLA; Fisher et al. 1982) 7 d after inoculation and dislodging the conidia. The conidial suspension of the isolate CBS 130351 was also applied to the crown of Myrtus communis plants (10 mL/plant). All plants were subsequently covered with plastic bags for 48 h and maintained.

**Taxonomy**

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**Fig. 1** Symptoms of leaf spot caused by Calonectria spp. on several ornamental plants. a. Myrtus communis; b. Metrosideros thomasi; c. Callistemon sp.; d. Callistemon sp.; e. Dodonaea viscosa; f. Callistemon viminalis; g. Metrosideros excelsa; h. Metrosideros excelsa cv. Aurea; i. Metrosideros sp.
in a growth chamber at 25 ± 1 °C for 14 d. Five plants for each isolate and host were used and the same number of control plants were treated using sterile water. Pathogenicity tests were evaluated 5, 10 and 25 d after inoculation.

RESULTS

Disease survey and isolates

During the survey, a total of 46 Calonectria isolates were collected from ornamental hosts sampled. Majority of the isolates (41) were associated with leaf spots or leaf blight of Callistemon spp. (18), D. viscosa (1), Metrostedoros spp. (17) and Myrtus communis (5), and the remaining (5) with crown and root rot of M. communis. Leaves showed minute brown spots, which often enlarged, forming a necrotic centre surrounded by a dark purple halo (Fig. 1). Young, non-lignified terminal shoots often exhibited dieback or lesions similar to those on the leaves. Severe defoliation was observed on M. communis and M. excelsa cv. Aurea. Several seedlings of M. communis had crown and root rot, and fungal sporulation occurred on the lower part of the crown. Initial symptoms were brown lesions that expanded rapidly to girdle the stem at the seedling crown, above and below the soil line, resulting in plant death.

DNA sequence comparisons

Amplicons of approximately 450 bases for HIS3 and 500 bases each for BT and TEF-1α were generated. The 70 % reciprocal bootstrap trees showed no conflict in tree topologies for the three gene regions and therefore they were combined in a dataset consisting of 1 532 characters including gaps. Of these characters, 1 187 were constant and parsimony uninformative. Analysis of the 345 parsimony informative characters yielded 16 equally most parsimonious trees (TL = 814, CI = 0.721, RI = 0.923, RC = 0.666), of which the first tree is presented (Fig. 2). A HKY+I model for BT, a GTR+I+G model for HIS3 and a GTR+G model for TEF-1α was selected for Bayesian analysis. The Bayesian consensus tree confirmed the tree topology obtained with maximum parsimony including bootstrap support.

The phylogenetic tree illustrates a number of well-supported clades containing the Calonectria isolates obtained during the survey. Some of the isolates clustered in a clade representing C. polizzi with a bootstrap value (BP) of 97 and a Bayesian posterior probability (PP) value of 1.00. Several isolates also grouped with and close to C. mexicana in two separate well-supported clades (BP = 68, PP = 0.95 and BP = 78, PP = 0.98, respectively), which could represent novel phylogenetic species.

Table 1 Calonectria isolates used in the phylogenetic analyses and pathogenicity trials.

| Species | Isolate number | β-tubulin | Histone H3 | TEF-1α | Host /substrate |
|---------|----------------|------------|------------|--------|----------------|
| C. brasiliensis | CBS 230.30 | GQ267241 | GQ267259 | GQ267328 | Eucalyptus sp. |
| C. ceriana | CBS 114257 | GQ267242 | GQ267260 | GQ267329 | Leaf litter |
| C. chinensis | CBS 123693 | FJ918510 | FJ918559 | E. grandis × urophylla |
| C. colombiana | CBS 112474 | FJ918511 | FJ918560 | E. grandis × urophylla |
| C. colombiensis | CBS 111220 | GQ267207 | AY725662 | AY725711 | Soil |
| C. hawksworthii | CBS 111870 | AF333407 | GQ190649 | FJ918558 | Nelumbo nucifera |
| C. insularis | CBS 114558 | AF210861 | FJ918526 | FJ918565 | Soil |
| C. leucothoe | CBS 109168 | FJ918508 | FJ918533 | Myrtus communis |
| C. mexicana | CBS 112689 | AF210863 | FJ918560 | Myrtus communis |
| C. pauciramosa | CBS 123402 | FJ972419 | FJ972438 | Callistemon citrinus |
| C. pseudomexicana | CBS 109166 | AN709270 | AN709276 | Callistemon laevis |
| C. pseudoscoparia | CBS 125256 | GQ267228 | GQ267277 | Myrtus communis |
| C. scoparia | CBS 105351 | JN067272 | JN067259 | Myrtus communis |
| C. spathulata | CBS 112869 | AF308463 | FJ918524 | Metrosteros thomassi |
| C. sulawesiensis | CBS 125248 | GQ267225 | GQ267272 | Callistemon citrinus |
| C. tunisiana | CBS 123558 | JN067277 | JN067262 | Metrosteros thomassi |
| C. variabilis | CBS 112691 | GQ267240 | GQ267284 | Metrosteros thomassi |
| C. zuluenis | CBS 114677 | AF333424 | GQ267286 | Metrosteros thomassi |
| D. viscosa | CBS 113091 | AF333429 | GQ267288 | Metrosteros thomassi |
| Eucalyptus | CBS 113091 | AF333429 | GQ267288 | Metrosteros thomassi |
| S. officinalis | CPC 416 | FJ918514 | FJ918531 | E. grandis |
| T. amazoniensis | CPC 416 | FJ918514 | FJ918531 | E. grandis |

1 CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Pedro Crous working collection housed at CBS; DISTEF: Dipartimento di Scienze e Tecnologie Fitosanitarie, Catania, Italy.
2 GenBank accession numbers.
3 Isolates used for the pathogenicity trials.
4 Ex-type cultures; Isolates in bold obtained during survey.
**Taxonomy**

DNA sequence and morphological comparisons of the Calonectria isolates obtained during the survey show that these isolates belong to *C. mexicana* and *C. polizzii* and also constitute two previously undescribed taxa. Based on morphological comparisons, isolate CBS 130353 agrees with *C. mexicana* (Schoch et al. 1999) and isolates DISTEF-TMC2, CBS 130351, DISTEF-TMEA1, DISTEF-TMN3 and CBS 130352 represent *C. polizzii* (Lombard et al. 2010b). The remaining isolates are newly described as follows:

**Calonectria pseudomexicana** L. Lombard, G. Polizzi & Crous, *sp. nov.* — MycoBank MB563138; Fig. 3

Teleomorph unknown.

*Calonectria mexicana* morphologic similes sed minus ramis conidiophorae. 

**Etymology.** Name reflects the fact that this species closely resembles *C. mexicana*.

*Conidiophores* with a stipe bearing penicillate suites of fertile branches, stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 38–69 × 5–9 µm; stipe extensions septate, straight to flexuous, 175–251 µm long, 3–6 µm wide at the apical septum, terminating in a fusiform to broadly ellipsoidal vesicle

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**Fig. 2** One of 16 most parsimonious trees obtained from a heuristic search with 1 000 random addition sequences of the combined sequences of β-tubulin, histone H3 and translation elongation factor 1α sequence alignments of the Calonectria isolates obtained during the survey and other closely related species. Scale bar shows 10 changes. Bootstrap support values (in **bold**) and Bayesian posterior probability values are shown at the nodes. Thickened lines indicate branches in the strict consensus tree and the consensus tree of the Bayesian analyses. The tree was rooted to *C. chinenis* (CBS 112744) and *C. colombiensis* (CBS 112220). Isolates in **bold** were obtained during the survey.
9–14 µm diam with papillate apex. **Conidiogenous apparatus** 38–68 µm long, 32–64 µm wide; primary branches aseptate or 1-septate, 21–43 × 4–7 µm; secondary branches aseptate, 13–26 × 4–7 µm; tertiary branches and additional branches (–4), aseptate, 10–18 × 2–6 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–14 × 2–6 µm; apex with minute periclinal thickening and inconspicuous collarette.

**Conidia** cylindrical, rounded at both ends, straight, (40–)43–48(–49) × (4–)5–6 µm (av. = 45 × 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. **Megaconidia** and microconidia not seen.

Culture characteristics — Colonies fast growing at 24 ºC on MEA, sienna to bay on surface, reverse sienna after 7 d; moderate white aerial mycelium with sparse to moderate sporulation; chlamydospores extensive throughout medium.

**Specimens examined.** **Tunisia.** Carthage, Tunis, from Callistemon sp., Nov. 2010, G. Polizzi, (CBS H-20685, holotype of *C. pseudomexicana*, culture ex-type CBS 130354 = DISTEF-TCROU1); Carthage, Tunis, from Callistemon sp., Nov. 2010, G. Polizzi, culture CBS 130355 = DISTEF-TCROU3; Carthage, Tunis, from Callistemon sp., Nov. 2010, G. Polizzi, culture DISTEF-TCROU4.

**Notes.** *Calonectria pseudomexicana* is morphologically similar to *C. mexicana*. *Calonectria pseudomexicana* has four or less conidiophore branches while *C. mexicana* has five as reported by Schoch et al. (1999).

**Calonectria tunisiana** L. Lombard, G. Polizzi & Crous, sp. nov. — MycoBank MB563139; Fig. 4

Telomorph unknown.

*Calonectria mexicana* morphologic similes sed minus conidiophorae ramis et fructibus breviore sunt stipe auge.

**Etymology.** Name refers to the country Tunisia, where the fungus was collected.

Conidiophores with a stipe bearing penicillate suites of fertile branches, stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 42–95 × 7–11 µm; stipe extensions septate, straight to flexuous, 147–199 µm long, 4–5 µm wide at the apical septum, terminating in a fusiform to broadly ellipsoidal vesicle 8–14 µm diam with papillate apex. **Conidiogenous apparatus** 40–68 µm long, 30–66 µm wide; primary branches aseptate or 1-septate, 17–41 × 5–7 µm; secondary branches aseptate, 10–22 × 4–7 µm; tertiary branches aseptate, 9–18 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–13 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. **Conidia** cylindrical, rounded at both ends, straight,
× 4–6 µm (av. = 49 × 5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Megaconidia and microconidia not seen.

Culture characteristics — Colonies fast growing at 24 °C on MEA, sienna to bay on surface, and reverse sienna after 7 d; sparse white aerial mycelium with sparse sporulation; chlamydospores extensive throughout the medium.

Specimens examined. Tunisia, Carthage, Tunis, from Callistemon laevis, Nov. 2010, G. Polizzi, (CBS H-20684, holotype of C. tunisiana, culture ex-type CBS 130357 = DISTEF-TCL1); Carthage, Tunis, from Callistemon sp., Nov. 2010, G. Polizzi, culture CBS 130356 = DISTEF-TCROU2; Carthage, Tunis, from Metrosideros excelsus, Nov. 2010, G. Polizzi, culture DISTEF-TME1.

Notes — Morphologically, C. tunisiana is similar to C. mexicana and C. pseudomexicana, but can be distinguished from both taxa by its shorter stipe extensions. The conidiophores of C. tunisiana (–3) also form fewer fertile branches than C. mexicana (–5) and C. pseudomexicana (–4) (Schoch et al. 1999).

Pathogenicity
All plants inoculated with the Calonectria spp. in this study developed leaf spot, leaf blight or crown and root rot symptoms. The first symptoms of leaf spot and leaf blight were observed 5 d after inoculation on all test plants inoculated with the Calonectria spp., resembling the symptoms observed during the survey. Isolates of C. pseudomexicana (CBS 130354, 130355), C. tunisiana (CBS 130356, 130357) as well as the single isolate of C. mexicana (CBS 130353) produced the most severe symptoms. Isolates of C. polizzii (CBS 130351, 130352) also caused leaf spot and leaf blight on all inoculated plants, but less severe than the other three Calonectria spp. tested. Ten days after inoculation, severe or moderate defoliation of M. communis and M. excelsa cv. Aurea plants was observed.

All inoculated plants of M. communis developed crown rot, basal stem rot and root rot 25 d after inoculation with the isolate representing C. polizzii (CBS 130351). All un-inoculated control plants remained healthy and re-isolations from the test plants consistently yielded the test fungi.

**DISCUSSION**

During a survey of diseased plants at an ornamental nursery in Tunis, Tunisia, a number of Calonectria spp. were isolated from plants exhibiting crown, root rot and leaf spots. DNA sequence and morphological comparisons allowed the identification of two of these isolates as C. mexicana and C. polizzii as well as the description of two new species, C. pseudomexicana and C. tunisiana, both in the C. scoparia complex (Schoch et al. 1999).
**Calonectria mexicana** resides in the *C. scoparia* complex (Schoch et al. 1999) and can be distinguished from the other seven *Calonectria* spp. in the complex based on their unique papillate vesicles (Schoch et al. 1999, Lombard et al. 2010b, C. Chen et al. 2011). Until now, *C. mexicana* has only been reported from soil samples collected in Mexico, and its pathogenicity was unknown (Schoch et al. 1999, Crous 2002). This study represents the first report of this fungus outside Mexico, and also demonstrates its pathogenicity on some plant hosts.

*Calonectria polizzi* has previously been reported from ornamental plants collected at a nursery in Sicily, Italy (Schoch et al. 2001, Lombard et al. 2010b), although its pathogenicity was not confirmed. This study represents the first confirmation of the pathogenicity of *C. polizzi* and widens its distribution to Tunisia. *Calonectria polizzi* is a member of the *C. scoparia* complex and can be distinguished from the other members by its smaller macroconidial dimensions (Lombard et al. 2010b).

The description of *C. pseudomexicana* and *C. tunisiana* adds two more species to the *C. scoparia* complex. This complex is characterised by 1-septate macroconidia and the formation of ellipsoidal to obpyriform terminal vesicles on the stipe extensions (Schoch et al. 1999, Crous 2002, Lombard et al. 2010b). Based on phylogenetic inference, both these newly described species are closely related to *C. mexicana*, which they also resemble in morphology. They can be distinguished from *C. mexicana* and each other by the number of fertile branches produced on the conidiophores. *Calonectria tunisiana* (av. = 49 × 5 μm) has slightly larger macroconidia than both *C. mexicana* (av. = 45 × 4 μm; Schoch et al. 1999) and *C. pseudomexicana* (av. = 45 × 5 μm).

The pathogenicity tests with isolates of *C. mexicana*, *C. polizzi*, *C. pseudomexicana* and *C. tunisiana* clearly showed that they are able to cause symptoms similar to those observed during the survey. *Calonectria polizzi* was less virulent than the other three species, but should still be regarded as an important nursery pathogen. This supports the view that most *Calonectria* spp. can induce leaf spots if the environmental conditions are favourable (Crous 2002). All four species caused similar disease symptoms on the nine inoculated plant species, suggesting that little is known about the host specificity and mechanisms of infection of this group of plant pathogens.

This study stresses the importance of *Calonectria* spp. as nursery pathogens. Their soil-borne nature has contributed to their ease of movement globally and little is known about their origins. Furthermore, it is not known if these fungal pathogens originated from Tunisia or were introduced, and more isolates are needed for a study of their population dynamics.

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