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A Critically Endangered Proteaceae in the Cape Florisitic Region threatened by an invasive pathogen

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Background: *Sorocephalus imbricatus* (Thunb.) R.Br. is a range-restricted species endemic to the Cape Floristic Region (CFR), South Africa. It is currently classified as Critically Endangered in accordance with the IUCN criteria. Like many other species endemic to the CFR, *S. imbricatus* is subjected to several major threats including habitat loss, habitat degradation and the impacts of invasive alien species. *Sorocephalus imbricatus* was recently identified as a species requiring improved representation in *ex-situ* collections. During field work undertaken to collect germplasm for this purpose, a concerning number of dead and dying plants were observed.

Objectives: To determine the cause of rapid death of individuals in a remnant subpopulation of *S. imbricatus*.

Method: A field visit to a subpopulation of the only extant population, Elandskloof, was conducted to examine the symptoms associated with *S. imbricatus* mortality, and to collect samples for isolation and identification of putative pathogens.

Results: Dead and dying plants showed clear symptoms of root and collar rot, with *Phytophthora cinnamomi* Rands recovered from all samples. The collections highlighted the severe impact of *P. cinnamomi* on *S. imbricatus*, with the size of the subpopulation being reduced from 62 to 37 individuals (a 40% reduction) between October 2021 and May 2022.

Conclusion: This study describes, for the first time, rapid mortality of the Critically Endangered Proteaceae species, *S. imbricatus,* likely caused by the invasive pathogen *P. cinnamomi*. This concerning discovery highlights the urgent need for greater recognition of the threat *P. cinnamomi* poses not only to *S. imbricatus,* but to the broader floristic diversity of the CFR. Importantly, it illustrates a need for a substantial body of work to be undertaken to address a significant lack of knowledge regarding the relative threat that *P. cinnamomi* poses to species of the CFR.

Keywords: Proteaceae; *Phytophthora cinnamomi*; root rot; susceptibility continuum; threatened species.

Introduction

The Cape Floristic Region (CFR) contains more than 9 300 species of vascular plants, 68% of which are endemic to the region (Manning & Goldblatt 2012). The exceptionally high level of species diversity and endemism, together with having one of the highest concentrations of threatened plants in the world, has led to the recognition of the CFR as one of the 'hottest hotspots' of biodiversity globally (https://whc.unesco.org/en/list/1007/). Habitat loss (due to infrastructure development, urban expansion, crop cultivation, timber plantations and mines), habitat degradation (by overgrazing and inappropriate fire

management) and the impacts of invasive alien plant species have all been identified as major threats to the biodiversity of the CFR (Richardson et al. 1996; Bomhard et al. 2005; Manning & Goldblatt 2012). Importantly, climate change is also likely to be a major future threat (Bomhard et al. 2005).

Target 8 of the Global Strategy for Plant Conservation (GSPC; a programme adopted under the Convention on Biological Diversity) sets a goal for 'at least 75 per cent of threatened plant species in *ex-situ* collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programmes'. In 2017, a full gap analysis of South African Proteaceae held in documented *ex-situ* collections (both living and seed), was undertaken by institutional members of Botanic Gardens Conservation International (BGCI), guided by Target 8 of the GSPC and the IUCN/SSC (2014) Guidelines on the Management of *Ex-situ* Populations for Conservation. Members of the genus *Sorocephalus* R.Br. were among those that stood out as horticulturally difficult to maintain (Blackhall-Miles 2020). *Sorocephalus* is a small genus, containing 11 species, five of which are Endangered and four that are Critically Endangered (IUCN 2022). At the time of the gap analysis, only two species of *Sorocephalus* were being conserved in *ex-situ* collections (Blackhall-Miles 2020).

One of the species for which the development of conservation measures was prioritised was *Sorocephalus imbricatus* (Thunb.) R.Br. Commonly known as the tile-leaf powder puff, *S. imbricatus* is a slender, single stemmed, sparsely branched shrub growing to 1.5 m, flowering from spring to midsummer (September to December) (Goldblatt & Manning 2000). Endemic to the CFR, with a limited distribution in mountains of the Western Cape, it occurs on sandstone fynbos and montane shale bands at 330–860 m altitude (Rebelo & Raimondo 2020). Historically, *S. imbricatus* occurred at three localities: Piketberg, Groot Winterhoek and Elandskloof mountains. Today, however, only the Elandskloof population remains extant, with a declining number of patches of plants occurring over a 14 km range (Rebelo & Raimondo 2020). Now Critically Endangered, several threats to the survival of this species have been identified. These include habitat loss due to dam construction and forestry plantations, and ongoing habitat degradation due to inordinately frequent fires and invasion by alien plants (Rebelo & Raimondo 2020).

In October 2021, one of the remaining Elandskloof patches of *S. imbricatus* was visted by members of the South African National Biodiversity Institute (SANBI), Cape Nature and the Millennium Seed Bank (MSB) (Figure 1B). The team monitored size and status of the population, collected cuttings for vegetative propagation trials, and hand pollinated and bagged flowers of several plants. Sixty-two individual plants were counted at the site. A follow-up visit to collect seed took place in February 2022. During this visit, it was noted that several *S. imbricatus* plants were dead, and others were dying, with the number of surviving plants reduced to 51 individuals. In May 2022, a site visit was conducted to determine the cause of death of individuals in this population. Symptoms of root and collar rot were immediately obvious, with plants appearing to have died rapidly. The observed symptoms appeared typical of *Phytophthora* infection. The aim of this study was to determine the cause of the root disease seriously threatening *S. imbricatus*.

Material and methods

Disease description, sample collection and isolations

The assessed Elandskloof *S. imbricatus* subpopulation was restricted to an area of approximately 2 500 square metres of the Witzenberg Local Municipality, Western Cape, South Africa (-33.389801, 19.100955). The area where the subpopulation occurs is surrounded by a plantation of *Pinus* (Figure 1A). The plants appeared to have died rapidly, with leaves still attached to stems and minimal decomposition of plant parts observed (Figure 1E).

Three dead plants were carefully removed from the ground and there was evidence of root and collar rot in all individuals. The diseased plants were collected together with rhizosphere soil, to determine the cause of their death in the laboratory. An additional two living but symptomatic plants were inspected (Figure 1C). Bark tissue of these two individuals was scraped back, revealing necrotic lesions in the collar and stem of both plants, typical of *Phytophthora* infection (Figure 1D). Root and soil samples were collected from these two plants.

Sorocephalus imbricatus root pieces were washed in distilled water, blotted dry and plated onto *Phytophthora* selective media, NARPH (50 mg nystatin, 200 mg ampicillin, 10 mg rifampicin, 25 mg pentachloronitrobenzene, and 50 mg hymexazol per 1 L deionised water and 17 g cornmeal agar, as described by Hüberli et al. 2000). Plates were kept in the dark at 22°C and examined daily. Preliminary identifications were made using microscopy. After three to five days, coralloid hyphae with hyphal swellings, typically characteristic of *P. cinnamomi,* emerged from root tissues of three of the five samples. Single hyphal tip isolations were made and transferred onto fresh half strength potato dextrose agar (½ PDA; 19.5 g PDA powder, Merck, South Africa, 7 g Difco™ agar, 1 L deionised water).

The soil and root samples from which *Phytophthora* was not recovered by direct plating were flooded with distilled water and 'baited' to further determine

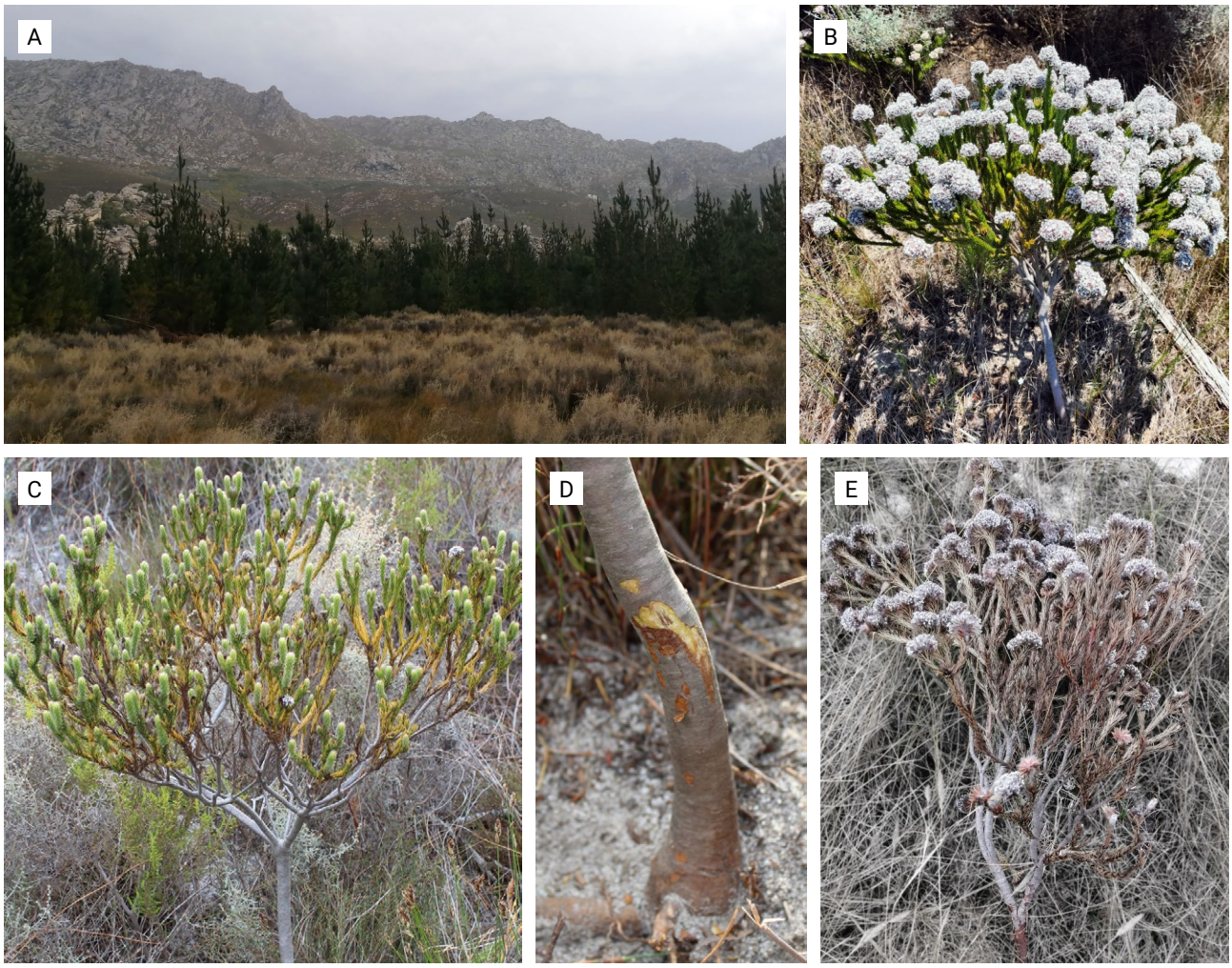

Figure 1. A, Study site – Elandskloof subpopulation of *Sorocephalus imbricatus,* surrounded by a *Pinus* plantation; B, healthy *S. imbricatus* in full flower, October 2021; C, symptomatic *S. imbricatus*; D, bark of plant in C removed to expose collar lesion; E, dead *S. imbricatus,* May 2022.

the presence of *Phytophthora*. Approximately 300 g of soil and roots was placed in a plastic tray containing 1 L of non-sterile distilled water. The floating plant litter was discarded and leaves of *Bauhinia galpinii* N.E.Br., *Hedera helix* L. and *Quercus suber* L., and petals of *Rosa hybrida* L. cv. 'Iceberg' were placed on the water surface as baits. The baits were maintained at room temperature and monitored regularly for lesion development over five days. Symptomatic baits with lesions were plated onto NARPH. Plates were stored, inspected and subcultured as described for the direct plated roots. Purified cultures were maintained on 10% clarified V8-agar (V8A; 0.1 L clarified V8 juice, Campbell Soup Company USA, 0.1 g $CaCO_3$, 15 g Difco™ Agar, Becton, Dickinson and Company, Sparks, USA, 0.900 L deionized water), and ½ PDA, at 22°C.

DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from seven-day-old isolates grown on ½ PDA using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocols. The internal transcribed spacer regions 1 and 2 (ITS), including the 5.8S rRNA region, were amplified using primers ITS6 (Cooke et al. 2000) and ITS4 (White et al. 1990). PCR reactions were prepared following the protocols described by Bose et al. (2021). Amplified fragments were treated with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The forward and reverse sequences were separately sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Thermo Fisher) following the manufacturer's protocol. The obtained products were cleaned and sequencing of the products was carried out at the Bioinformatics and Computational Biology Unit, University of Pretoria. CLC Main Workbench v. 8.0.1 (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analy sis-and-visualization/qiagen-clc-main-workbench/) was used to assemble and trim the raw sequences, which were deposited in GenBank (Table 1). Consensus sequences were aligned to *P. cinnamomi* strain Ex-type CPHST BL 12 internal

transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (length 866 bp) MG865473.

Permitting

Permission to collect samples was provided by the Western Cape Nature Conservation Board. Collections were made under permit number CN35-28-14709.

Results

Disease description and isolations

During the May 2022 visit, the number of surviving plants was further reduced from 51 in February 2022 to 37 individuals. *Phytophthora* was isolated from direct plated roots of the two living plants, and roots of one of the dead plants. Baiting confirmed the presence of *Phytophthora* in the remaining two samples. The morphology of the *Phytophthora* isolates recovered from the *S. imbricatus* root and rhizosphere samples was consistent with that known for *P. cinnamomi* Rands. Five isolates, one originating from each of the sampled *S. imbricatus* plants, were purified and have been preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). The five isolates were further identified by DNA sequencing.

Pathogen identification

For all five *Phytophthora* isolates, amplicons of approximately 840 bp were generated for the ITS region. The five isolates had identical sequences, and were identical to the sequence of the ex-type isolate of *P. cinnamomi* (CPHST BL 12). The molecular identification thus supported the morphological identification of *P. cinnamomi*.

Discussion

A site visit to investigate rapid death in a subpopulation of *S. imbricatus* identified root and collar rot as the cause of mortalility. The symptoms were typical of *Phytophthora* infection. Laboratory isolation and subsequent DNA sequencing identified isolates as *P. cinnamomi*. It was not possible to undertake pathogenicity tests due to the Critically Endangered status of *S. imbricatus* in its natural habitat and a lack of plants currently growing in *ex-situ* sites. However, despite the inability to conduct Koch's postulates, the consistent recovery of the invasive soilborne pathogen *P. cinnamomi* from dead and dying individuals, together with the observation of symptoms typical of those known to be caused by this pathogen, provide convincing evidence that it was the causal agent of the observed deaths.

The Global Invasive Species Database lists *P. cinnamomi* as one of the 100 worst invasive alien species (GISD 2022). It is a globally important plant pathogen, causing root and crown rot, cankers, dieback and mortality of approximately 5 000 woody plant species (Hardham & Blackman 2018). The association of *P. cinnamomi* with death of a species in the Proteaceae is not surprising. The first report of mortality in natural ecosystems of South Africa was by Van Wyk (1973), who provided an account of *P. cinnamomi* causing 'quick decline' of *Leucadendron argenteum* (L.) R.Br. in the Western Cape. Subsequent studies highlighted the importance of *P. cinnamomi* as a root rot pathogen of numerous native species (Von Broembsen 1984; Von Broembsen & Brits 1985; Von Broembsen & Kruger 1985; Hulbert et al. 2019). Despite this initial evidence for *P. cinnamomi* being a notable threat to the flora of the CFR, particularly members of the Proteaceae, very few studies have investigated the relative susceptibilities of the Cape flora to this pathogen by artificial inoculation. Much of the limited pathogenicity work that has been conducted dates back to the 1970s and 1980s (Van Wyk 1973; Von Broembsen 1984; Von Broembsen & Brits 1985).

The identification of *P. cinnamomi* as a disease-causing agent is an important component regarding the

Table 1. GenBank accession numbers of *Phytophthora cinnamomi* isolates obtained from *Sorocephalus imbricatus*

Species	Isolate[a]	Host	Substrate	ITS
Phytophthora cinnamomi	CMW58750	*S. imbricatus* dead	rhizosphere soil & roots (baited)	OP748937
Phytophthora cinnamomi	CMW58751	*S. imbricatus* dead	rhizosphere soil & roots (baited)	OP748938
Phytophthora cinnamomi	CMW58752	*S. imbricatus* living	roots (direct plated)	OP748939
Phytophthora cinnamomi	CMW58753	*S. imbricatus* living	roots (direct plated)	OP748940
Phytophthora cinnamomi	CMW58754	*S. imbricatus* dead	roots (direct plated)	OP748941

[a] CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

management of threatened species such as *S. imbricatus*. Like South Africa's CFR, the Southwest Australian Floristic Region (SWAFR) is a global biodiversity hotspot, characterised by a Mediterranean climate and old, weathered, nutrient-deficient landscapes (Hopper & Gioia 2004). Similar to the CFR, the biodiversity of the region is threatened by habitat loss, habitat degradation, the impacts of invasive alien plant species and climate change (Monks et al. 2019). In addition, *P. cinnamomi* is considered to be one of the most significant threats to the conservation of the floral diversity of the SWAFR (Shearer et al. 2007; Cahill et al. 2008; Barrett & Rathbone 2018). Several of the plant families most severely impacted by *P. cinnamomi* in the SWAFR (e.g. Ericaceae, Fabaceae and Proteaceae), are families that are important components of the CFR (Goldblatt 1997; Barrett & Yates 2015). There is a pressing need to assess the risk *P. cinnamomi* poses to conservation of the Cape flora.

Rebelo et al. (2019) noted high mortality of immature and mature individuals in remaining subpopulations of *S. imbricatus*. They highlighted the threats posed by afforestation, alien plant invasion, too frequent fire and dam construction. It seems plausible, however, that *P. cinnamomi* has been overlooked as the cause of the observed mortality. Similarly, while the IUCN assessment of Rebelo and Raimondo (2020) lists threats by invasive species, this is limited to the impacts of invasive plants, and disease threats by invasive pathogens (including *P. cinnamomi*) are not currently considered. The presence of this pathogen represents a very concerning threat to the viability of this subpopulation, as demonstrated by the rapid and substantial (40%) reduction in population size observed between October 2021 (n=62) and May 2022 (n=37). Left unchecked, *P. cinnamomi* will likely continue to cause mortality of *S. imbricatus* individuals within this subpopulation. A hygiene plan should be developed to minimise the risk of further spread of *P. cinnamomi* within the area where the population occurs. Ideally, the health status of additional *S. imbricatus* subpopulations should be determined.

The use of the systemic biodegradable fungicide, phosphorous acid (phosphite), may assist in maintaining the health of remaining *S. imbricatus* individuals. While this treatment cannot eradicate *Phytophthora* from the soil, it induces a defence response in *Phytophthora*-challenged plants and has been shown to increase the resistance of susceptible plant species to infection by *P. cinnamomi* (Hardy et al. 2001; Shearer et al. 2012; Eshraghi et al. 2014). The application of phosphite could buy time to allow the collection of additional *S. imbricatus* germplasm to be stored in seed banks, or propagated and grown in botanical gardens for future translocation programs.

In 1975, Knox-Davies warned of the potential susceptibility of many components of the fynbos vegetation to *P. cinnamomi*. Importantly, he emphasised the need for an intensive local research programme to, among other things, determine the relative susceptibilities of fynbos species to *P. cinnamomi*. Today, near 50 years on, very little progress has been made in this regard. Considering the alarmingly high number of rare and threatened taxa occurring in the CFR, and with many of these taxa such as *S. imbricatus* belonging to families known to be susceptible to *P. cinnamomi,* it is imperative that a research programme similar to that proposed by Knox-Davies (1975), be established. Methods to position taxa on a *P. cinnamomi* resistance-susceptibility continuum have been well developed by researchers working with threatened flora of the SWAFR (Shearer et al. 2013). Understanding the relative susceptibility of taxa can inform strategies for *in-situ* conservation, as well as informing where conservation priorities and recovery efforts should be directed. Substantial efforts have been made to identify and address many of the threats to the CFR. However, the rapid decline in population numbers of the Critically Endangered *S. imbricatus* highlights the urgent need to better understand the role of the invasive pathogen, *P. cinnamomi,* as a key threatening process to the flora of this region.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

MN raised the alarm after observing mortality in the *Sorocephalus imbricatus* population. MN and TP collected samples, TP conducted the diagnostics and wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

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