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Cypress canker: An important disease discovered for the first time on a native South African tree

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Abstract

Cypress canker is a branch and stem canker disease of Cupressaceae trees, particularly those in the genera *Cupressus* and *Hesperocyparis*. These trees have been planted in many parts of the world as ornamentals and the *Seiridium* species that cause the disease, consequently, also have an almost global distribution. The taxonomy of *Seiridium* species causing cypress canker has recently been revised and numerous species are now believed to cause the disease. This study describes, for the first time, cypress canker on the native South African Cupressaceae tree, *Widdringtonia nodiflora*. The aim was to identify the causal agent and confirm its pathogenicity. Phylogenetic analyses of sequence data for four regions identified the fungus as *Seiridium neocupressi*, a species previously known only from Australia, New Zealand and Italy. Field inoculations of *W. nodiflora* branches resulted in distinct cankers within 6 weeks and the fungus could be reisolated from the treated trees. Cypress canker has been known in South Africa for many decades, where it causes a serious disease on nonnative species of *Cupressus*, but it has never been found on native Cupressaceae. The newly discovered disease caused by a probable alien pathogen is of particular concern because only three species of *Widdringtonia* occur in South Africa and are important components of the native flora. The two other species, *W. wallichii* and *W. schwartzii*, occur in small endemic and threatened populations. The origin of *S. neocupressi* in South Africa and the relative susceptibility of the three *Widdringtonia* species, consequently, requires urgent attention.

KEYWORDS
cedar, Cupressaceae, pathogen, *Seiridium*, *Widdringtonia*

1 | INTRODUCTION

Cypress canker is arguably the most serious disease of trees in the Cupressaceae family. The disease is caused by several different species of *Seiridium*, of which *Seiridium cardinale* is considered the most aggressive and important (Graniti, 1998). This fungus was first discovered and described in the western United States in 1927, where it caused a devastating disease on the Monterey cypress, *Hesperocyparis* (previously *Cupressus*) *macrocarpa* (Adams et al., 2009; Wagener, 1939). It subsequently spread to many other parts of the world, resulting in a canker disease on both ornamental and plantation-grown *Cupressus* species (Danti et al., 2013; Graniti, 1986). *S. cardinale* is native to California, but some of the most serious damage due to this pathogen has been

in the Mediterranean regions of Europe, where it is an invasive alien species (Della Rocca et al., 2019). In that region, species such as the Italian cypress *Cupressus sempervirens* is highly susceptible and the climatic conditions are particularly conducive to infection (Xenopoulos & Diamandis, 1985).

The taxonomy of the *Seiridium* species that cause cypress canker has been controversial for many years. The three best-known species, *S. cardinale*, *S. cupressi* and *S. unicorne*, all cause cankers on Cupressaceae trees and were described based on the morphological characteristics of their conidial appendages (Graniti, 1998). This variable characteristic led to their being treated by different authors either as a single, two or three distinct species (Boesewinkel, 1983; Swart, 1973).

The first study to apply DNA sequence data to resolve the taxonomy of *Seiridium* species associated with cypress canker concluded that these fungi represented one morphologically variable species (Viljoen et al., 1993). This was followed by a more detailed study that increased the number of sequenced gene regions and suggested the existence of two species, namely *S. cardinale* and a morphologically variable species comprising both *S. cupressi* and *S. unicorne* (Barnes et al., 2001). The most recent taxonomic treatment of these fungi considered sequence data for four regions and confirmed that *S. cardinale*, *S. cupressi* and *S. unicorne* are distinct species (Bonthond et al., 2018). These authors also described four additional species occurring on Cupressaceae trees, although the connection of these fungi to disease based on experimental data was not clear in many cases.

Canker disease is commonly found on *Cupressus* species in South Africa. *S. cardinale* and *S. unicorne* were the first species to be collected from diseased trees in the country (Wingfield et al., 1988; Wingfield & Du Toit, 1986). One of the two isolates that Bonthond et al. (2018) used to describe their new species, *S. cancrinum*, was from a canker on a *Cupressus* species collected in the Mpumalanga Province in 1984 by the first author of the present study. In addition, two isolates described by Bonthond et al. (2018) as *S. pseudocardinale* are closely related to a South African isolate (CMW 805) in the Barnes et al. (2001) study and *S. pseudocardinale* may, therefore, also be present in South Africa. On the other hand, the report of *S. cupressi* by Barnes et al. (2001) was shown to represent *S. unicorne* (Bonthond et al., 2018) and *S. cupressi*, according to current knowledge, remains absent from South Africa. Consequently, for the present, we accept that *S. cardinale*, *S. cancrinum*, *S. unicorne* and probably *S. pseudocardinale*, are associated with cypress canker in South Africa. With the exception of *S. unicorne*, which has a wide host range beyond the Cupressaceae and is considered to be only mildly pathogenic (Boesewinkel, 1983; Danti et al., 2013; Graniti, 1998), the relative importance of these pathogens in South Africa is unknown.

Cupressus species are popular ornamental trees in South Africa where they are commonly grown as nonnatives in parks and gardens, but only three Cupressaceae species are native to the country (Kerfoot, 1966). These are all members of the iconic African cedar genus *Widdringtonia*, namely *W. wallichii* (previously *W. cedarbergensis*), *W. schwartzii* and *W. nodiflora*. The former two species have very restricted natural occurrences, whereas *W. nodiflora* occurs substantially more widely, with a distribution extending from the south-west of the Western Cape Province, along the eastern coast of Africa and northwards into Malawi (Farjon & Filer, 2013). Very little is known regarding the diseases of these trees, although some attention has been paid to the Cedarberg cedar, *W. wallichii*, which is in decline and listed by the IUCN as critically endangered (Farjon et al., 2013; White et al., 2016). A brief survey of diseases of *W. wallichii* by Wingfield et al. (1988) specifically considered whether canker caused by *Seiridium* species might occur on these trees. While a fungus identified as *S. cardinale* was found on a nonnative *Cupressus* species in the area, there was no evidence of cypress canker on the native trees.

During a recent inspection of the health of *W. nodiflora* growing in the Franschoek Valley of the Western Cape Province of South Africa, damaging cankers were commonly found on a small population of these trees. A fungus having a morphology typical of a *Seiridium* species was visible on the surface of the cankers. The aim of this study was to describe the disease and identify its causal agent.

2 | MATERIALS AND METHODS

2.1 | Disease description and isolations

The affected *W. nodiflora* trees were restricted to an area of approximately 1000 m^2 on an east-facing slope of the Franschoek Valley, Western Cape Province, South Africa (33.9346° S, 19.1640° E). Cankers, typically exuding copious amounts of resin, were common on most trees and were found on both stems and branches (Figure 1). Removal of the outer bark showed distinct necrosis of the cambium at the leading edges of the lesions and cross-sections of the cankers showed deep resin impregnation of the wood (Figure 1).

Spore masses typical of *Seiridium* species were observed in acervuli on the surfaces of the cankers. These were lifted from the acervuli using a sterile hypodermic needle and transferred to the surface of malt extract agar (MEA; Biolab) amended with 1% streptomycin sulphate (Sigma-Aldrich). On diseased tissue where acervuli were not evident, isolations were made from the leading edges of the developing lesions. Here, branch sections containing lesions were surface disinfested with 70% ethanol for 1 min after which approximately 5 mm^3 sections of stained tissue were cut from the lesions and plated onto MEA. Cultures were purified by transferring single hyphal tips to clean MEA plates and maintained at 25°C.

2.2 | Morphology

For morphological characterization, the top sections of the conidiomata immersed in canker tissue were removed using a scalpel.

FIGURE 1 *Widdringtonia nodiflora* trees in the Franschhoek Valley. (a,b) Trees with symptoms in the field. (c–e) Cankers. (f) Vertical section through conidioma. (g) Conidiophores and conidiogenous cells. (h) Conidia. Scale bars: f = 100 μm; g, h = 25 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

After moistening, the inner structures containing parts of the conidiomatal walls and conidiogenous apparatuses were extracted and placed on microscope slides in water. For measurements, the water was replaced with 85% lactic acid and images were captured using a Nikon Eclipse N*i* and SMZ18 microscope mounted with a DS-Ri2 camera. Measurements were presented as min–max with average ± standard deviation. Canker tissues containing a few conidiomata were cut into small pieces (5 mm diameter) to observe the configuration of the conidiomata in the tissue. The pieces were boiled for a few seconds to soften the bark tissue, mounted in tissue freezing medium (Leica) and cut into 10–12 μm sections using a cryomicrotome (Leica). The sections were mounted in 85% lactic acid for further observation.

2.3 | DNA isolation, sequencing and phylogenetic analyses

DNA was isolated from 7-day-old cultures of three different strains of *Seiridium* using the Prepman Ultra Sample Preparation Reagent (Thermo Fisher Scientific). The internal transcribed spacer regions 1 and 2 (ITS), including the 5.8S rRNA region, were amplified using

primers ITS1 and ITS4 (White et al., 1990). Parts of the genes for of the DNA-dependent RNA polymerase II second largest subunit (*RPB2*) was also amplified with primer pair RPB2-5F2 and fRBP2-7cR (Liu et al., 1999; Sung et al., 2007), a fragment of the translation elongation factor 1–α (*TEF1*) using primers EF1-728F and EF2 (Carbone & Kohn, 1999; O'Donnell et al., 1998), and partial fragments of the β-tubulin (*TUB2*) using primers T1 and BT2b (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997).

PCR mixtures were prepared following the protocols described by Pham et al. (2019) and reaction conditions followed those used by Bonthond et al. (2018). Amplified fragments were treated with ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific). The purified products were sequenced in both directions with the same primers used for amplification, using the BigDye terminator sequencing kit v. 3.1 on an ABI Prism 3100 DNA sequencer (both from Thermo Fisher Scientific) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. Geneious Prime v. 2022.1.1 (https://www.geneious.com) was used to assemble and trim the raw sequences, which were deposited in GenBank (Table S1).

Sequences of species closely related to those emerging from this study were sourced from GenBank (Table S1). All sequences were aligned with MAFFT v. 7 (Katoh & Standley, 2013) and inspected manually using MEGA v. 7 (Kumar et al., 2016). A concatenated data set comprising ITS, *RPB2*, *TEF1* and *TUB2* sequences was generated. Maximum-likelihood (ML) analysis was conducted using RaxML v. 8.2.4 (Stamatakis, 2014) on the CIPRES Science Gateway 3.3 (Miller et al., 2010), with default GTR substitution matrix and 1000 rapid bootstraps. *Neopestalotiopsis protearum* (CBS 114178) was used as the outgroup taxon.

2.4 | Pathogenicity tests

Pathogenicity of the isolated *Seiridium* species was tested in a population of *W. nodiflora* trees adjacent to the population from which the fungus was initially isolated. Inoculations were initiated during summer (January) of 2022 using two strains (CMW 57798 and CMW 57803). Ten trees were randomly selected for the inoculations. On each individual tree, three branches were selected and inoculated with either one of the two test isolates or an uninoculated MEA control. A 7 mm diameter cork borer was used to remove the bark and expose the cambium on each selected branch. Similar sized discs, taken from the actively growing margins of 2-week-old cultures on MEA, were inserted into these wounds with the mycelial growth facing the xylem. Wounds were covered with Parafilm to prevent desiccation and contamination. To account for the fact that branch size may influence fungal growth rates, branch diameter was measured and an attempt was made to use branches of uniform diameter.

Branches were harvested after 6 weeks by cutting them from the trees at the points of insertion into the main stems. After removing the bark, the length of the lesions (mm) associated with each inoculation point was measured with digital callipers. To confirm the identity of the inoculated fungi, inoculation points were first inspected using a 10× magnification hand lens for the presence of *Seiridium* acervuli. Isolations were made from spore masses in acervuli or the leading edges of the developing lesions, as described above. Cultures were incubated in the dark at 25°C for approximately 5 days and identified based on morphology.

Lesion length data were not normally distributed after implementing a Shapiro–Wilk test ($W = 0.82659$, $p < 0.001$) in R v. 3.6.3 (R Core Team, 2020). The influence of treatment (different isolates and controls), branch diameter and their interaction (as fixed effects) on lesion length data were therefore tested with generalized linear mixed-effect models using the lme4 package (Bates et al., 2015) in R. Lesion length data best fitted a gamma distribution with log link function (Bolker et al., 2009). Tree individual was used as a random variable. Because these analyses showed no overdispersion of variances compared to the models, a χ^2 statistic and p values were calculated (Bolker et al., 2009). The model followed the format: glmer (lesion length – treatment + stem diameter + cultivar*stem diameter + [1|Tree individual], family = Gamma [link = "log"], data = data). Significant main effects were separated using a conservative Tukey post hoc test in the multcomp package in R (Hothorn et al., 2008). A probability level of 5% was considered significant.

3 | RESULTS

3.1 | Disease description and pathogen identification

A *Seiridium* species was commonly found sporulating on the surface of the *W. nodiflora* cankers (Figure 1). Pure cultures were easily obtained from these spores or from the leading edges of lesions associated with the cankers. Three isolates (CMW 57797, CMW 57798 and CMW 57803), originating from separate trees, were purified and have been preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. All three cultures were used for further morphological study and molecular identification and two (CMW 57798 and CMW 57803) were used in the pathogenicity trials.

The *Seiridium* species associated with the affected *W. nodiflora* trees had a consistent morphology, both in culture and from structures on naturally infected tissues. The conidiomata in vivo were stromatic, pycnidioid, subperidermal in origin, innate, erumpent, broadly conical in vertical view. Conidiophores were borne on the cavity walls of the conidiomata, and were filamentous, paraphysoid, hyaline, septate and branched near their base. Conidiogenous cells were hyaline, tubular, 4–19 × 2–3 μm (11.5 ± 3.4 × 2.6 ± 0.3 μm, $n = 25$) with two to four annellations. Conidia were fusiform to ellipsoid, straight or slightly curved, 23–31 × 8–17 μm (26.3 ± 1.7 × 9.9 ± 1.3 μm, $n = 50$) bearing appendages. The basal cells were hyaline, obconic with truncate bases, 2–7 μm long (3.5 ± 0.8 μm, $n = 50$) and the median cells were brown to dark brown, doliiform to short cylindrical and 3-septate. These cells were thick-walled, unequal, with smooth

walls, sometimes slightly constricted at the septa, having septal pores distinctly visible and together 10–25 μm long (20.2 ± 2.2 μm, *n* = 50). The apical cells were hyaline, short conical, 1.5–4 μm long (2.7 ± 0.6 μm, *n* = 50) and the appendages tubular, attenuated with single, unbranched, apical appendages that were 2–11 μm long (7.7 ± 2.1 μm, *n* = 50). A basal appendage when present, was single, positioned at the centre or obliquely, unbranched and 1–10 μm long (4.3 ± 2.4 μm, *n* = 50).

For all three *Seiridium* isolates, amplicons of approximately 580 bp were generated for the ITS region, 950 bp for *RPB2*, 520 bp for *TEF1* and 750 bp for *TUB2*. The concatenated data set used in the phylogenetic analyses included 27 ingroup taxa and contained 2569 characters, including alignment gaps. The three isolates had identical sequences and formed a well-supported (97%) monophyletic clade in the ML tree (Figure 2) that included the ex-type isolate of *S. neocupressi* (CBS 142625). The isolates were thus identified as *S. neocupressi*.

3.2 | Pathogenicity tests

When removing the bark of inoculated branches, distinct lesions were evident around the points inoculated with the two *Seiridium* test isolates (Figure 3). Lesions consisted of reddish-brown vascular staining in the form of streaks. Controls showed similar staining, but these were small and did not extend far from the inoculation points (Figure 3a). Fruiting structures (acervuli) of a *Seiridium* species were evident on the bark around the inoculation points but were not found on the bark of the control inoculations. Fungal cultures with a morphology identical to the inoculated *Seiridium* species were consistently reisolated from all inoculations but never from the controls.

Only the treatment (pathogen or control), and not the branch diameter, had a significant effect on lesion length (Table 1). Lesions caused by the two isolates were significantly longer than those in the controls, but there was no difference in lesion length between the two isolates (Figure 4; Table 1). The overall model had an Akaike information criterion (AIC) value of 183.8 with a deviation of 167.8, *df* = 22.

4 | DISCUSSION

This study has recorded, for the first time, a serious stem and branch canker disease on one of the only three native members of the Cupressaceae in South Africa. The affected *W. nodiflora* trees were in a relatively confined area of the Franschoek Valley, surrounded

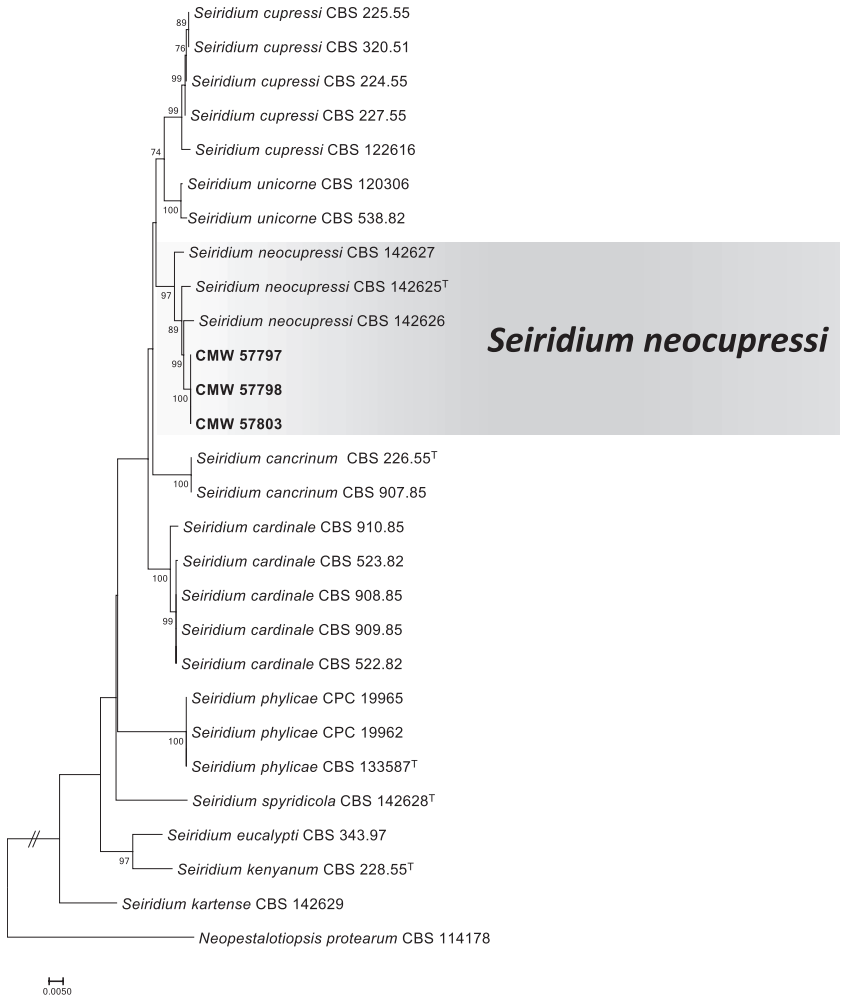

FIGURE 2 Phylogenetic tree based on maximum-likelihood (ML) analysis of a combined DNA data set of ITS, *RPB2*, *TEF1* and *TUB2* sequences for *Seiridium* species. Isolates sequenced in this study are presented in boldface. Bootstrap values (≥70%) for ML analyses are indicated at the nodes. Isolates representing ex-type cultures are marked with a 'T'. *Neopestalotiopsis protearum* (isolate CBS 114178) represents the outgroup taxon. See Table S1 for GenBank accession numbers.

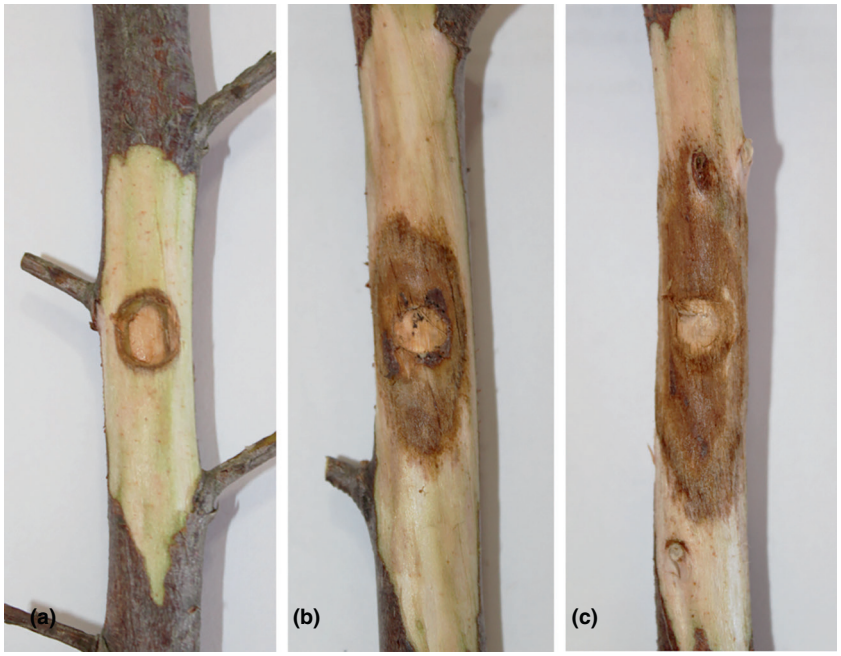

FIGURE 3 Stripped branches of *Widdringtonia nodiflora* showing lesions 6 weeks after inoculation. (a) Control. (b,c) Inoculated with a 6-day-old mycelial plug of *Seiridium neocupressi*, CMW 57803 (b) and CMW 57798 (c). [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 The effect of treatment and branch diameter on the development of *Seiridium* lesions on *Widdringtonia nodiflora*

Variable	*df*	χ^2	*p*	Post hoc
Treatment	4	100.88	<0.001	CMW57798 = CMW57803, both ≠ control, Figure 4
Branch diameter	3	0.8634	0.8343	n.a.
Treatment × Branch diameter	2	0.8091	0.6673	n.a.

by a larger dispersed area of trees showing no evidence of infection. The disease was not observed on these trees during prior visits to the area over the last 5 years (author's personal observation). This suggests that the disease has emerged recently. A *Seiridium* species was consistently associated with the cankers and was identified as *S. neocupressi* based on DNA sequence data. Two isolates of the fungus caused distinct cankers in a relatively short period after inoculation.

The discovery of a new canker disease on *W. nodiflora* caused by *S. neocupressi* provides no indication that the fungus was previously present in South Africa. Prior to the comprehensive taxonomic re-evaluation (Bonthond et al., 2018), three *Seiridium* species associated with *Cupressus* cankers in various parts of South Africa had been recorded in the country (Barnes et al., 2001; Viljoen et al., 1993) and these isolates were shared with the culture collection of the Westerdijk Fungal Biodiversity Institute by the first author of the present study. Using these isolates, Bonthond et al. (2018) recognized three species occurring in South Africa, namely *S. cardinale*, *S. cancrinum* and *S. unicorne*. In this regard, *S. neocupressi* could well be a new pathogen in the country that has found a susceptible host in *W. nodiflora*.

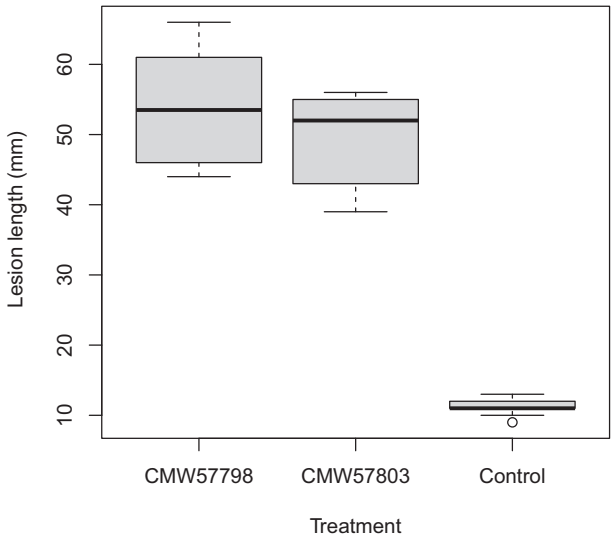

FIGURE 4 Lesion length 6 weeks after incubation of two isolates of *Seiridium neocupressi* inoculated into *Widdringtonia nodiflora* branches. Boxes indicate 25%–75% data range, whiskers indicate 1.5× the interquartile range.

The dimensions of some key morphological structures of *S. neocupressi* that were observed in this report varied in comparison to the species description (Bonthond et al., 2018). For example, the conidia in our collections were wider (up to 17 µm) than those previously recorded (up to 10.5 µm; Bonthond et al., 2018). Another notable difference was that the septal pores in the conidia were previously described as invisible, whereas they were obvious in our specimens. However, our observations considered *S. neocupressi* from living *W. nodiflora* material, whereas the original description was based on in vitro cultures of the ex-holotype strain (CBS 142625), originating from *C. sempervirens* in Italy. It is thus reasonable to expect some variation in these morphological characteristics.

S. neocupressi, reported in this study, is relatively closely related to the important pathogens *S. cardinale* and *S. cancrinum* that are known to occur on *Cupressus* species in South Africa. *S. cardinale* has a wide global distribution but is believed to have originated in the United States (Della Rocca et al., 2019). In contrast, *S. cancrinum* is known only from Africa (Kenya, Morocco and South Africa), where it has been collected on nonnative *Cupressus* species (Bonthond et al., 2018; Jones, 1953). It seems unlikely to be a native pathogen in those countries, although it is possible that it could have undergone a host shift from a native member of the Cupressaceae.

The discovery of a serious canker disease on native South African *Widdringtonia* trees is of considerable concern, especially because *Seiridium* species are aggressive pathogens of cypress (Cupressaceae; Danti et al., 2013). Although *W. nodiflora* has a relatively wide distribution in southern Africa (Farjon & Filer, 2013), it is an important component of the natural ecosystem. It is also closely related to the iconic and highly endangered *W. wallichii* and *W. schwartzii*. The apparently newly emerging canker disease could provide a source of infection for those trees, which occur in areas geographically close to those where the new disease has appeared. A previous, but relatively limited, inoculation study by Wingfield et al. (1988) showed that *W. wallichii* seedlings were not highly susceptible to *S. cardinale*. However, the results of the present investigation suggest that this question deserves more serious attention and that their susceptibility to *S. neocupressi* should be urgently considered.

Before this report, *S. neocupressi* was known only from Australia, New Zealand and Italy (Bonthond et al., 2018), causing disease on a related tree genus, *Cupressus*. Its emergence in South Africa is significant because similar situations underpin some of the most serious diseases of trees globally. These diseases are typically caused by introduced fungal pathogens that have suitable susceptible hosts on related but different tree species. Classic examples are those of Dutch elm disease caused by *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier, 2000) and chestnut blight caused by *Cryphonectria parasitica* (Rigling & Prospero, 2018) that have devastated native populations of *Ulmus* and *Castanea*, respectively, in Europe and North America. There are currently only three well-documented pathogens known to be introduced into South Africa and causing serious damage to native tree populations. These include the root pathogens *Phytophthora cinnamomi* (Nagel et al., 2013), *Armillaria mellea* (Coetzee et al., 2018) and the more recently discovered myrtle rust pathogen *Austropuccinia psidii* (Roux et al., 2016). *S. neocupressi* causing a canker disease on native *W. nodiflora* appears to be the fourth example of this category of disease.

There are many questions that remain to be answered regarding the serious canker disease on *W. nodiflora* discovered in this study. Of these, one of the most intriguing is where the pathogen might have originated. One possibility is that it has been on species of *Cupressus* in South Africa for some time and that it has now undergone a host shift (Slippers et al., 2005) to a native tree genus. Alternatively, the disease represents a new introduction of an invasive alien fungal pathogen. Either way, this is an important problem that deserves to be addressed with host range studies and population genetic analyses.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers ON892039–ON892041 and ON924453–ON924461, as listed in Table S1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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