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Seiridium cardinale on *Juniperus* species in Greece

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Summary

Seiridium cardinale, the cause of cypress canker disease, was found on *Juniperus foetidissima, J. excelsa, J. oxycedrus* and *J. phoenicea*, in a number of natural juniper woodlands in Greece. The presence of infections was sporadic in most cases, with a limited number of plants affected by the pathogen. At one locality in the Prespes Lakes region of northern Greece, however, the incidence of infection was very high, especially on *J. foetidissima* and *J. excelsa*. The identity of *S. cardinale* was confirmed using morphological characters and comparisons of DNA sequences for the β-tubulin gene region. The pathogenicity of *S. cardinale* isolates from *Juniperus* and *Cupressus* was verified in cross-inoculation trials on both potted and field grown plants.

1 Introduction

The fungus *Seiridium cardinale* (Wag.) Sutton & Gibson causes a serious canker disease on cypress trees and other members of the Cupressaceae. It was originally reported in California on Monterey cypress (*Cupressus macrocarpa* Hartweg.; Wagener 1928) and it is believed that the disease was spread from North America to many other areas of the world. The fungus has been found in South America, South Africa, Europe, Asia, Australia and New Zealand (Grasso and Ponchet 1979; Graniti 1998). Two other species, *Seiridium unicorne* (Cke. & Ell.) Sutton and *Seiridium cupressi* (Guba) Boesew. [teleomorph: *Lepteutypa cupressi* (Natrass et al.) Swart], also cause canker diseases on species of Cupressaceae (Graniti 1998).

Juniperus spp. are evergreen shrubs or small trees in the family Cupressaceae. They are widely distributed throughout Greece growing among other forest species usually in the understorey, but they also dominate in certain areas forming shrubby juniper woodlands. They usually occur on dry rocky sites playing a significant role in the protection of soils against erosion and providing important habitats for birds and mammals.

Little attention has been given to *Juniperus* spp. as hosts of *S. cardinale*. Wagener (1939) reported *S. cardinale* on *J. chinensis* L., *J. sabina* L. var. *tamariscifolia* Ait., *J. virginiana* L. and *J. scropulorum* Sargent. The fungus was successfully inoculated on plants of *J. communis* L., in Britain and Italy (Strouts 1973; Parrini and Panconesi 1981). There are also reports of *S. unicorne* on a number of *Juniperus* spp. from different countries (Sasaki and Kobayashi 1975; Tisserat et al. 1991).

In a preliminary report, Xenopoulos (2001) recorded the presence of *S. cardinale* on three *Juniperus* spp., *J. foetidissima* Willd., *J. excelsa* Bieb. and *J. oxycedrus* L. in a natural stand close to the Prespes Lakes in northern Greece. The present study provides a more detailed account of the presence of *S. cardinale* on *Juniperus* species growing in natural woodlands in various areas of Greece. The identity of the fungus was confirmed based on

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morphological characters and on DNA sequence comparisons and the pathogenicity of *S. cardinale* isolates from *Juniperus* and *Cupressus* was tested in cross-inoculation trials.

2 Materials and methods

2.1 Collection sites, isolation and morphological examination

The presence of *S. cardinale* on various *Juniperus* spp. growing under natural conditions was investigated between 2001 and 2004. Surveys were made in the areas of the Prespes Lakes region in northern Greece, and the Parnassos and Giona mountains in central Greece, parts of the Peloponnese and the Attica regions as well as the island of Lefkada in the Ionian Sea. Samples from branches with cankers and die-back symptoms were collected and transferred to the laboratory. The species of *Juniperus* examined in these areas are listed in Table 1.

Branches with cankers were examined under the dissecting microscope to detect acervuli of *S. cardinale*. Isolations were made by transferring spore masses from acervuli onto Petri dishes containing potato dextrose agar (PDA; Oxoid, Hampshire, UK). Where acervuli were absent, isolations were made by first removing the outer tissues near the canker margins with a sterile scalpel and then aseptically transferring small pieces of the underlying affected bark onto PDA. Cultures were incubated at 25°C and exposed to diffuse daylight. In some of the cultures, sterilized cypress (*Cupressus sempervirens* L.) seeds were added to facilitate the formation of acervuli. Growth characteristics of isolates from *Juniperus* spp. were compared with isolates from *C. sempervirens* and *C. macrocarpa* trees from Greece and one isolate (CMW 18794) from Italy.

Conidia from acervuli on infected juniper branches and those that developed in culture were examined in water using a bright-field microscope at 400× magnification. The lengths and widths of 50 conidia were measured for two isolates, CMW 18794 from *C. sempervirens* and CMW 18606 from *J. excelsa.*

2.2 DNA extraction, sequencing and phylogenetic analyses

Isolates used for DNA extraction were grown on 2% malt extract agar (MEA; Biolab, Midrand, Johannesburg) for 2 weeks. Aerial mycelium was scraped from the surface of the cultures, placed in sterilized 1.5 ml Eppendorf tubes and lyophilized. Lyophilized mycelium was then crushed into a fine powder using liquid nitrogen. DNA extraction was carried out using the phenol/chloroform method described by Barnes et al. (2001).

Two areas of the β-tubulin gene were amplified using primer combinations Bt1a/Bt1b and Bt2a/Bt2b (Glass and Donaldson 1995). PCR and sequencing conditions and

Table 1. **Occurrence of *Seiridium cardinale* on *Juniperus* spp. in different areas of Greece**

Locality	*Juniperus* sp.	Latitude (N)/ longitude (E)	Altitude (m a.s.l.)	Abundance
Prespes	*J. excelsa*	40°49′/21°01′	900	Common
	J. foetidissima	40°49′/21°01′	900	Common
	J. oxycedrus	40°49′/21°01′	900	Sporadic
Giona Mt	*J. foetidissima*	38°28′/22°26′	1100	Sporadic
Parnassos Mt	*J. foetidissima*	38°32′/22°12′	1200	Sporadic
	J. oxycedrus	38°36′/22°22′	500	Sporadic
Parnis Mt	*J. oxycedrus*	38°08′/23°41′	800	Sporadic
Korinthia	*J. oxycedrus*	37°58′/22°38′	750	Sporadic
Lefkada	*J. phoenicea*	38°40′/20°33′	30	Sporadic

reactions were the same as those described in Barnes et al. (2001) with two exceptions. Amplicons were purified using Sephadex G-50 columns (Sigma-Aldrich, Steinnheim, Germany) and sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied Biosystems, Foster City, CA, USA). Sequences obtained in this study (see GenBank numbers in Table 2) were aligned with, and compared to, other sequences obtained from GenBank, available through NCBI at http://www.ncbi.nlm.nih.gov/Genbank/index.html (Katoh et al. 2005). Alignments were carried out online using mafft Version 5.8 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server) with the L-INS-I strategy and the gap opening penalty set at 1.53. A partition homogeneity test (PHT) with 100 replicates was performed in paup* Version 4.0b10 (Phylogenetic Analysis Using Parsimony; Swofford 2002) to determine the combinability of the sequence data sets. Parsimony analyses were carried out using the Heuristic search option with tree bisection-reconnection and 100 random stepwise additions in paup. All characters were given equal weight and gaps were treated as a fifth character. Confidence levels of the branching points were determined using a bootstrap consensus of 1000 replicates. *Seiridium papillatum* Z.Q. Yuan was used as the outgroup and was treated as a monophyletic sister group to the ingroup.

2.3 Inoculation trials

In two inoculation trials, four *Juniperus* species were used in total. In the first trial, 2- to 4-year-old seedlings of *J. oxycedrus* (Attika seed source), *J. macrocarpa* Sm. (Attika seed source) and *J. phoenicea* L. (Milos island seed source) were used in 12-l plastic pots containing a mixture of farm soil and sphagnum peat (2 : 1 v/v). In this trial, 2-year-old cypress (*C. sempervirens*) saplings were also used in pots. The plants were placed outdoors in the Amygdaleza nursery (15 km from Athens) and irrigated twice a week during the dry seasons. In the second trial, mature trees of *J. oxycedrus* and *J. foetidissima* present in a natural woodland on Mount Giona, at elevation 1250 m, where natural infections by the fungus were found previously, were inoculated.

Inoculations in both trials were performed in October of 2002 and the experiment was terminated after 7 months, in May 2003. In potted plants, inoculations were made on the lower stem of the plants. Stem diameters at the inoculation points ranged from 6 to 11 mm. After inoculation, pots were arranged in a completely randomized design. Each mature tree received two inoculations on separate branches of 11–16 mm in diameter. Details of plant number, age and stem diameter at inoculation points are listed in Table 3.

Two isolates of *S. cardinale* were used in the inoculation experiments. These were an Italian isolate from *C. sempervirens* (CMW 18794) and an isolate from *J. foetidissima* (CMW 18605). In the trial with potted plants, both isolates were used, whereas in the field trial on mature trees, only isolate CMW 18605 was used. Inoculum was prepared from stems of *Sorghum vulgare* Pers. Stems approximately 1 mm in diameter were cut into pieces 15 mm in length and autoclaved for 30 min at 121°C. Fungal isolates were grown in Petri dishes containing PDA for 1 week at 25°C after which the autoclaved *S. vulgare* stems were added and then further incubated for 3 weeks. For inoculations, a hole, 1.5 mm in diameter, was made on the bark of the stems and branches and the fungus-covered stems inserted (Ponchet and Andréoli 1984). Sterile *S. vulgare* stems were used in five plants of each species in the trial with potted plants, and also on a separate branch of all the inoculated mature trees to serve as controls.

Inoculated plants in the nursery were examined monthly and the development of cankers and other symptoms noted. Mature plants in the natural woodland were examined once before harvesting, 5 months after inoculation. At sampling, stems of all harvested plants were examined under the dissecting microscope and the presence of acervuli in the infected area of the bark recorded. Lesion (canker) lengths were measured for all inoculated stems

Table 2. ***Seiridium*** **isolates for which** *β*-**tubulin sequence data were generated in this study**

Species	Isolates[1]	Host	Origin	*β*-Tubulin 1[2]	*β*-Tubulin 2[2]
S. cardinale	CMW 18602	*Cupressus sempervirens*	Arkadia, Greece	DQ926968	DQ926974
S. cardinale	CMW 18603	*Cupressus macrocarpa*	Attika, Greece	DQ926969	DQ926975
S. cardinale	CMW 18604	*Juniperus foetidissima*	Prespes Lakes, Greece	DQ926967	DQ926973
S. cardinale	CMW 18605	*Juniperus foetidissima*	Prespes Lakes, Greece	DQ926970	DQ926976
S. cardinale	CMW 18606	*Juniperus exelsa*	Prespes Lakes, Greece	–	DQ926978
S. cardinale	CMW 18794 (ATCC 38654)	*Cupressus sempervirens*	Italy	DQ926971	DQ926977
S. cupressi	CMW 18607	*Cupressus sempervirens*	Kos Island, Greece	DQ926972	DQ926979

[1]CMW, Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria; ATCC, American Type Culture Collection (provided by P. Raddi, CNR-Italy).
[2]GenBank accession numbers.

Table 3. **Mean lesion (canker) length of *Seiridium cardinale* on *Juniperus* and *Cupressus* species, 7 months after inoculation (mm)**

Inoculated plants	Fungal isolate[1]	Number of inoculations	Number of infections[2]	Age of plants (years)	Diameter at inoculation point (mm)	Lesion (canker) length[3] (mm)
Potted plants						
J. oxycedrus	1	10	9 (10)	4	8–11	46.1a ± 2.3
J. oxycedrus	2	9	8	4	8–10	44.9a ± 1.9
J. macrocarpa	1	11	9 (10)	4	7–10	48.6a ± 4.6
J. macrocarpa	2	9	8	4	8–10	47.63a ± 1.9
J. phoenicea	1	11	9 (11)	2	5–7	52.33a ± 3.9
C. sempervirens	1	10	10	2	7–15	84.4b ± 5.0
C. sempervirens	2	10	9 (10)	2	8–12	85.2b ± 4.2
Field grown						
J. oxycedrus	2	16	16	>20	11–15	81.1c ± 9.9
J. foetidissima	2	16	16	>20	12–15	45.1a ± 3.0

[1]1 = isolate CMW 18794; 2 = isolate CMW 18605.
[2]Numbers in brackets show total number of infected plants, including those with dead tops, which were not considered in the statistical analysis.
[3]Values are mean ± SE. Numbers followed by the same letter do not differ significantly according to Duncan's multiple range test ($p = 0.05$).

and branches by lightly scraping the bark from the periphery of the canker and noting the discolouration of the inner bark tissues and sapwood. Re-isolation of the fungus was attempted from the canker margins on a representative sample of plants from each treatment to confirm the presence of *S. cardinale*, especially from cankers that did not develop acervuli of the fungus.

Analysis of variance was performed on lesion lengths in every treatment and Duncan's multiple range test ($p = 0.05$) was used to compare the mean values, using SPSS software (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Disease occurrence, symptoms and morphological characteristics

Infections by *S. cardinale* were found on four different *Juniperus* species in natural woodlands, in a number of different localities of Greece (Table 1). In most areas, the presence of infections was sporadic, with a limited number of plants affected at each locality. However, in an area close to Psarades village in the Prespes Lakes region of northern Greece, the intensity of infection was very high, especially on *J. foetidissima* and *J. excelsa*. *Juniperus oxycedrus* was also found to be infected by the fungus in this area, but disease severity was lower on this species. In Parnassos and Giona Mts of central Greece, *S. cardinale* infections were detected on a limited number of *J. foetidissima* plants at high altitudes. At a different locality of Parnassos Mt, at lower altitudes, the fungus was also detected on *J. oxycedrus* plants. Infections on *J. oxycedrus* were also found in Parnis Mt and Korinthia Prefecture (Kaliani village), in southern Greece. On the west coast of Lefkada Island (Porto Katsiki), *S. cardinale* was detected on *J. phoenicea.*

Most infected junipers were found in close proximity to infected cypress trees, the main hosts of *S. cardinale* in Greece. However, in Prespes Lakes region in northern Greece and at high altitudes of Parnassos and Giona mountains, *S. cardinale* infections were found only on junipers.

Affected junipers showed symptoms of die-back on one or more top or lateral branches. In some cases a major part of the plant crown was dead. On closer examination, elongated cankers were evident on branches and/or the main stem. These were similar to the cankers formed on cypress trees, with abundant resin exudation. They became more evident by removing the outer bark to expose the dark brown, dead tissue, which was usually impregnated with resin, while the surrounding healthy tissue was pearl white. Acervuli of the pathogen appeared scattered over the cankered areas of the bark as small black dots that were barely visible to the naked eye.

Seiridium cardinale was consistently isolated from bark tissue as well as from spore masses in acervuli. Isolates from *Juniperus* spp. were very similar in cultural morphology to those from *C. sempervirens* and *C. macrocarpa* trees in Greece and the isolate from Italy. Colonies on PDA showed a dense floccose aerial mycelium, whitish to grey, turning to greyish olive-green in older cultures. The colour on the reverse side of cultures varied from pinkish-salmon to light orange. Radial growth at 25°C was 10–12 mm/week.

Black conidial masses were formed on the cypress seeds, 2–3 weeks after their placement on cultures. Conidial masses were rare on cultures lacking cypress seeds. Conidia from *Juniperus* isolates were indistinguishable from those of the isolates from *Cupressus* spp. The characteristic 5-septate, oblong-fusiform conidia of *S. cardinale* (20–28 × 8–9 μm) were observed, with characteristic hyaline, short (approximately 1 μm) and conical apical cells.

3.2 DNA extraction, sequencing and phylogenetic analyses

Sequencing the β-tubulin 1 and 2 PCR products using the forward and reverse primers produced sequences of approximately 450 and 510 bp long, respectively. Translation of the consensus sequences into amino acids revealed one intron present in the β-tubulin 1 region (56 bp long) flanked by exons of sizes 141 and 267–276 bp depending on the quality of the ends of the sequences. The sequences of the β-tubulin 2 region contained an intron of between 78 and 112 bp followed by an exon of 42 bp, an intron of 67 bp for the *S. cardinale* isolates and 66 bp for the *S. cupressi* isolates and an exon of 182 bp. After alignment in MAFFT with additional *Seiridium* sequences, the individual data sets consisted of 406 and 478 characters for β-tubulin 1 and 2 regions, respectively.

The PHT with the heuristic search option in PAUP showed significant congruence with a p-value of 0.65. Individual data sets were, therefore, combined in the phylogenetic analyses. Of 877 total characters: 729 characters were constant, 82 variable characters were parsimony-uninformative and 66 characters were parsimony-informative. Fourteen most parsimonious trees were generated with a tree length of 170, a consistency and retention index value of 0.93 and 0.95, respectively, and a g1 value of −0.71. The slight variation observed in the topology of the trees occurred in the placement of some of the *S. cardinale* isolates. The rest of the tree topology remained identical in all 14 trees. One most parsimonious tree is illustrated in Fig. 1.

All isolates obtained from *Juniperus* and *Cupressus* species in this study grouped with the previously identified *S. cardinale* clade, including the authenticated isolate from ATCC 38654 (CMW 18794) from *C. sempervirens* in Italy. The one isolate (CMW 18607) collected from *C. sempervirens* and identified as *S. cupressi* based on morphology, fell within the *S. cupressi* clade. It had identical sequence to isolate (CMW 1646) collected from the same region.

3.3 Inoculation trials

All *Juniperus* and *Cupressus* species used in the inoculation trials became infected by both isolates of *S. cardinale*. In the field trial, all plants developed cankers on the inoculated branches. In potted plants, 67 of 70 inoculated plants developed infection symptoms

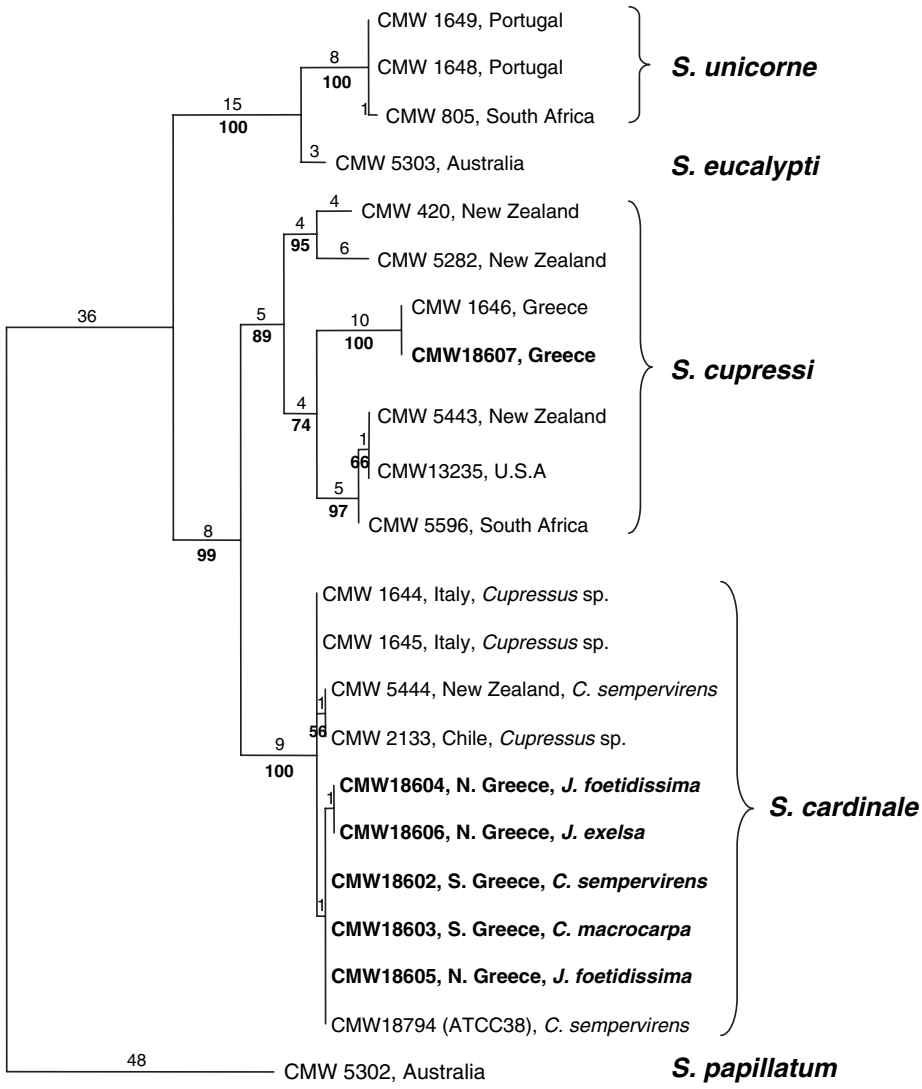

Fig. 1. Phylogenetic tree of *Seiridium* species based on *β*-tubulin sequence data. The most parsimonious tree was obtained using the heuristic search option in PAUP. The isolates from *Juniperus* and *Cupressus* fall within the *S. cardinale* clade based on sequence data thus supporting the morphological identification of these isolates as *S. cardinale*. An additional isolate collected from *C. sempervirens* falls within the *S. cupressi* clade showing that both pathogens, *S. cardinale* and *S. cupressi*, are present within Greece. Branch lengths are indicated above the branches while bootstrap confidence levels are represented below the branches

(95.7%). Of the remaining plants, one died of unknown cause and two saplings failed to become infected. Most plants survived during the 7-month incubation period. In five potted plants, mainly those with small diameters (≤ 6 to 7 mm), the terminal shoots died 3–4 months after inoculation because of stem girdling and had to be excluded from lesion

measurements made at the end of the experiment. Acervuli were present in almost all the infected trees in the nursery at harvest in May. These structures were rare, however, on the infected branches of *Juniperus* trees inoculated in the natural woodland.

In the nursery trial, no significant differences ($p = 0.05$) were found in lesion length for two isolates of *S. cardinale*, from *Juniperus* (CMW 18605) and *Cupressus* (CMW 18794) on any of the *Juniperus* spp. inoculated (Table 3). Similarly, there were no significant differences in virulence between these two isolates on the *Cupressus* saplings inoculated. Significant differences in lesion length were, however, found between potted *J. oxycedrus* plants and those of the same species growing in natural conditions. Lesion lengths on branches of the field trees ranged between 32 and 160 mm (average 81 mm), compared with 29–55 mm (mean 45 mm) on potted plants of the same species. Significant differences in lesion length were also observed between *J. oxycedrus* and *J. foetidissima* field grown plants, averaging 81 mm and 45 mm, respectively. In potted plants, lesion length was greater on *Cupressus* plants (ranging between 62 and 111 mm, mean 85 mm) than all *Juniperus* species that averaged 45–52 mm.

4 Discussion

This study represents the first detailed assessment of the presence of *S. cardinale* in natural juniper woodlands in various areas of Greece. The pathogen was previously recorded on *Juniperus foetidissima, J. excelsa* and *J. oxycedrus* in a brief communication (Xenopoulos 2001), but this is the first record of its occurrence on *J. phoenicea*. This study also represents the first pathogenicity tests with the fungus on *Juniperus* spp. in Greece. There are also no reports of *S. cardinale* or any other *Seiridium* sp. on these *Juniperus* spp. elsewhere in the world.

Of the three *Seiridium* spp. known to be associated with cypress cankers, *S. unicorne* and *S. cupressi* are morphologically very similar to each other. They are thus often confused with each other or misidentified (Swart 1973; Chou 1989). DNA-based methods have, however, provided a means to conclusively identify these species (Barnes et al. 2001). Isolates CMW 420 and CMW 5443 used in this study were previously identified as representing *S. unicorne,* based on morphology but were clearly shown to represent *S. cupressi* by Barnes et al. (2001). The isolate CMW 18607 collected from *C. sempervirens* in Greece was originally identified based on morphology as *S. cupressi* and this was confirmed here using DNA sequence comparisons. On the other hand, *S. cardinale* can be identified based on conidial morphology, with short and conical, hyaline apical cells or appendages (Boesewinkel 1983). It was, therefore, not surprising that the isolates collected from *Juniperus* and *Cupressus* in this study resided in the *S. cardinale* clade in the phylogram based on β-tubulin sequences.

The pathogenicity of *S. cardinale* from *Juniperus* and *Cupressus* was verified in inoculation tests on three of the species found to be naturally infected in this study. Inoculation tests with *J. excelsa* were not performed, because seedlings of this species were not available. Another juniper, *J. macrocarpa,* which was not found infected under natural conditions and has not been reported before as a host of *S. cardinale,* was also successfully inoculated in the trials and can be considered susceptible to the pathogen.

Statistical analysis of the lesion lengths showed that the two isolates of *S. cardinale* (from juniper and cypress) did not differ significantly in virulence when inoculated on the same plant species under the same conditions. There were also no significant differences in virulence of different *S. cardinale* isolates used in inoculation tests on clones of *C. sempervirens* in Italy (Raddi and Panconesi 1984).

Inoculated plants showed some differences in relative susceptibilities to *S. cardinale.* However, the number of trees tested was small and general conclusions cannot be drawn on the basis of these results. Lesion length was significantly greater on *C. sempervirens*

seedlings than on any of the *Juniperus* spp. grown under the same conditions, while differences in lesion length among the different *Juniperus* spp. were not significant. Lesion lengths on branches of mature *J. oxycedrus* trees were significantly greater than those on *J. foetidissima* inoculated with the same isolate of *S. cardinale*. However, under natural conditions in the Prespes Lakes, the pathogen was more virulent on *J. foetidissima* than on *J. oxycedrus*.

Significant differences were observed in lesion length between *J. oxycedrus* trees growing in the nursery at low elevation and the mature plants growing under natural conditions at high elevation where relative humidity is high and fungal growth was greater. These results are consistent with those of previous studies on cloned *C. sempervirens* (Santini et al. 1997; S. Xenopoulos, unpublished data), where susceptibility of trees to *S. cardinale* was significantly greater in moist areas than in where conditions were drier.

Seiridium cardinale has been known in Greece since the early 1960s and has caused significant damage to *C. sempervirens* in plantations in many areas of the country (Xenopoulos and Diamandis 1985; Tsopelas and Xenopoulos 2006). In some areas, the infection level has been >90%. However, in natural stands of this species on some of the Aegean islands, infection levels have been very low (Xenopoulos and Diamandis 1985; P. Tsopelas, unpublished data). This is similar to the situation in California, where *C. macrocarpa* in natural stands has not been affected while those grown in gardens and plantations have been severely infected by *S. cardinale* (Wagener 1939). Results of the present study reflect a similar pattern with *Juniperus* spp., which are only mildly affected in natural woodlands. In only one locality of the Prespes Lakes region, the disease has caused considerable damage to junipers, causing extensive die-back on branches and in many cases killing whole trees. The environmental conditions in this area favour the spread of the pathogen as the region receives heavy rain and the relative humidity of the atmosphere is very high for long periods of time.

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