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Research Papers

Ceratocystis ficiicola causing a serious disease of *Ficus carica* in Greece

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Summary. *Ceratocystis ficiicola* causes vascular wilt of fig trees in Japan, invading root systems and the main stems eventually leading to tree death. In surveys from 2018 to 2020 in fig orchards in Greece, this fungus was detected in two separated regions. The fungus was consistently isolated from infected wood and from rhizosphere soil. The isolates were identified based on multi-locus phylogenetic analyses of *rpb2*, *bt1* and *tef1* gene regions and detailed morphological characteristics, including comparisons with an ex-type isolate of *C. ficiicola* from Japan. The pathogenicity of Greek isolates was proven on *Ficus carica* and *F. benjamina* plants. *Ceratocystis ficiicola* is a soil-borne pathogen, and the occurrence of vascular wilt outbreaks suggest that the pathogen spreads within and between orchards with infested soil and wood debris during ploughing. The pathogen is also spreading in Greece with infected propagation material. This is the first detailed report of *C. ficiicola* outside Japan, and there is concern over potential spread of the pathogen to other Mediterranean countries, where approx. 70% of the world fig production occurs.

Keywords. *Ceratocystis ficiicola*, *Ficus carica*, soil-borne pathogen, vascular wilt, Greece.

INTRODUCTION

Fig (*Ficus carica* L.) is one of the oldest domesticated tree crops globally. According to Kislev *et al.* (2006) this tree was cultivated for its fruit during the Early Neolithic period (11,400 to 11,200 years ago), preceding cereal domestication. *Ficus* (Moraceae) includes more than 700 species, native to the tropics or sub-tropics. *Ficus carica* is indigenous to the Middle East and Asia Minor, and is mostly cultivated in warm and dry areas of the Mediterranean region (Condit, 1955; Flaishman *et al.*, 2008). *Ficus carica* has been cultivated in Greece since ancient times, as mentioned by Homer (8th century BC) *The Odyssey*, and Theophrastus (4–3rd century BC) described cross-

fertilization of wild and domesticated figs (Condit, 1955). Fig continues to be an important crop, in Greece, where production in 2019 was 19,730 t (FAO, 2019). Thus, any negative impact on these trees due to serious disease is considered important from both a cultural and an economic standpoint.

Since the 1970s, a serious disease caused by a *Ceratocystis* species has been reported in Japanese fig orchards. The causal agent of this disease was initially attributed to *Ceratocystis fimbriata sensu lato* (Kato *et al.*, 1982). Kajitani and Masuya (2011) described the pathogen as a new species, *Ceratocystis ficicola* Kajitani and Masuya, based on discernable morphological characteristics and DNA sequence data. Like many other *Ceratocystis* species, *C. ficicola* is a soil-borne pathogen that infects roots and main stems of susceptible hosts, causing vascular wilt and eventually tree death. The pathogen is dispersed by human activities, mainly with contaminated plant material, and has spread to many fig-growing areas of Japan (Kajitani and Masuya, 2011; Kajii *et al.*, 2013).

In 2018, a *Ceratocystis* species was isolated from two neighbouring fig orchards in the Attica region of Greece. The affected trees showed symptoms of wilt and extensive crown defoliation, and some trees had died. The morphological characteristics of the isolated fungus were similar to those of *C. ficicola* (Tjamos *et al.*, 2018). In 2019 and 2020, the same fungus, based on morphology, was also isolated from diseased fig trees at different localities on Euboea Island, where considerable damage was occurring in fig orchards.

The objective of the present study was to identify *Ceratocystis* species, having similar morphology to *C. ficicola*, found causing disease on *F. carica* in the two regions of Greece. This was achieved based on multi-locus phylogenetic analyses and detailed observation of morphological characteristics of the fungus, including

comparisons with the ex-type isolate of *C. ficicola*. Pathways of dispersal of the pathogen were also considered and the extent of damage to fig orchards in Greece is reported. Pathogenicity tests were also conducted with the fungus on plants of *F. carica* and *Ficus benjamina* L.

MATERIALS AND METHODS

Sampling and fungus isolations

Field surveys for disease symptoms were conducted from April to early September, corresponding to the time of year that fig trees retain full foliage. In the fig orchards of the Markopoulo Mesogaia municipality in East Attica, surveys took place in 2018 and 2019, and in the Istiaia-Aidipsos municipality on the Euboea Island, in 2019 and 2020 (Table 1). These two regions are more than 200 km apart.

Trees with symptoms including crown defoliation and leaf wilt were sampled. The lower parts of the main stems were examined for the presence of necrotic bark, which was then removed to expose diseased cambial tissues and discoloured wood (Figure 1C–E). Samples of stained wood were then cut from the lesions using a surface-disinfected chisel. Some of the young plants were uprooted; the root systems examined for disease symptoms, and these were collected for laboratory examination. Soil samples were also collected from a depth of 15–20 cm near the bases of symptomatic trees from most of the surveyed orchards.

For isolation of fungi, PARPH V8 agar medium was used (containing 5% clarified V8 juice, 5 µg mL⁻¹ pimaricin, 250 µg mL⁻¹ ampicillin, 10 µg mL⁻¹ rifamycin, 50 µg mL⁻¹ PCNB, and 50 µg mL⁻¹ hymexazol). This selective medium is widely used for the isolation of *Phytophthora* species (Ferguson and Jeffers, 1999) but was also effective for the isolation of *Ceratocystis* species.

Table 1. Occurrence of *Ceratocystis ficicola* in fig orchards of Greece.

Locality	Fig cultivar	Dates investigated	Number of orchards	Number of trees sampled
<i>Regional Unit of East Attica</i>				
Municipality of Markopoulo Mesogaia	Vasilika honey-white	May 2018	1	4
	Vasilika black	May 2018	1	2
	Vasilika honey-white	April 2019	1	2
<i>Euboea Island</i>				
Municipality of Istiaia-Aidipsos				
Avgaria village	Smyrna	April, September 2019	2	3
Taxiarchis village	Smyrna	September 2019	1	2
Oreoi village	Smyrna	September 2020	2	4
Kastaniotissa village	Smyrna	September 2020	2	3



Figure 1. Field images of infected fig trees. A, infected mature tree of Vasilika honey-white cultivar with symptoms of leaf wilt and defoliation; B, infected young tree of Smyrna cultivar defoliated at the end of summer, with some of the unripe fruit still on the tree; and C, D and E, cankers at the bases of *Ficus carica* trees, with extensive wood staining under the dead bark.

Direct isolation from wood samples was performed after they had been surface-disinfested with 70% ethanol and air dried in a laminar flow cabinet. Small pieces of stained wood were aseptically removed from the samples with a sterile scalpel and transferred to Petri dishes containing PARPH V8A medium.

A carrot baiting method was also used for a sub-set of samples. Small pieces of stained wood were placed between two slices (each 5 mm thick) of carrot root, as described by Moller and De Vay (1968). The carrot baits were then placed in empty Petri dishes with moistened filter paper and incubated for 7–10 d at 25°C. The carrot discs were subsequently examined for the presence of ascomata under a dissecting microscope, and when these

were present, masses of ascospores were transferred to PARPH V8A medium.

Isolations from soil samples were performed using a modification of the trapping technique described by Grosclaude *et al.* (1988) for *Ceratocystis platani* (Walter) Engelbrecht and Harrington. Soil samples (40–50 g) were placed in 250 mL capacity conical flasks, which were filled with 200 mL of sterile deionized water. Freshly cut twigs from fig trees (approx. 10 cm long and 6–10 mm in diameter) with the bark removed were placed in the flasks. Air was continuously pumped into the flasks through a Pasteur pipette, using an aquarium pump (Aqua-Air AP4, Interpet). The flasks were incubated at room temperature (23–25°C). After 7–10 days, the

twigs were examined under a dissecting microscope for the presence of ascomata and, where present, masses of ascospores were aseptically transferred to PARPH V8A medium. In some cases, this trapping technique was also used with pieces of infected wood in the flasks rather than soil. In all cases, resulting, agar medium colonies having *Ceratocystis* morphology were sub-cultured onto V8A medium.

Growth in culture

Growth rates of fungal cultures were determined on 10% V8A at different temperatures from 10 to 40 °C at 5°C intervals. Two measurements of colony diameters were made perpendicular to each other on each culture plate after 7 and 14 d incubation at 25°C.

Measurements of fungal structures were made from 7 to 10d old pure cultures on V8A and malt extract agar (MEA) with fungal structures mounted in water or 85% lactic acid. A Zeiss Axioskop light microscope and SC30 camera (Olympus) or a Nikon Eclipse Ni microscope and DS-Ri2 camera (Nikon) were used to capture images and to take measurements. Ascomata were examined at 50x or 100x magnification and the conidia and ascospores at 400x or 1000x. For each of the two isolates from Greece (CMW 56935 and CMW 54794), the dimensions of 50 conidia of each type were measured.

Besides the isolates from Greece, the ex-holotype culture of *C. ficicola* from Japan (MAFF 625119 = CMW 38543) was also examined.

DNA extraction, sequencing and phylogenetic analyses

To confirm the identity of the *Ceratocystis* species isolated from infected fig trees, two cultures from infected wood and two from the soil baiting (Table 2) were subjected to DNA sequencing. Mycelium from the cultures grown on 2% MEA for approx. 2 weeks was scraped from the surfaces of the agar culture plates and DNA was extracted using the Fungal DNA MiniPrep kit (Zymo Research).

Three gene regions were PCR-amplified and sequenced. Primers RPB2-5Fb and RPB2-7Rb were used to amplify the second largest subunit of the RNA polymerase II (*rpb2*) (Fourie *et al.*, 2015), primers β t1a and β t1b (Glass and Donaldson, 1995) to amplify the β -tubulin 1 (*bt1*) region, and primers EF1-728F and EF1-986R (Jacobs *et al.*, 2004) for the Translation Elongation Factor 1- α (*tef1*) gene region.

PCR reactions and conditions, including optimal MgCl₂ concentrations and annealing temperature for the three gene regions, were carried out as described by Fourie *et al.* (2015), with an Expand-PCR programme of 96°C for 10 min, (94°C for 30 s, primer specific anneal-

Table 2. Details of the *Ceratocystis ficicola* isolates from Japan and Greece analysed in the present study.

Species ¹	Isolate No. ²	Substrate	Country	Locality	Year collected	GenBank accession numbers		
						bt1	tef1	rpb2
<i>C. ficicola</i> ^T	MycoBank 518749; CMW 38543; BPI 843724, MAFF 625119	<i>Ficus carica</i>	Japan	Fukuoka Prefecture	1990	KY685077	KY316544	KY685082
<i>C. ficicola</i>	CMW 38544; NCF0801	<i>Ficus carica</i>	Japan	Fukuoka Prefecture	1991	KY685078	KY685079	KY685083
<i>C. ficicola</i>	CMW 54793; FCE-1	<i>Ficus carica</i> cultivar Smyrna	Greece	Istiaia-Aidipsos, Avgaria village	2019	Same as KY685077	Same as KY316544	Same as KY685082
<i>C. ficicola</i>	CMW 54794; FCE-2	Soil under infected <i>Ficus carica</i> cultivar Smyrna	Greece	Istiaia-Aidipsos, Avgaria village	2019	Same as KY685077	Same as KY316544	Same as KY685082
<i>C. ficicola</i>	CMW 54795; FCE-4	Soil under infected <i>Ficus carica</i> cultivar Smyrna	Greece	Istiaia-Aidipsos, Avgaria village	2019	Same as KY685077	Same as KY316544	Same as KY685082
<i>C. ficicola</i>	CMW 54796; FCM-1	<i>Ficus carica</i> cultivar Vassilika honey white	Greece	Municipality of Markopoulo Mesogaia	2018	Same as KY685077	Same as KY316544	Same as KY685082

¹ T = Ex-type material of *Ceratocystis ficicola*;

² CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; BPI = US National Fungus Collections, Beltsville, Maryland, USA; MAFF = Ministry of Agriculture, Forestry and Fisheries Culture Collection, Tsukuba, Ibaraki, Japan; FCE, FCM= Institute of Mediterranean Forest Ecosystems culture collection numbers

ing temperature for 45 s, 72°C for 1 min) for 10 cycles, (94°C for 30 s, primer specific annealing temperature for 45 s, 72°C for 1 min + 5 s/cycle increase) for 30 cycles, and 72°C for 10 min. All amplicons were purified using 6% Sephadex G-50 columns (Sigma-Aldrich), were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems), and the products were run on an ABI 3100 Genetic Analyzer (Applied BioSystems, Thermo Fisher).

The forward and reverse sequence reads were assembled into contigs in CLC Bio Main workbench v.6 (CLC Bio, www.clcbio.com), and the consensus sequences generated were used in BLAST analyses against the NCBI GenBank database (NCBI; <http://www.ncbi.nlm.nih.gov>) to determine the closest similarity matches with authenticated *Ceratocystis* species.

Sequences were added to the combined *rbp2*, *bt1* and *tef1* *Ceratocystis* database obtained from Barnes *et al.* (2018) (TreeBASE No. S22005), with a focus on retaining all the *Ceratocystis* species in the Asian-Australian Clade (AAC) in the phylogenetic analyses. *Ceratocystis albifundus* was used as the outgroup taxon. Sequences were re-aligned using the online version of MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/server/>). Maximum parsimony (MP) analyses performed in PAUP v. 4.0 (Swofford 2002) and Maximum likelihood (ML) analysis in RAxML (Stamatakis 2014) were carried out on the combined dataset with the same parameters used by Barnes *et al.* (2018).

Inoculation tests

Two isolates of *C. ficicola* collected from different localities were used for inoculation trials. One of these (CMW 56935) from the Attica region was used in the first test (2018) and the other (CMW 54794) from Euboea Island was used in the second test (2019). The isolates used in the inoculation tests were grown for 2 weeks at 25°C in Petri dishes containing V8A. For inoculations, a 3 mm diam. cork-borer was used to remove the bark from stems or branches of test trees (see below), and a mycelium plug of similar size was inserted into each wound which was then wrapped with Parafilm®. Control plants were inoculated in the same way with sterile V8 agar plugs.

The two inoculation tests were carried out at the Institute of Mediterranean Forest Ecosystems in Athens, Greece. In the first test, 11 branches (diam. 1–4 cm) of a wild *F. carica* tree were inoculated in June 2018 with isolate CMW 56935. In the second test conducted in September 2019, a total of 13 *F. carica* saplings (1–2 years-

old) of different size and grown in 7.5 L capacity pots, were inoculated with isolate CMW 54794. Stem diameters of these plants at the inoculation points was from 5 to 20 mm. Non-inoculated control plants were not used in the first test, while in the second test six plants were inoculated with sterile V8 agar plugs.

In September 2019, inoculations were also performed on branches of two *Ficus benjamina* trees, in the same area with isolate CMW 54794. A total of 16 branches on the two trees were inoculated. A third *F. benjamina* tree was used as a control, by inoculating seven branches with sterile V8 agar plugs.

After inoculation, the plants were monitored regularly for expression of disease symptoms. In the first inoculation test (2018), the inoculated branches were harvested after 5 weeks. In the second test (2019), most of the plants were harvested 6 weeks after inoculation, although some of the plants displaying symptoms of wilt were harvested at an earlier time to allow for lesions to be measured. All inoculated stems and branches were sliced open vertically above and below the inoculation points, and the lengths of internal xylem staining were measured. Re-isolations from these lesions were made at different distances from the inoculation points, by aseptically transferring pieces of stained wood onto the selective V8A medium.

Statistical analyses were performed to compare the means of internal lesion lengths for the inoculations on *F. carica* and *F. benjamina* branches or stems of the *F. carica* sapling plants. In these comparisons, only the saplings of *F. carica* that were harvested after 6 weeks were included. A Shapiro-Wilk test was used to test normality of the data, and a Levene's test was used to assess equality of variances. A Kruskal-Wallis H test was then applied to compare the means of lesion lengths between *F. carica* and *F. benjamina* plants, followed by Dunn-Bonferroni *post hoc* method.

RESULTS

Disease symptoms and distribution

In the Markopoulo Mesogaias municipality in East Attica, wilt of fig trees was detected at one location with orchards of mature fig trees (20–30 years-old) of the 'Vasilika honey-white' and 'Vasilika black' cultivars, distributed over approx. 10 ha (Table 1). In the Istiaia-Aidipsos municipality, in the northern part of Euboea Island, the disease was found in fig orchards at four locations (villages) more than 10 km apart, on young as well as mature trees. All the wilt-affected trees on Euboea Island were of the 'Smyrna' cultivar (Table 1).

Wilted mature trees were usually located in one part of each orchard, and the disease appeared to have spread to neighbouring fig trees. In some cases, more than one disease centre was observed. Infected mature trees of all three cultivars showed thinning and yellowing of the foliage, either on some of the branches or over the entire crowns (Figure 1A). In some trees, most of the leaves had dropped by the end of summer, while some of the unripe fruits were retained on the trees indicating rapid death (Figure 1B). In many of the affected mature trees, complete defoliation was observed by the end of the summer, although full foliage had been present on these trees early in the spring. The affected trees eventually died, and in many cases, where dead trees had been replaced with new young plants; these were also dying within 1 to 2 years after replacement.

On mature trees, extensive bark cankers were observed at the bases of the trunks. Necrotic dark brown sapwood was obvious after removal of the bark in the lower part of the trunks (Figure 1C–E), and these lesions extended upwards. Necrotic roots were also observed on affected trees. In some symptomatic young (2–3 years-old) trees, wood staining was not always present at the trunk bases, although in these cases necrotic roots were observed. Wood staining was not found on the branches of wilted trees with chlorotic leaves, or on defoliated trees. Infestations by wood boring insects were not observed on any of the wilted fig trees examined.

In a newly planted 6 ha orchard on Euboea Island, 3 years after planting, approx. 40–50% of the trees were dead or dying with evident disease symptoms. This land had not previously been used for fig tree cultivation.

Fungus isolations

A *Ceratocystis* species was consistently isolated directly from stained fig tree wood on the selective PARPH V8A medium. The fungus was also isolated on plain V8A, but in most of these cases it was overgrown by saprotrophic fungi. On selective medium, colonies were suppressed and scarcely formed ascomata, but they resumed normal growth when transferred to plain V8A medium and ascomata developed after 7–10 d.

The *Ceratocystis* species was also isolated using the carrot baiting technique, but in many cases the carrots were contaminated with bacteria and isolations were inconsistent using this technique. The trapping technique with fig twigs as baits was effective for isolating the *Ceratocystis* species from soil and from wood samples. For twig baiting, the fungus had colonized the twigs and formed ascomata (Figure 2A) after 7–10 d, at the water level. The fungus was easily isolated from

ascospore masses transferred to selective medium. In a few cases, it was not possible to isolate the fungus from wood samples taken from symptomatic trees, especially where these trees had died, but the fungus was readily isolated from soil samples collected near the bases of these trees using the twig baiting technique.

Fungus morphology

Colonies in V8A, after 7–10 d in culture, were circular with slightly undulate margins and were dark oliveaceous green. The optimum growth temperature was 25°C, with a radial growth of 20–23 mm week⁻¹. The fungus grew well at 30°C but no growth was observed at 35 or 40°C. When the cultures were transferred to 25°C after 2 weeks incubation at 35°C, the colonies resumed normal growth, but incubation for 2 weeks at 40°C killed the fungus and no growth was observed on the plates after transferring these to 25°C.

Black ascomata (Figure 2A, B) were observed after 7–10 d incubation on V8A, and were partially embedded in the agar. These measured 400–600 µm diam. and had globose bases and long necks (1150–2200 µm long). They had ostiolar hyphae at the tips of the necks (Figure 2C) which were 180–300 µm long. Ascospores exuding from the tips of the ascomata necks formed sticky masses of creamy colour (Figure 2A). Ascospores were one-celled, ellipsoidal in top view, measured 5–7 × 4–5 µm, with the characteristic “bowler-hat” shape typical of *Ceratocystis* spp. (Figure 2D). The fungus produced abundant cylindrical endoconidia (12–40 × 4–7 µm) with rounded apices, and these varied slightly as bulged to straight, from less to more round apices (Figure 2F, G). The conidia were extruded in chains from tubular conidiogenous cells (Figure 2H). Aleurioconidia (Figure 2E) were formed singly or in short chains on short conidiophores, and these were ovoid to subglobose (9–13 × 7–12 µm), pigmented and thick-walled. Doliform endoconidia were not observed.

Morphological characteristics of the Greek isolates were identical to those of the Japanese isolate (exholotype MAFF 625119 = CMW 38543), except that the dimensions of the endoconidia were different. The Japanese isolate had cylindrical endoconidia with dimensions of 5–9.5 × 4.5–8 µm, whereas those made in the present study were 12–40 × 4–7 µm.

Sequencing and phylogenetic analyses

Amplicons of the *rpb2*, *bt1* and *tef1* gene regions produced fragments of approx. 1123, 592 and 754 bp,



Figure 2. Microscopic features of *Ceratocystis ficicola*. A, ascomata on a twig bait; B, ascoma; C, ostiolar hyphae; D, ascospores; E, an aleurioconidium; F and G, cylindrical endoconidia; H, phialides. Scale bars: A = 1.5 mm; B = 500 μ m; C = 100 μ m; D to H = 10 μ m.

respectively. For each of the three gene regions, the sequences for all four isolates were 100% identical to each other. Blast searches against the NCBI GenBank database resulted in 100% identity matches to *C. ficicola* ex-type isolate from Japan [Mycobank MB518749, CMW 38543 for *rpb2* (KY685082), *bt1* (KY685077) and *tef1* (KY316544) gene regions (Table 2)].

The combined dataset (*rpb2*, *bt1* and *tef1*) of the sequences used for the phylogenetic analyses consisted of 2127 aligned characters. MP analyses resulted in eight most parsimonious trees with TL = 299, CI = 0.863, RI = 0.945 and RC = 0.815, with 238 parsimony-informative characters and six uninformative characters. For the ML analyses, using GTR-GAMMA as the best fit model,

the log-likelihood of the most likely tree obtained was -5139.12. MP and ML analyses placed the isolates from Greece, collected from infected *F. carica*, and those obtained from the soil, in the same clade as those of *C. ficicola* from Japan with 100% bootstrap support (Figure 3). These isolates form part of the Asian Australasian Clade (ACC) of *Ceratocystis*, as defined by Li *et al.* (2017).

Inoculation tests

All the plants of *F. carica* and *F. benjamina* inoculated with *C. ficicola* developed symptoms of infections (Figure 4), while no symptoms were observed on any

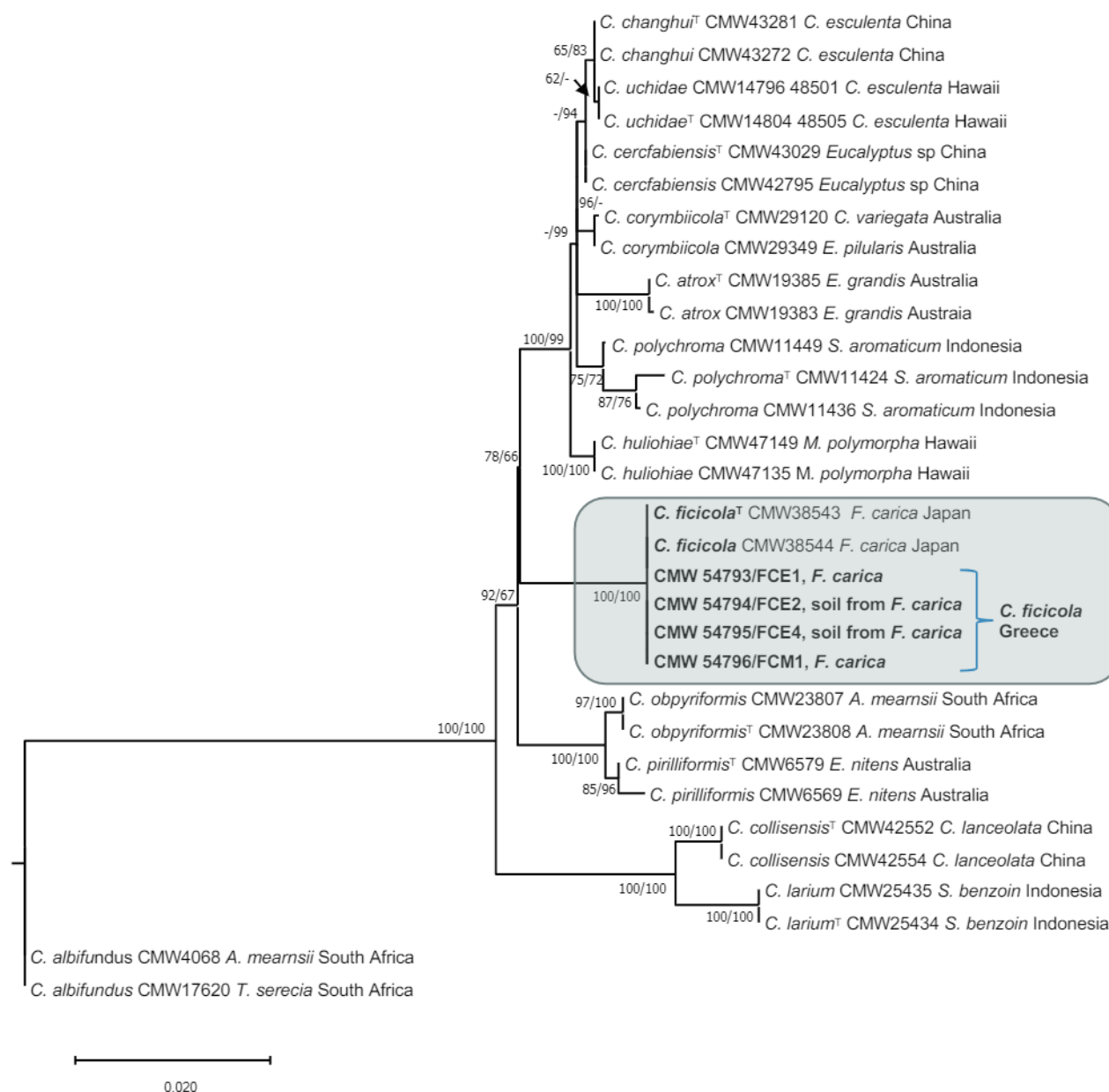


Figure 3. Phylogenetic placement of the isolates from Greece with *Ceratocystis ficicola* from Japan in the Asian-Australian *Ceratocystis* Clade. The ML phylogeny, constructed from the combined regions of the *rpb2*, *bt1* and *tef1* gene sequences, is presented with MP and ML bootstrap values >60 % indicated on the branch nodes. T indicates ex-type representatives of each species. *Ceratocystis albifundus* was used as the outgroup.

of the control plants. Lesions on the inoculated plants included necrotic bark around the inoculation point (Figure 4B), which, in some cases, was evident as early as 2 weeks after inoculation. Exposure of the cambium in stems showed brown-coloured streaked discolouration of the woody tissues, extending longitudinally in the xylem beyond the necrotic bark (Figure 4D, E).

Branch inoculations on *Ficus carica*

The internal lesions associated with the stained wood on the *F. carica* branches inoculated in 2018, and measured after 5 weeks, extended up to 12 cm from the inoculation points. The lesion lengths were 10–23 cm (mean = 14.2 cm).



Figure 4. Inoculations with *Ceratocystis ficicola*. A, *Ficus carica* symptoms of leaf wilt 6 weeks after inoculation; B, canker formed on a branch twig of *F. carica* 5 weeks after inoculation; C, canker formed on a branch twig of *F. benjamina* 6 weeks after inoculation; D and E, main stem of a *F. carica* sapling with xylem discoloration extending longitudinally beyond the necrotic bark 6 weeks after inoculation. Arrows in E show the edges of the lesion.

Inoculations on *Ficus carica* saplings

Three of the *F. carica* saplings inoculated in 2019 that had small diameters (57 mm), showed stem girdling and symptoms of wilt 2 weeks after inoculation. For these three plants, the lesions were 6–12 cm long. For the other ten saplings, the internal lesion lengths were up to 21 cm long at 6 weeks after inoculation. The total lengths of the stained wood associated with these inoculations was from 2.6–37.8 cm (mean = 12.9 cm) (Figure 5).

Branch inoculations on *Ficus benjamina*

Inoculated-branches developed necrotic lesions around the inoculation sites (Figure 4C). On this host, wood staining was very limited, extending only 0.8–1.5 cm from the inoculation points with total length 1.4–3 cm (mean = 2.17 cm). Symptoms of wilt were not evident on branches 6 weeks after inoculation.

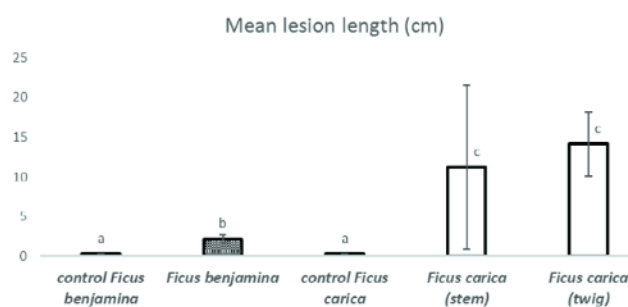


Figure 5. Mean internal lesion lengths (cm), after inoculation of *Ficus carica* sapling stems and branch twigs, or *F. benjamina* branch twigs, with *Ceratocystis ficicola*. Columns accompanied by standard deviations of means, and those accompanied by the same letters are not significantly different ($P \leq 0.05$).

There were no statistically significant differences in the mean internal lesion lengths between *F. carica* saplings (stems) and branches (twigs) in the two experiments, according to Kruskal-Wallis H test and Dunn-

Bonferroni post hoc method ($P \leq 0.05$) (Figure 5). In contrast, the mean lesion lengths on the *F. benjamina* branches differed significantly from both of the *F. carica* inoculations. There were also significant differences between lesion lengths for the control plants and those inoculated with the *C. ficicola* on both hosts (Figure 5). For *F. carica*, wood discolouration was limited to approx. 3 mm around the inoculation sites.

The inoculated fungus was consistently re-isolated from stained wood from *F. carica* and *F. benjamina* plants, and at different distances (up to 17 cm) from the inoculation points. The re-isolated fungus had the typical morphology of the inoculated fungus. The fungus could not be isolated from plants inoculated as experimental controls.

DISCUSSION

This study has provided clear evidence for the presence of *C. ficicola* in fig orchards in two separate regions (Attica and Euboea Island) of Greece. These two areas separated by large distance. This is the first detailed report of the presence of this fungus outside of Japan, in this case also supported by morphological and DNA sequence comparisons. This study also included inoculation trials that confirmed the pathogenicity of Greek isolates of *C. ficicola*.

A similar disease to that caused by *C. ficicola* in Japan and Greece has been reported in Brazil on cultivated fig trees. In that case the pathogen was reported as *Ceratocystis fimbriata* Ellis and Halsted *sensu lato* (Pasura and Harrington, 2004). Kajitani and Masuya (2011) showed that *C. ficicola* is different to the *Ceratocystis* species from fig trees in Brazil, based on morphological differences and ITS sequences. The distinct nature of the fungus killing fig trees in Brazil and Japan was also confirmed by Harrington *et al.* (2011). Results of the present study based on morphological comparisons and DNA sequence data for three gene regions also confirmed that *C. ficicola* from Greece is in the Asian-Australian clade of *Ceratocystis* (Li *et al.*, 2017). In contrast, isolates from fig trees in Brazil form part of the Latin American Clade (Harrington *et al.*, 2011).

Observations in the present study agree with those of Kajitani and Masuya (2011), confirming that *C. ficicola* has morphology that is distinct from other species of *Ceratocystis*. This is relevant as many species of *Ceratocystis*, as defined by De Beer *et al.* (2014), cannot easily be distinguished based on morphology. The most characteristic structures in *C. ficicola* that distinguish it from other *Ceratocystis* species are the large ascomata with

very long necks ($>2000 \mu\text{m}$). Ascospores are very similar in shape and size to those of *Ceratocystis fimbriata sensu stricto* (Marincowitz *et al.*, 2020). Doliform endoconidia were not observed in the present study, and these were also absent from observations of Kajitani and Masuya (2011). However, there was a difference in the size of the cylindrical endoconidia in the present study isolates compared with those described by Kajitani and Masuya (2011). This was confirmed in comparisons of the Greek isolates and the Japanese ex-holotype isolate (CMW 38543) made in the present study.

In Japan, the disease of fig caused by *C. ficicola* is commonly referred to as “*Ceratocystis* canker”, but Kajii *et al.* (2013) stated that the disease is more typical of vascular wilt. The pathogen invades the roots and the main stems of host plants, causing xylem dysfunction and wilt symptoms on infected fig trees. In cross sections of main stems, dark brown radial discolouration of the sapwood tissues was evident at the bases of affected trees, extending upwards and downwards to the roots (Kajii *et al.*, 2013). Consistent with the view of Kajii *et al.* (2013) that infection by *C. ficicola* should be defined as a wilt disease, Morita *et al.* (2016) and Sumida *et al.* (2016) in inoculation studies on mature fig trees, as well as young seedlings, reported that xylem discolourations were correlated with xylem dysfunction. The water supply to leaves decreased when infection progressed causing leaf wilting and extensive xylem dysfunction leading to plant death.

Ceratocystis ficicola also kills host cambium and the bark tissues causing cankers, which were present at the bases of the main stems on most symptomatic trees in Greece. In our surveys, wood discolouration was not evident in the branches of trees displaying wilt of the leaves, and similar observations were made in Japan by Kajii *et al.* (2013). However, the inoculated branch twigs in our experiments developed cankers and extensive wood discoloration. Overall, these symptoms are similar to those of many other tree diseases caused by *Ceratocystis* species, and the disease is best described as canker-wilt (Tsopelas *et al.*, 2017; Nasution *et al.*, 2019).

In the present study, inoculation experiments, showed that *F. benjamina* and not only *F. carica* plants can become infected with *C. ficicola*. However, this species was less susceptible to the pathogen than *F. carica*. In Japan, *Ficus erecta* Thunb., which is an indigenous species, has been proven resistant to *C. ficicola* and has been used in breeding programmes to develop resistant fig tree rootstocks (Yakushiji *et al.*, 2019).

In several studies carried out in Japan, it has been shown that *C. ficicola* is a soil-borne pathogen. It has also been suggested that the fungus persists in the soil as thick-walled aleurioconidia, and newly planted trees in contami-

nated soil are soon infected by the pathogen (Kajii *et al.*, 2013; Yakushiji *et al.*, 2019). Our field observations, in both regions of Greece considered, also suggest soil-borne disease problems, where trees were planted to replace those that had died also became infected. We consistently isolated *C. ficicola* from soils associated with dying trees.

Detection of *C. ficicola* in the soil at an early stage will be important for pathogen detection and application of canker-wilt control strategies. In the present study, using twig baiting, the fungus was readily isolated from soil, even in cases where the pathogen could not be directly isolated from wood samples from nearby symptomatic trees. This baiting method was originally described by Grosclaude *et al.* (1988) for detection and isolation of *Ceratocystis platani*, which causes the canker stain of plane trees (*Platanus*). The method was also successfully used to isolate *C. platani* from adult *Platypus cylindrus* Fab. beetles, a vector of *C. platani* (Soulioti *et al.*, 2015). The present study is the first to use baiting technique to isolate *C. ficicola* from soil and wood samples.

According to Kajitani and Masuya (2011), the dispersal biology of *C. ficicola* is not fully understood. One of the suggested pathways of disease spread in Japan was with infected propagation material (Kajii *et al.*, 2013). This was also evident in one of the orchards examined on Euboea Island. The pathogen was widespread in a new plantation over an area of 6 ha, 3 years after the fig trees had been planted. Since there was no previous use of this land for fig cultivation, the most probable means of pathogen dissemination over such a short period would be with infected nursery stock.

Every effort should be made to avoid further spread of *C. ficicola* with planting material and via contaminated soil. This is a probable pathway for introduction and spread of the pathogen into other areas and countries. The pathogen can survive in soil associated with plants for planting, when symptoms are not evident. While nursery stock should be carefully examined for the presence of *C. ficicola*, disease symptoms may not be always evident, especially during winter when new plants are being established. This problem is exacerbated with fig trees having no foliage during this period, and infected plants may escape the attention during inspections.

Ceratocystis ficicola may also spread with tractors used for ploughing that move infested soil and wood debris within or between orchards. Deep ploughing also causes root wounding, allowing pathogen infection sites. The use of infested ploughing machinery is likely to be a major pathway for pathogen dissemination in the fig orchards of Euboea Island. A major pathway of disease spread for *C. platani* in many areas of Europe has been from terracing machinery that transmit the pathogen

over short and long distances (Tsopelas *et al.*, 2017). Some *Ceratocystis* species can also survive for long periods as aleurioconidia in infected wood. This spore form was also observed by Kajii *et al.*, (2013) in the vessel elements of fig trees infected with *C. ficicola*. Contaminated pruning and cutting tools may therefore be involved in the spread of the pathogen to healthy trees. Precautionary measures, including disinfection of pruning tools and the machinery used in fig orchards have been recommended to avoid further spread of *C. ficicola* in Greece.

Ambrosia beetles (*Euwallacea interjectus* Blandford) have been suggested as possible vectors of *C. ficicola* in Japan. These insects also contribute to expansion of the fungus in sapwood of infected trees (Kajii *et al.*, 2013). The frass produced by the insects may also disseminate the pathogen, as with other *Ceratocystis* species (Harrington, 2013; Tsopelas *et al.*, 2017). Frass can be dispersed by wind, rain splash or running water over short distances, and can contaminate soil or initiate new infections in pruning wounds (Harrington, 2013). In Greece, infestation by wood boring insects in *C. ficicola*-infected fig trees has not been observed. However, future surveys should include careful inspection for the presence of such insects. The ambrosia beetle *Platypus cylindrus* has been reported in Greece to attack *Platanus orientalis* trees already infected by *C. platani*, and this insect may be involved in the spread of that pathogen (Soulioti *et al.*, 2015).

The origin of *C. ficicola* is unknown. Kajitani and Masuya (2011) have suggested two possibilities; one that the fungus is invasive in Japan, or alternatively, that it is native to that country and has, over time, come into contact with the highly susceptible *F. carica*. All evidence emerging from the present study indicates that *C. ficicola* is an alien invasive pathogen in Greece. This because *F. carica* has been cultivated in Greece since ancient times, but canker-wilt has only been detected in recent years and at a small scale. An important question remains as to how the pathogen was introduced into this country, either from Japan or from another source in the Mediterranean region where it is yet to be detected.

Ceratocystis ficicola causes a very important disease of fig trees in Japan, resulting in severe outbreaks of canker-wilt (Kajitani and Masuya, 2011; Yakushiji *et al.*, 2019). The presence of the pathogen in Greece is particularly relevant. Fig is a very important crop in Mediterranean countries, because it is adapted to the dry climatic conditions of the region. About 70% of total world fig production occurs in Mediterranean countries (Flaishman *et al.*, 2008). Consequently, the disease could result in severe losses if the pathogen were to be disseminated to other countries, such as Turkey, Egypt or Morocco where fig production is also important.

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