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Research Papers

Fig rust caused by *Phakopsora nishidana* in South Africa

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Summary. Fig rust, caused by *Cerotelium fici*, was first recorded in South Africa in 1927. Recent observations have revealed high incidence of rust and untimely defoliation of fig trees (*Ficus carica*) in residential gardens and commercial orchards. Using phylogenetic analysis, the causal organism of a fig rust isolate (PREM63073) collected in 2020 was confirmed as *Phakopsora nishidana*. Inoculation and microscope studies showed that mulberry plants were immune to *P. nishidana* isolate PREM63073. Infection of fig leaves occurred through stomata on the abaxial leaf surfaces. Very long germ tubes were observed for *P. nishidana*, often with no clear contact with the leaf surfaces and an apparent lack of directional growth towards stomata. Inoculated plants from 15 fig cultivars varied in their severity of leaf infection, whereas fruit of the cultivar Kadota developed reddish-brown blemishes without sporulation. Currently, *C. fici* and *P. nishidana* are recognised as occurring on *F. carica* in South Africa. This suggests a need to resolve the worldwide distribution and identity of the rust species involved.

Keywords. *Cerotelium fici*, *Ficus carica*, host response, infection, phylogenetic analysis.

INTRODUCTION

Ficus belongs to the *Moraceae*, which contains 60 genera and at least 1400 species (Gerber, 2010). The edible fig, *Ficus carica* L., one of approximately 750 *Ficus* species (Lötter, 2014), is native to the Middle East and Mediterranean regions and thrives where winters are mild and summers are hot and dry (Verga and Nelson, 2014). In 2019, the top fig producing countries, with a combined harvest of just over 1.1 million tons, were Turkey, Egypt, Morocco, Iran, Algeria, Spain, Syria, United States (mostly California), Tuni-

sia, and Afghanistan (https://fao.org/faostat, accessed 16 Feb 2021). According to the South African Fig Producers Association, South Africa (SA) ranks 33rd in global production of figs, exporting 336 tons in 2018/19 and 239 tons in 2019/20, to various destinations (https://www.safigs.co.za/links/, accessed 28 April 2021).

According to Karsten (1951), Godée Molsbergen mentioned figs as one of the many cultivated plants introduced to the South African Cape region between 1652 and 1662. Initially, fig production was restricted to individual trees or single rows or avenues on farms and residential gardens. In the absence of commercial production during the 18th and 19th centuries, fresh figs were used mostly for local consumption, but also as dried products and different types of preserve (Lötter, 2014). In 1902/03, the Cape Department of Agriculture imported 140 'Smyrna' and 'Capri' trees of different cultivars (e.g., Calimyrna, Kassaba, Bardajie, and Capri No. 1, No. 2 and No. 3), providing momentum to the local industry. Once the trees were established, 'Capri' cuttings with fruit containing *Blastophaga* wasps were imported under carefully controlled conditions for the insect to serve as a pollinating vector. South African fig production, located mainly in the Western Cape Province, varied considerably over the years (Lötter, 2014). Over the past 50 years attempts to revitalize the fig industry were launched by establishing experimental blocks and importing additional cultivars from California and France.

Doidge (1927; 1950) listed fig rust specimens collected from Mozambique, Zimbabwe, and the Eastern Cape, Free State, KwaZulu-Natal, North West, and Western Cape provinces in SA as belonging to *Cerotelium fici* (Castagne) Arthur. In addition, Verwoerd (1929) reported the occurrence of *C. fici* in the Western Cape, specifically in Wynberg, Paarl and Stellenbosch, and also from drier localities such as Barrydale, Ladismith and Oudtshoorn.

Fig rust symptoms are first visible as flecks on upper leaf surfaces, followed by the appearance of sporulating pustules on the lower surfaces. Pustules can occur over entire leaf blades, but are usually clustered in areas where moisture accumulates (McKenzie, 2013). Eventually, the spots on the upper surface of each affected leaf become brown and angular and may coalesce to form larger necrotic areas, leading to defoliation under conditions of severe infection. Fruit infections are visible as blister-like rust pustules (Verga and Nelson, 2014). Mulberry (*Morus* spp.) has also been reported as a host for *C. fici*, although Laundon and Rainbow (1971) doubted the conspecificity of the rust fungi on fig and mulberry.

In 2020, a fig tree heavily infected with rust was observed in a residential garden (Figure 1). The vis-

ible impact of the disease on tree health prompted an investigation to: (i) confirm the identity of the causal organism; (ii) understand the infection process; and (iii) evaluate methods to determine the host range and response of fig cultivars to the rust isolate under controlled conditions.

MATERIALS AND METHODS

Molecular identification of the fig rust isolate

Infected fig tree leaves (cultivar 'Parisian') containing uredinia were collected on 9 February 2020 in the coastal town of Vermont, Western Cape, SA. Infected leaf tissue, accessioned as PREM63073 in the National Collection of Fungi, Pretoria, was freeze-dried. A modified cetyltrimethylammonium bromide (CTAB) method (Visser *et al.*, 2009) was used to extract genomic DNA from ground leaf tissue. The 5.8S rRNA-ITS2-28S rRNA [Rust2Inv: 5'-GATGAAGAACACAGTGAAA-3' (Aime, 2006) and LR6: 5'-CGCCAGTTCTGCTTACC-3' (Vilgalys and Hester, 1990)] locus was PCR amplified, where each 10 µL reaction contained 10 ng DNA, 0.25 µM primers, a 1 × concentration of KAPA Plant PCR buffer and 0.5 U KAPA3G Plant DNA polymerase (KAPA Biosystems). The amplification regime consisted of a single 3 min denaturation step at 94°C, followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min, and a final 5 min elongation step at 72°C.

The amplicon was cloned into the pGem-T® Easy plasmid vector (Promega Corporation) and transferred into *Escherichia coli* JM109 competent cells. Cloned inserts of four recombinant plasmids were sequenced with the Rust2Inv, primer LR6, and internal primers LR0R (Moncalvo *et al.*, 1995) and LR3 (Vilgalys and Hester, 1990), using the BigDye™ Terminator v 3.01 Sequencing kit (Thermo Fisher Scientific). Sequenced products were separated on a 3130x1 Genetic Analyzer (Applied Biosystems), using the StdSeq50_POP7 sequencing run module. Once all ambiguous nucleotides were corrected, the four overlapping sequences of each recombinant clone were assembled into a single contiguous sequence, using the online CAP3 Sequence Assembly Program (Huang and Madan, 1999). The final consensus sequence was deposited on GenBank (accession No. MZ047090).

The two allelic sequences were aligned with selected reference sequences (Table 1), using the online MAFFT web interface (Katoh *et al.*, 2019). *Helicobasidium longisporum* was used as the outgroup (Maier *et al.*, 2016). After trimming the 5' and 3' extending sequences, the resulting 1036 bp ITS2-28S rRNA sequences were used

Figure 1. Rust damage on leaves of a fig tree in a residential garden in Vermont, Western Cape, South Africa, in February 2020.

to determine the appropriate nucleotide substitution model with the Akaike Information Criterion (AIC) within jModelTest v 2.1.1 (Darriba *et al.*, 2012; Guindon and Gascuel, 2003). The suggested TIM2 + G + I (n = 5) nucleotide substitution model was replaced with the GTR + G + I (n = 5) model (Lecocq *et al.*, 2013) for Bayesian inference (BI) analysis in MrBayes v 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). BI analysis was started from a random tree using four Markov Chain Monte Carlo (MCMC) chains. The search was limited to 5,000,000 searches, where every 500th generation was sampled. The average standard deviation of split frequencies was examined and the analysis stopped at a value below 0.01. The first 1000 trees were discarded as burnin before analysis.

Maximum parsimony (MP) analysis was carried out in PAUP* v. 4.0b10 (Swofford, 2003), where all characters were weighted equally. The heuristic search was carried out with 1000 addition-sequence replicates, with tree bisection and reconnection (TBR) branch swopping. Ten trees per replicate were saved. Bootstrap support for proposed branches was evaluated with 1000 replicates

with 100 random addition-sequence replicates and TBR branch swopping.

Spore and pustule morphology

The widths and lengths of 60 urediniospores of isolate PREM63073 were measured with an Olympus BX53 light microscope, fitted with a DP72 digital camera for image capturing with Analysis LS Research version 2.2 software (Olympus Soft Imaging System). Free-hand cross-sections of pustules (uredinia and telia) were made after soaking small pieces of dried leaves in hot water for a minimum of 30 min. Spores and pustules were examined with either a Zeiss Axioscope or a Nikon E600 microscope.

Host infection studies

During early spring 2020, young plants (< 1 m height) of 15 edible fig cultivars and a Queensland Red mulberry plant (*Morus rubra* L.) were grown in 10 L

Table 1. Taxa used in the molecular identification of the Vermont fig rust isolate PREM63073. Allelic variants of the isolate identified in the present study are indicated in bold font.

Taxon	Host	Isolate	Country	GenBank	Reference
Batistopsora crucis-filii	*Annona* sp.	BPI:863563	Guyana	DQ354539	Aime, 2006
Batistopsora crucis-filii	*Annona tomentosa*	PUR:87629	[a]-	KF528023	Beenken, 2014
Batistopsora pistila	*Annona sericea*	BPI:863563	-	KF528028	Beenken, 2014
Batistopsora pistila	*Annona spraguei*	PUR:66577	-	KF528029	Beenken, 2014
Cerotelium fici	*Ficus coronulata*	BRIP:56890	Australia	MH047209	Unpublished
Cerotelium fici	*Ficus coronulata*	BRIP:59463	Australia	MH047210	Unpublished
Cerotelium fici	*Ficus* sp.	BRIP:58068	Australia	KP753385	Maier *et al.*, 2016
Cerotelium fici	*Ficus* sp.	LAH20019AM	Pakistan	MK135779	Unpublished
Cerotelium fici	*Ficus* sp.	LAH20025AM	Pakistan	MK135780	Unpublished
Coleosporium plumeriae	*Plumeria rubra*	BRIP:55387	Australia	KM249866	McTaggart *et al.*, 2014
Helicobasidium longisporum	-	AFTOL-ID	-	AY885168	Unpublished
Crossopsora fici	*Ficus* sp.	BRIP:56872	Australia	MH047208	Unpublished
Crossopsora fici	*Ficus virens* var. *sublanceolata*	BRIP:58118	Australia	MH047207	Unpublished
Phakopsora annonae-sylvaticae	*Annona sylvatica*	PUR:87311	Brazil	KF528008	Beenken, 2014
Phakopsora cherimoliae	*Annona cherimola*	ZT:RB3096	-	KF528011	Beenken, 2014
Phakopsora cherimoliae	*Annona cherimola x squamosa*	PUR:89695	-	KF528012	Beenken, 2014
Phakopsora cingens	*Bridelia tomentosa*	BRIP:55628	Australia	KP729474	Maier *et al.*, 2016
Phakopsora gossypii	*Gossypium* sp.	BPI:910191	USA	KY764073	Unpublished
Phakopsora jatrophicola	*Jatropha* sp.	BPI:910194	USA	KY764078	Unpublished
Phakopsora jatrophicola	*Jatropha curcas*	BPI:910195	USA	KY764079	Unpublished
Phakopsora meibomiae	*Aeschynomene* sp.	R188	Colombia	EU851164	Zuluaga *et al.*, 2011
Phakopsora myrtacearum	*Eucalyptus grandis*	UP:217	Mozambique	KP729471	Maier *et al.*, 2016
Phakopsora myrtacearum	*Eucalyptus* sp.	[b]PREM:61156	Kenya	KP729472	Maier *et al.*, 2016
Phakopsora myrtacearum	*Eucalyptus grandis*	PREM:61155	Kenya	KP729473	Maier *et al.*, 2016
Phakopsora myrtacearum	*Eucalyptus grandis*	PREM:61155	Kenya	NG060142	Maier *et al.*, 2016
Phakopsora nishidana	*Ficus carica*	BPI:910197	USA	KY764080	Unpublished
Phakopsora nishidana	*Ficus carica*	BPI:842289	USA	KY764081	Unpublished
Phakopsora nishidana (as *Cerotelium fici*)	*Ficus carica*	UACH-107	Mexico	MF580676	Solano-Báez *et al.*, 2017
Phakopsora pachyrhizi	*Glycine max*	BPI:871755	Zimbabwe	DQ354537	Aime, 2006
Phakopsora pachyrhizi	*Desmodium* sp.	BRIP:56941	Australia	KP729475	Maier *et al.*, 2016
Phakopsora phyllanthi	*Phyllanthus acidus*	BPI:910198	USA	KY764082	Unpublished
Phakopsora phyllanthi	*Phyllanthus acidus*	ZT:RB8581	-	KF528025	Beenken, 2014
Phakopsora rolliniae	*Annona exsucca*	NY:3237	Trinidad and Tobago	KF528036	Beenken, 2014
Phakopsora tecta	*Commelina diffusa*	BPI:843896	Costa Rica	DQ354535	Aime, 2006
Phakopsora tecta	*Commelina* sp.	BRIP:56943	Australia	KP729476	Maier *et al.*, 2016
Prospodium lippiae	*Aloysia polystachya*	U152	Argentina	DQ354555	Aime, 2006
Puccinia graminis	*Glyceria maxima*	BRIP:60137	Australia	KM249852	McTaggart *et al.*, 2016
Puccinia junci	*Juncus tenuis*	PDD:99243	New Zealand	KX985745	Padamsee and McKenzie, 2017
UFS20_1	***Ficus carica***	**PREM:63073**	**South Africa**	**MZ047090**	**This study**
UFS20_2	***Ficus carica***	**PREM:63073**	**South Africa**	**MZ047090**	**This study**

[a] Information not available.
[b] Mycological collection at the South African National Collection of Fungi, Pretoria, South Africa.

bags filled with a mulch mixture, and acclimatized for 3 weeks pre-inoculation in a greenhouse cubicle with a temperature range of 18 to 25°C. The plants were watered every second day with reverse osmosis water, and were each fertilised once a week with 100 mL of a 0.2% (w/v) Multifeed-Classic water-soluble fertilizer [Effekto®, NPK analysis 19:8:16 (43)]. Cuttings representative of *Morus alba* L., including common or white mulberry, as well as weeping mulberry, were sampled a few hours before inoculation from confirmed sources. These cuttings were placed in 10 L containers filled with reverse osmosis water before and after inoculation.

Urediniospores of isolate PREM63073, kept at -80°C, were used in all experiments. Three weeks before inoculation, the urediniospores were increased through the inoculation of leaves of the fig cultivar Kadota. Procedures described by Boshoff *et al.* (2020) were used to heat-shock the spores and for inoculation of plants and subsequent incubation. After 3 weeks, urediniospores were collected from the Kadota leaves into size 00 gelatine capsules, by connecting an air vacuum to a cyclone spore collector (Pretorius *et al.*, 2019).

The response of fig and mulberry plants, including the mulberry cuttings, to the local rust isolate, was studied in two independent trials. During the first trial, a urediniospore concentration of 98×10^4 spores mL^{-1} in 0.3 mL Soltrol® 130 isoparaffinic oil, was applied per leaf. Two leaves were inoculated per cultivar/cutting, one inoculated on the abaxial surface and the other on the adaxial surface. In the second trial, one leaf per cultivar/cutting was inoculated on the abaxial surface, using a urediniospore concentration of 87.7×10^4 spores mL^{-1} in 0.3 mL Soltrol® 130 oil, as described above. During the second trial, replicate young developing figs from the cultivar Kadota were also inoculated using the above urediniospore concentration. Inoculated leaves on plants, young figs and cuttings were regularly observed for development of first symptoms of infection. Three weeks after inoculation, the number of rust pustules per cm^2 was counted on the abaxial leaf surfaces. This was achieved by placing a rectangular template with a 1 cm^2 opening at five random positions on each assessed leaf.

Analysis of variance (ANOVA; α = 0.05) of host response data was performed for the number of rust pustules counted per cm^2, using 'base' R functions, where the ANOVA model 'pustules ~ cultivar + trial' was applied (N = 150). Means were separated on the Minimum Significant Difference (MSD) test, using the *LSD.test* function from the 'agricolae' package, with an adjusted Bonferroni *P*-value to account for potential family-wise error rates (De Mendiburu, 2020). Data processing and analyses were performed with R version 4.0.2 (R Core Team, 2020) within R Studio version 1.2.5042 (R Studio Team, 2020). Data exploration, wrangling and visualisation were conducted using the 'Tidyverse' package (Wickham *et al.*, 2019). Data scripting was conducted in rmarkdown (Allaire *et al.*, 2020).

Fluorescence and scanning electron microscopy (SEM) were used to describe the host penetration process by the pathogen. Material for microscopy included newly sprouted leaves from the susceptible fig cultivars Cape White and Kadota, as well as young developing Kadota figs. Young leaves from the mulberry cultivar, Queensland Red, and freshly collected cuttings from a weeping mulberry plant, were included for fluorescence microscopy. The abaxial surfaces of three leaves per plant/cutting were each sprayed with 0.8 mL of urediniospore suspension (±1 mg mL^{-1} urediniospores in Soltrol® 130 oil), whereas dry spores were applied with a brush to young fruit. Post-inoculation treatment, dew chamber incubation and greenhouse conditions were as described above.

Fluorescence microscopy

Leaf and fig peel segments were sampled at 48 and 96 h (hpi), and 16 d post-inoculation (dpi), and were cut into 10 mm segments. Half of the samples were prepared using a modified method of Rohringer *et al.* (1977) as described by Moldenhauer *et al.* (2006), and stained leaf segments were stored in 50% (v/v) glycerol containing a trace of lactophenol to prevent deterioration and drying of fungal material.

The remaining leaf segments were processed according to the modified method (Maree *et al.*, 2020) of Ayliffe *et al.* (2011), using *Triticum vulgaris* Vill. lectin (wheat germ agglutinin) fluorescein isothiocyanate conjugate (WGA-FITC) (Sigma-Aldrich). The leaf segments were stained for 16 h at room temperature with 8.3 µg mL^{-1} (w/v) of WGA-FITC probe before viewing with an Olympus AX70 microscope (Rohringer *et al.*, 1977; Kuck *et al.*, 1981).

For the segments stained with Uvitex 2B, the blue wavelength epifluorescence cube with an excitation filter of 330-385 nm and a barrier filter of 420 nm showed fluorescence of stained fungal tissue. Observations of WGA-FITC stained segments were made with the ultraviolet wavelength (WU) epifluorescence cube, with an excitation filter of 450-480 nm and a barrier filter of 515 nm. The microscope was fitted with a CC12 digital camera for image capturing with Analysis LS Research version 2.2 software (Olympus Soft Imaging System).

Scanning electron microscopy

Leaf and fig peel segments were sampled at 48 and 96 hpi, and 16 dpi, and were cut into 5 mm segments. Samples were fixed according to the protocol of Glauert (1974). The dried samples were directly mounted on 12.2 mm diam. metal stubs (Cambridge pin type) using double-sided carbon tape, for observations of fruit or abaxial leaf surfaces. The mounted segments were coated with gold (± 60 nm thickness) in a sputter coater (Bio-Rad), and the specimens examined with a JSM-7800F Extreme-resolution Analytical Field Emission SEM.

RESULTS

Molecular identification of the fig rust isolate

Sequencing of multiple recombinant clones identified two ITS2-28S rRNA allelic variants of isolate PREM63073, which differed with four nucleotides within the ITS2 region over the 1054 bp amplicon. A

BLASTn search with the ITS2-28S rRNA locus showed that the isolate shared closest identity to *C. fici* isolate UACH-107 (MF580676; 99% identity, 994/1002 bp), *P. nishidana* voucher BPI:910197 (KY764080; 99% identity, 1019/1021 bp) and *Phakopsora myrtacearum* voucher UP:217 (KP729471; 99% identity, 1052/1059 bp). Five *C. fici* accessions (KP753385; MH047209; MH047210; MK135779; MK135780) shared between 93 and 91% identity with the fig rust isolate.

The fig rust isolate was identified using a phylogenetic approach where the topologies of the MP (results not shown) and BI (Figure 2) trees were almost identical, except for the placement of *Phakopsora gossypii*. A clade with excellent support (99% bootstrap (BS); 1.0 posterior probability (PP)) contained several isolates of *Phakopsora cherimoliae*, *P. rolliniae*, *P. annonae-sylvaticae*, *Batistopsora pistila* and *B. crucis-filii*. These were all collected from different *Annona* species hosts from the pawpaw/sugar apple family (*Annonaceae*).

Sister to this, was a clade that grouped both allelic variants of PREM63073 with one *C. fici* (likely incorrectly identified) and six other *Phakopsora* isolates. This clade
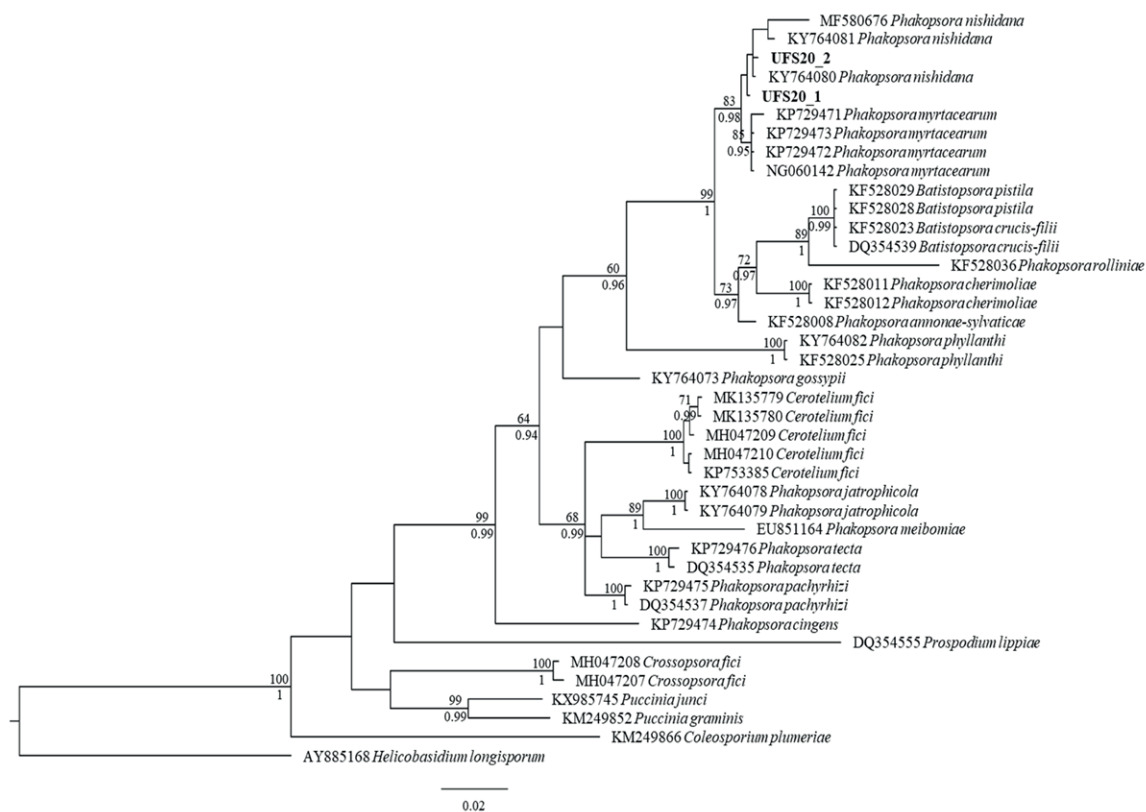

Figure 2. Identification of the Vermont fig rust (isolate PREM63073) using Bayesian inference (BI) and Maximum parsimony (MP) analyses within MrBayes and PAUP*, using the ITS2-28S rRNA locus. The two allelic variants for rust isolate PREM63073 are indicated in bold font. Bootstrap (BS) support values (>60%) are indicated above the nodes, and posterior probability (PP) values (>0.9) are indicated below the nodes.

was divided into two sub-clades (83% BS; 0.98 PP). The first sub-clade contained both PREM63073 variants, and three *P. nishidana* accessions that were all collected from *F. carica* trees from, respectively, SA, the USA and Mexico. The second sub-clade contained four *P. myrtacearum* isolates, all collected from *Eucalyptus* trees in eastern Africa.

The other five *C. fici* isolates grouped as a separate sub-clade (68% BS; 0.99 PP), within a larger clade (64% BS; 0.94 PP) containing *P. jatrophicola* (collected from *Jatropha* sp.), *P. pachyrhizi* (*Glycine max* and *Desmodium* sp.), *P. meibomiae* (*Aeschynomene* sp.) and *P. tecta* (*Commelina* sp.). The first of these five *C. fici* isolates (KP753385) formed part of a study on *P. myrtacearum* on eucalyptus trees in southern and eastern Africa, where the pathogen origin was indicated as a *Ficus* sp. in Australia (Maier *et al.*, 2016). The other four were direct submissions to GenBank as unpublished studies. The first two of these accessions (MH047209 and MH047210) were part of a study of *Uredo morifolia* on mulberry in Australia, where these isolates were collected from *F. coronulata*. The other two *C. fici* accessions (MK135779 and MK135780) were collected from *Ficus* spp. in Pakistan.

Spore and pustule morphology

Uredinia were scattered over the abaxial leaf surfaces, singly but frequently becoming crowded. The surrounding leaf tissue was darkly melanised. Telia were visible as dark brown crusts surrounding many uredinia in two of the specimens examined (Figure 3, A). The uredinia were sub-epidermal, erumpent (Figure 3, B), and telia were subepidermal and non-erumpent (Figure 3, C). Paraphyses (Figure 3, D) were present at the peripheries and scattered through the uredinia. The paraphyses were hyaline, cylindrical, wall ≈0.5 μm thick, 25–48 μm long, 5–8 μm wide. The telia were typical *Phakopsora*-like, forming subepidermal, non-erumpent crusts two to five cells deep. Telial cells were light brown, not in distinct columns, 12–20(–28) × 6–10 μm, with smooth walls 1 μm thick though slightly thickened in the uppermost cells to 1.5–2 μm (Figure 3, E). Urediniospores (Figure 3, F and G) were hyaline, and globose, ellipsoid or obovoid in shape, and measured (17.4–)19–26(–32.8) × (14.9–)16–19(–20.8) μm (means = 17.5 × 23.5 μm). Their walls were ≈1 μm thick, with no readily visible germ pores.

Infection studies

No symptoms or signs developed on the inoculated leaves of mulberry cultivar Queensland Red or on the cuttings from the common or weeping mulberry plants.

This apparent immunity of the tested mulberry plants was confirmed by fluorescence microscopy. Observations at either 48 or 96 hpi showed random distribution of appressoria, occasionally over stomata, on the mulberry leaves. Here, abortion of substomatal vesicles without development of haustorium mother cells (HMCs) (Figure 4, A) or non-penetrating appressoria (Figure 4, B), were observed.

Fig leaves inoculated on the adaxial surfaces did not develop meaningful infections (zero to trace infection), and the leaves remained mostly symptomless. The first signs of infection became visible as minute reddish-brown flecks for leaves inoculated on the abaxial surfaces, 7 dpi. The latent period, indicated by the first signs of sporulation, varied among cultivars, and was 10 dpi for Brunswick and Noire de Caromb. This was followed by 11 dpi for Tiger, Black Mission, Dalmatie, Ronde de Bordeaux, Col de Dame Noir, Parisian, Deanne, Adamsvy and Cape White at 11 dpi; 12 dpi for Kadota and Cape Brown; 13 dpi for Black Genoa; and 14 dpi for White Genoa. The ANOVA indicated no statistically significant differences ($F = 3.7$, $P = 0.06$) between the two experimental trial replicates for mean numbers of pustules per cm^2. However, significant differences were detected between the host cultivars ($F = 156.3$, $P < 0.05$). Cape White, Noire de Caromb (mean >20 pustules per cm^2) and Tiger (mean > 15 pustules per cm^2) were the most severely affected cultivars (Figure 5). While the Cape White and Noire de Caromb cultivars did not differ ($P > 0.05$) from one another, they were different from Tiger. The remaining cultivars, except White Genoa, produced mean numbers of pustules per cm^2 between 5.0 and 13.6, with three clusters of responses indicated by mean separation. White Genoa responded the least to inoculation, producing a mean of 3.2 pustules per cm^2. Rust severity (27 dpi) of infected leaves for cultivars Noire de Caromb (Figure 6, A), Brunswick (Figure 6, B), Adamsvy (Figure 6, C), Black Genoa (Figure 6, D), and 50 dpi for Brunswick on the adaxial (Figure 7, A) and abaxial (Figure 7, B) leaf surfaces, indicated severe disease on some cultivars.

Inoculated figs did not show typical rust signs or symptoms. However, as the fruit matured, reddish-brown blemishes became visible on the peels (Figure 8, A). Although germinated urediniospores were visible with SEM at 48 hpi on the skin of young Kadota fruit (Figure 8, B), sometimes close to stomata, no appressoria were observed. At 96 hpi, the germ tubes were collapsed with no signs of infections. Instead, blister-like structures, usually associated with host stomata in the urediniospore treated areas, but not necessarily with presence of rust spores, were observed at 96 hpi (Figure
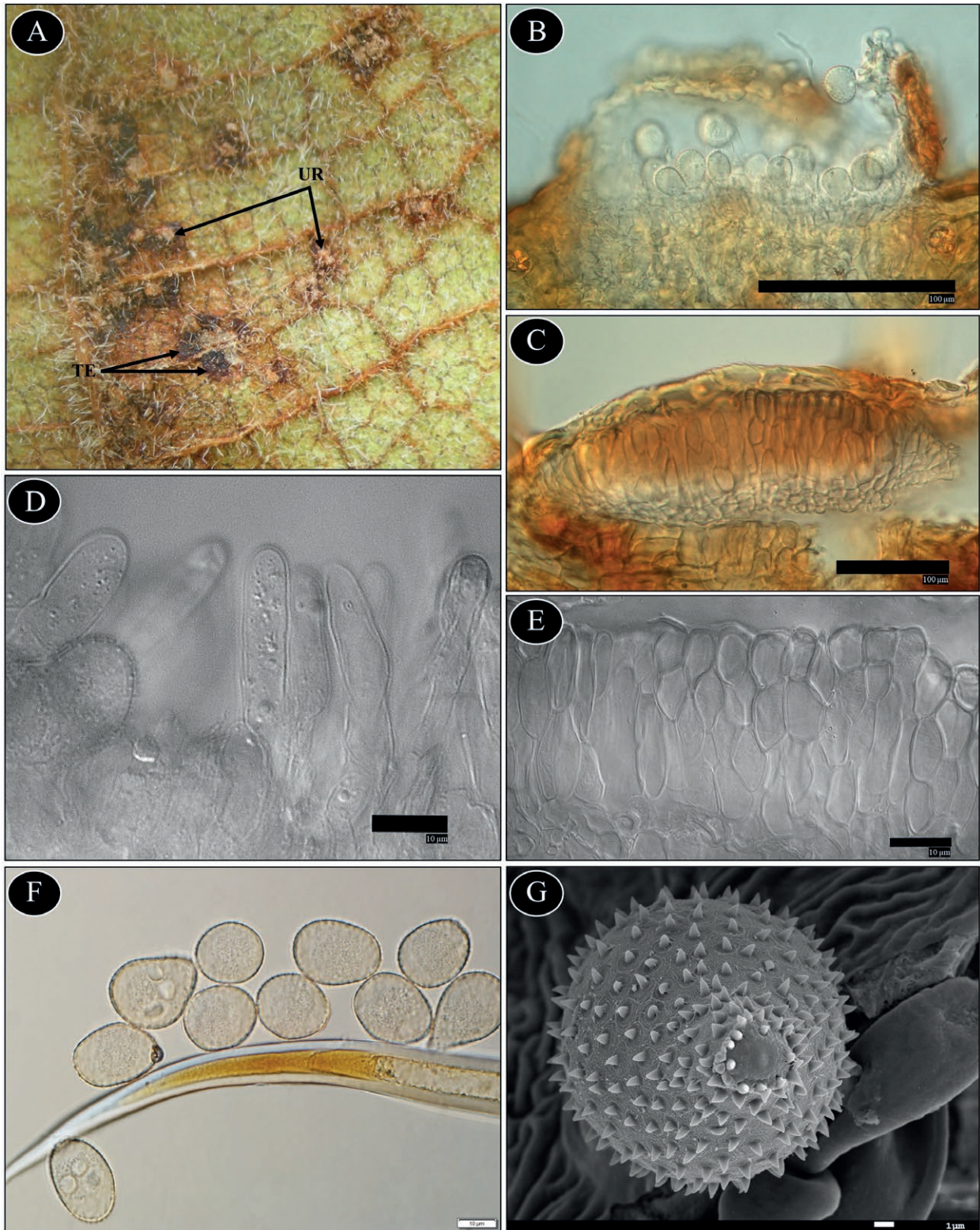
Figure 3. A, Uredinia (UR) and telia (TE) of *Phakopsora nishidana* on the abaxial surface of a leaf of *Ficus carica*. B, transverse cross section through a subepidermal erumpent uredinium, and C, a non-erumpent telium. D, thin-walled cylindrical paraphyses in a uredinium of *P. nishidana*. E, teliospores, F, thin-walled urediniospores (germ pores are obscure), and G, scanning electron micrograph showing ornamentation and hilum of a *P. nishidana* urediniospore. Scale bars in B to E represent values indicated below each bar.
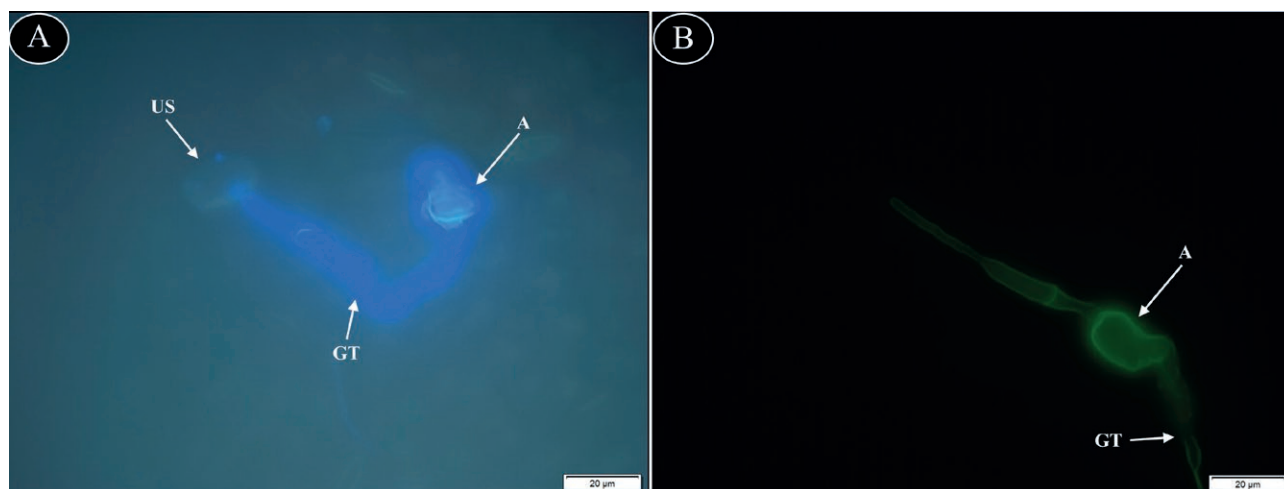
Figure 4. Microscope images of *Phakopsora nishidana* urediniospore germination, taken at 96 h post-inoculation, from A, Queensland Red mulberry leaf, indicating an early abortion, and B, mulberry, showing a non-penetrating appressorium. The urediniospore (US), germ tubes (GT) and appressoria (A) are indicated.
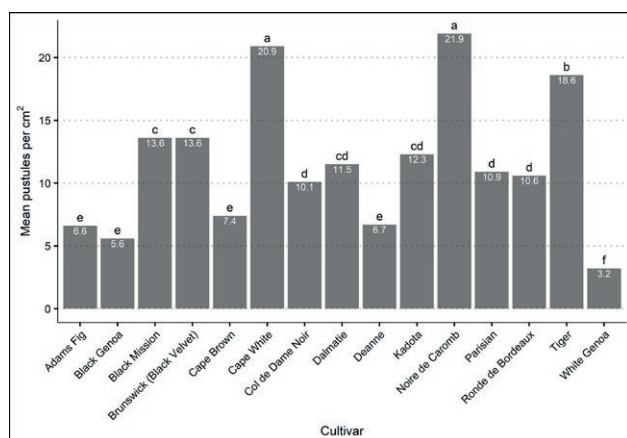

Figure 5. Mean numbers of rust pustules per cm² for 15 fig cultivars after inoculation with *Phakopsora nishidana*. Mean values, indicated by annotation in the bar, followed by the same letter for each cultivar did not differ ($\alpha < 0.05$), according to Fishers MSD, where MSD = 2.24 pustules per cm².

8, C). Inoculated areas of the fruit surfaces had sunken lesions at 14 dpi (Figure 8, D).

Light microscopy showed that leaves of Cape White and Kadota all had infection structures, typical of those formed on leaf surfaces by rust fungi during the first phases of infection cycles. At 48 hpi, these structures included germinated urediniospores, germ tubes, and appressoria forming over stomata on the abaxial leaf surfaces (Figure 9, A and B). As each appressorium matured, it was delimited from the germ tube by a septum (Figure 9, B and C) followed by collapse of the germ tube. Most of the appressoria were collapsed on top of stomata at 96 hpi (Figure 9, C). The *P. nishidana* germ tubes were very long, often extending from one trichrome to another with no apparent contact with the leaf surface (Figure 9, D). Formation of appressoria was not necessarily on the first encountered stomata (Figure 9, E), and appeared to be random. With fluorescence microscopy (not shown), only a few HMCs (mostly one or two) were visible at 48 hpi, whereas at 96 hpi, small colonies (approx. 30 HMC's) were observed. At 14 dpi, established colonies filled with urediniospores were common (Figure 9, F).

DISCUSSION

Phakopsora nishidana S. Ito is here recorded from SA, the first confirmation of this species occurring in Africa. This is the second rust fungus to be reported on cultivated fig in SA; the other species is the widely distributed fig rust, caused by *C. fici* (Doidge, 1927, 1950). The phylogenetic analysis of the present study showed that both these species belong to the *Phakopsoraceae*, as currently delimited (Aime and McTaggart, 2020). *Phakopsora nishidana* is in a distinct clade to that containing the type species, *P. pachyrhizi* Syd. & P. Syd. (Aime and McTaggart, 2020), and therefore should be assigned to a new genus. This has not been conducted in the present study, as more species need to be included in analyses before generic limits within the *Phakopsoraceae* can be accurately assessed. *Cerotelium fici* is, in the present analysis, a sister clade to *Phakopsora sensu stricto*, and
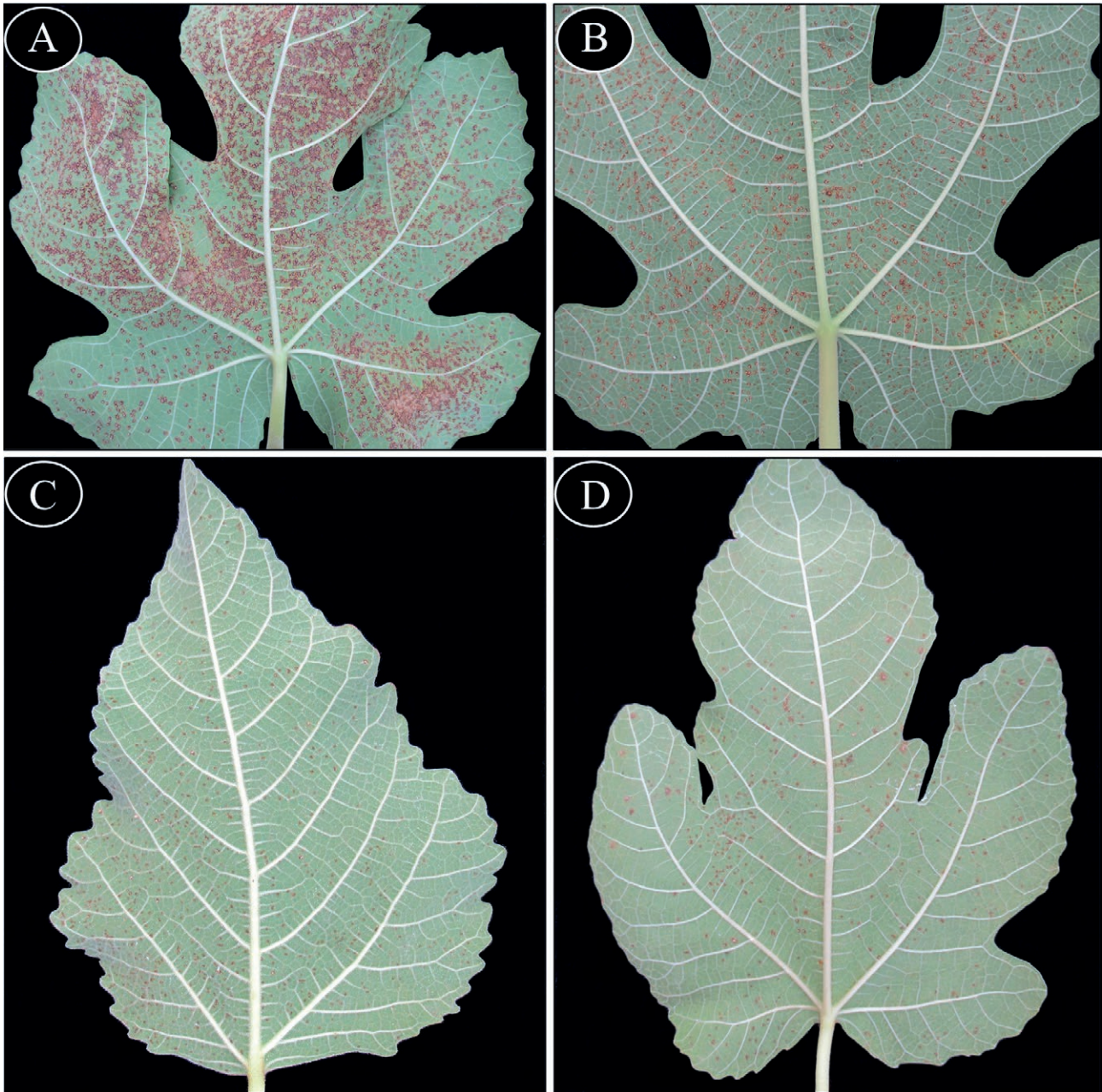
Figure 6. Fig rust symptoms on the abaxial surfaces of leaves from the fig cultivars Noire de Caromb (A), Brunswick (B), Adamsvy (C) and Black Genoa (D), 27 d post inoculation with urediniospores of *Phakopsora nishidana*.

may therefore be transferred to this genus depending on results of additional analyses.

The application of the names *Cerotelium fici* and *Phakospora nishidana* has been confused, with some workers applying the *C. fici* for the widely distributed fig rust pathogen (e.g. Laundon and Rainbow, 1971; McKenzie, 1986), whereas others have applied *P. nishidana* (e.g. Buritica, 1999; Hennen *et al.*, 2005; De Carvalho *et al.*,

2006). This resulted from differing interpretations of the species to which the first described spore stage, *Uredo fici* Castagne, belongs. Here, this is assigned to *C. fici*. The paraphyses of *P. nishidana* were described as both peripheral and intermixed with the urediniospores (Ito and Homma, 1938). In contrast, the uredinia of *U. fici* are surrounded by paraphyses (malupa-like) (Arthur, 1906), as described for *C. fici* by Butler (1914). In addi-
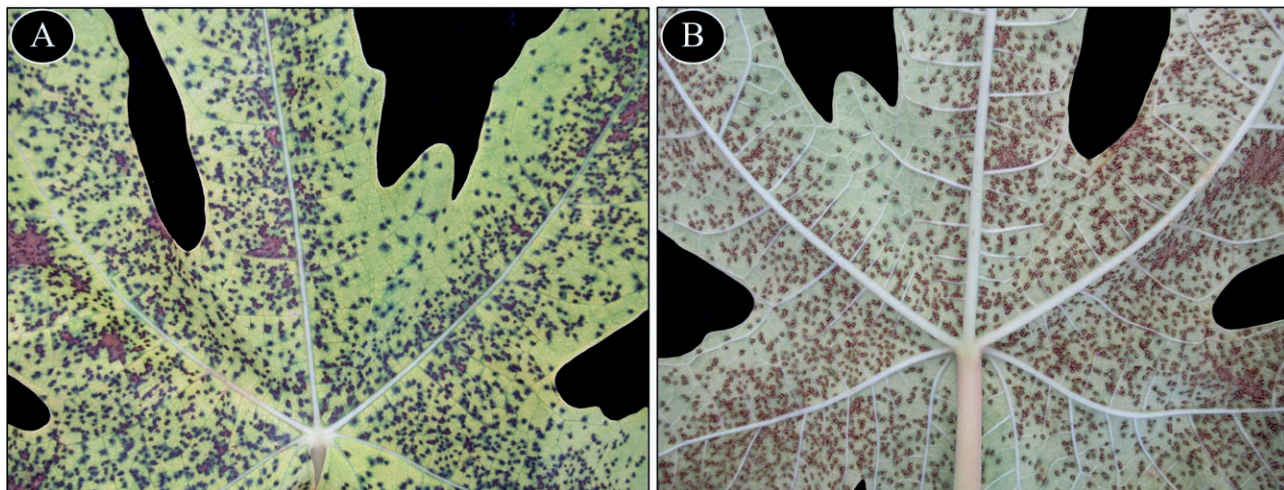
Figure 7. Fig rust symptoms on adaxial (A) and abaxial (B) leaf surfaces of the fig cultivar Brunswick, 50 d post inoculation of the abaxial surfaces with urediniospores of *Phakopsora nishidana*.
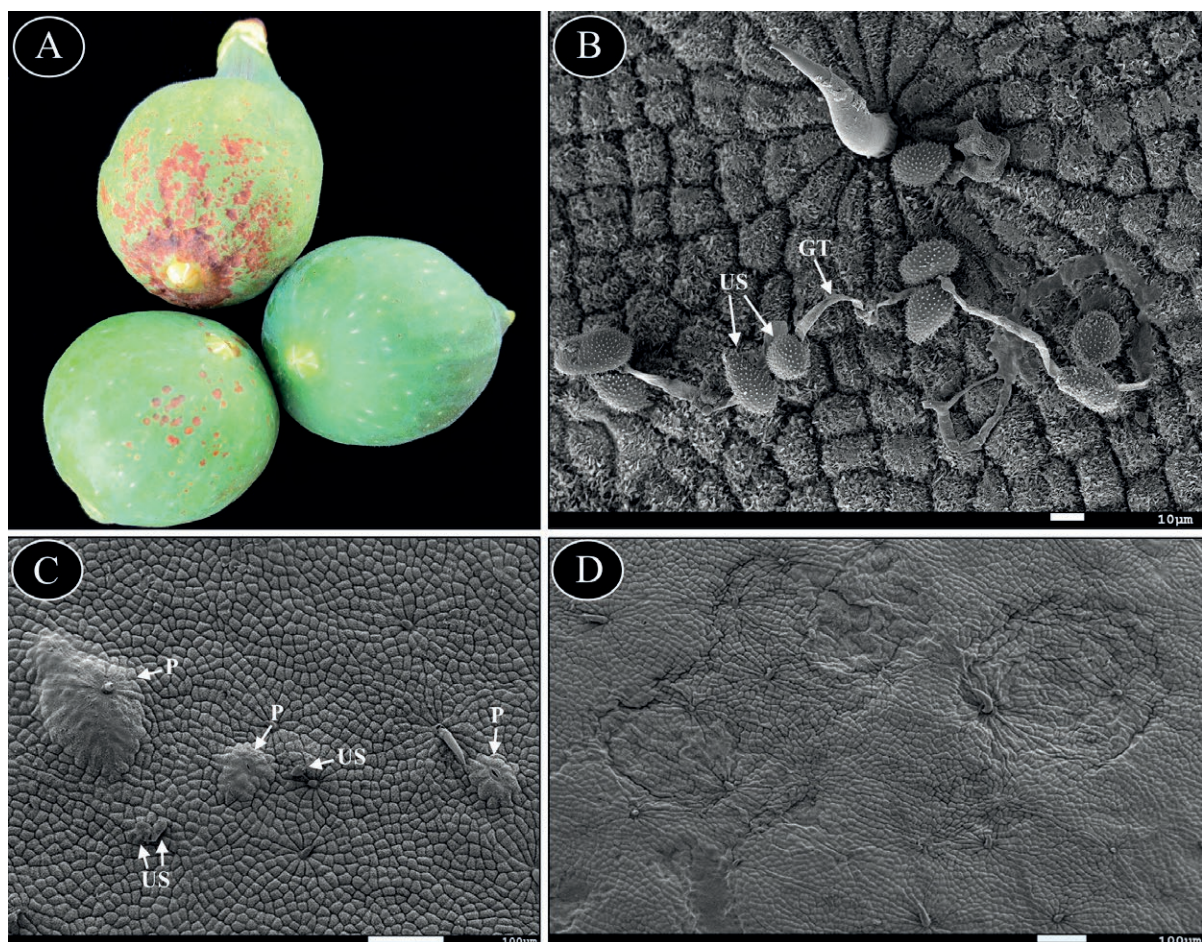

Figure 8. A, Fruit of fig cultivar Kadota after applying dry urediniospores of *Phakopsora nishidana* to young developing figs, showing blemishes (top and bottom left fruits), while the fig on the right represents an uninoculated control. B and C, scanning electron micrographs (SEMs) 96 h post inoculation, of a germ tube (GT), urediniospores (US) and blister-like pustules (P). D, SEM of sunken leaf lesions at 16 d post inoculation.
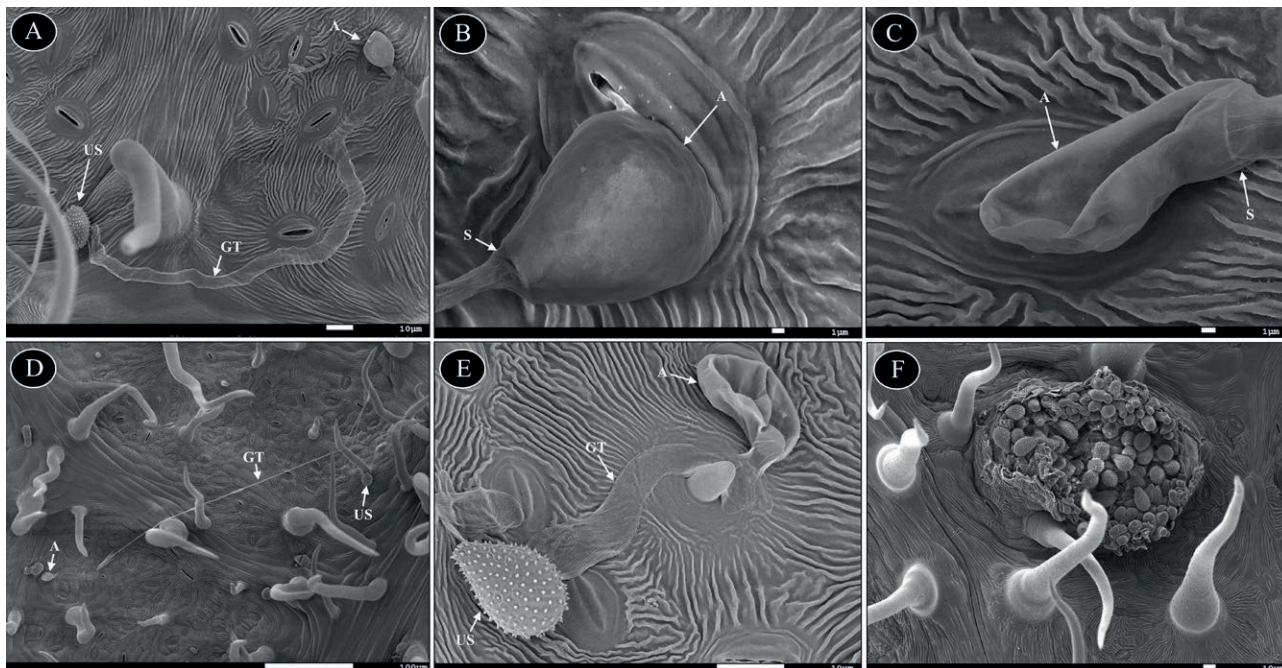
Figure 9. Scanning electron micrographs of *Phakopsora nishidana* on abaxial fig leaf surfaces, at 48 hpi (A and B), 96 hpi (C, D and E) and 16 dpi (F). Appressoria (A), germ tubes (GT), septum (S) and urediniospores (US) are indicated.

tion, Ito and Murayama (1949) assigned *U. fici* as the uredinial stage of *Phakopsora fici-erectae* S. Ito & Y. Otani ex S. Ito & Muray., not *P. nishidana*. However, *P. nishidana* was not recorded from *F. carica* (Ito and Murayama, 1949), the type host of *U. fici*. The uredinial morphology of *P. fici-erectae* should be re-examined and contrasted to that of *C. fici*, as this was beyond the scope of the present study.

The correct synonymy for the two species considered, is as follows:

Cerotelium fici (Castagne) Arthur, Bull. Torrey Bot. Club 44: 509 (1917)
basionym: *Uredo fici* Castagne, Pl. Crypt. Nord France, Edn 3 34: no. 1662 (1848)
≡*Malupa fici* (Castagne) Buriticá, Revta ICNE, Instit. Cienc. Nat. Ecol. 5(2): 175 (1994)
Physopella fici (Castagne) Arthur, Résult. Sci. Congr. Bot. Wien 1905: 338 (1906)
Kuehneola fici (Castagne) E.J. Butler, Annls mycol. 12(2): 79 (1914)

Note: under the previous rules of Nomenclature, a teleomorph name could not be based on an anamorph type specimen, and therefore this species was correctly cited as *Cerotelium fici* (E.J. Butler) Arthur, as Butler's *K. fici* was then the correct basionym. However, since the

principle of 'one fungus one name' has been applied, the first correctly published name becomes the basionym, even if for an anamorph.

Phakopsora nishidana S. Ito, Trans. Sapporo Nat. Hist. Soc. 15: 117 (1938)
Phakopsora fici Nishida, Engei no Tome 7: 881 (1911) *nomen nudum*

Besides these species, another, *Uredo sawadae* S. Ito (Farr and Rossman, 2021) has been described from *F. carica*. Other rust species described from various *Ficus* species, other than *F. carica*, include *Phakopsora fici-elasticae* T.S. Ramakr., *Cerotelium ficicola* Buriticá & J.F. Hennen, *Crossopsora fici* Arthur & Cummins (Farr and Rossman, 2021) and various *Uredo* species that must be assigned to one of these listed telial species or as yet undescribed species.

Cerotelium fici often occurs wherever cultivated figs are produced (Laundon and Rainbow, 1971; McKenzie, 1986; Latinović *et al.*, 2015). Except for the reports of Doidge (1927; 1950), Verwoerd (1929) and Lötter (2014), no published information on fig rust in SA could be found. As the description provided by Doidge (1927) corresponds to other accounts of *C. fici* (Laundon and Rainbow, 1971; McKenzie, 1986, 2013), the historical context and urediniospore morphology suggested that

the Vermont rust isolate could be *C. fici*. Phylogenetic analysis, however, clearly differentiated between *C. fici* and *P. nishidana*. The close association of molecular data of the local fig rust examined with two *P. nishidana* accessions confirmed that isolate PREM63073 was *P. nishidana* and not *C. fici*. The placement of the Mexican *C. fici* isolate UACH-107 (GenBank accession MF580676; Solano-Báez *et al.*, 2017) within this subclade indicated that it was incorrectly identified, due to a lack of adequate reference sequences. The morphology described for this isolate (Solano-Báez *et al.*, 2017) is consistent with that of the uredinial state of *P. nishidana*, though the urediniospores were slightly larger (23–27 × 16–19 μm) than originally described for this species (18–24 × 16–18 μm; Ito and Homma, 1938). One of the reference sequences for *P. nishidana* (KY764080) was of a specimen that originate from Mexico (BPI 910197), confirming the presence of this species in that country. Furthermore, a general assumption that fig rust is caused by *C. fici*, as well as morphological similarities between the urediniospores of *C. fici* and *P. nishidana*, may prevent clear visual distinction between the two species.

Teliospore morphology is distinct between *C. fici* and *P. nishidana* (Ito and Homma, 1938), with the telia of *C. fici* being typical of *Cerotelium*, as erumpent and producing teliospores in short chains which are not laterally adherent. The telia of *P. nishidana* are not erumpent, with the teliospores forming crusts of laterally adherent teliospores. Telia are seldom produced by *C. fici*, having only been recorded three times (Butler, 1914; Patil and Thirumalachar, 1971; Huseyin and Selcuk, 2004), whereas telia are readily produced by *P. nishidana* (Ito and Homma, 1938). The urediniospores of these two fungi are morphologically almost identical, being hyaline, thin-walled and with inconspicuous germ pores in both species. They differ in that they are slightly smaller in *P. nishidana* (18–24 × 16–18 μm; Ito and Homma 1938: typically 19–26 × 16–19 μm, though occasionally larger or smaller in the present study), compared to *C. fici* (20–35 × 14–25 μm: Butler, 1914; McKenzie, 1986; 2013; Huseyin and Selcuk, 2004; Latinović *et al.*, 2015). However, as stated above, the uredinia in *P. nishidana* are uredo-like, with paraphyses intermixed with the urediniospores, whereas those of *C. fici* are malupalike with only peripheral paraphyses (Butler, 1914; Doidge, 1927; Ellis, 2020). As the present study collection had *Phakopsora*-like telia present, and the uredinia had paraphyses intermixed through these pustules, this confirmed the rust's identity as *P. nishidana*, despite some of the urediniospores being larger than previously described for this species.

The taxonomy of rust fungi on figs remains confused, and there is need to fully resolve the species involved and their distribution, both within and beyond fig production regions. *Phakopsora nishidana* has been recorded from eastern Asia and the Americas (Hennen *et al.,* 2005; Solano-Báez *et al.*, 2017; Farr and Rossman, 2021), and there is a possibility that some of the records of *C. fici* from southern Africa, as well as elsewhere in Africa, may also be *P. nishidana*. The species with *Phakopsora*-like telia described from *Ficus* hosts, including *P. nishidana*, *P. fici-erectae* and *P. fici-elasticae*, as well as *U. sawadae*, need to be compared by phylogenetic analyses. The description of the fig rust identified as *P. nishidana* from South America (Buritica, 1999; Hennen *et al.*, 2005) fits the description of *P. fici-erectae* better than that for *P. nishidana*. It is possible that several species are involved, but are not recognised, as the urediniospore morphology for all is almost identical.

In SA, the Western Cape Province, specifically around the towns of Porterville, Malmesbury, Paarl and Wellington, and Napier in the Overberg, is considered as the most important fig producing region. The production conditions in this province are diverse and vary from more rust-conducive areas, with regular conditions of high humidity along the coastal regions, to semi-arid and less disease-prone areas such as Worcester, Ladysmith, Oudtshoorn and Prince Albert. Although rust regularly occurs towards the end of the production season in fig orchards in the Overberg, infection levels are generally low and without apparent economic impacts. In April 2021, however, rust incidence in orchards near Napier was high, with several trees defoliating due to rust infections. Similar to the observations of McKenzie (2013), rust pustules were typically clustered alongside leaf veins or on leaf sections exposed to moisture accumulation and retention. Seasonal variation in severity and distribution of rusts is influenced by inoculum levels, environment, and cultivar susceptibility. Wind and splashed-dispersed fig rust urediniospores require at least 14 h of wetness to germinate and infect fig leaves or fruit (McKenzie, 2013). Rogovski Czaja *et al.* (2021) showed that urediniospores of *C. fici* could germinate after 6 h, whereas successful infection of fig leaves was achieved between 18°C and 28°C.

The mulberry plants tested in the present study were all highly resistant to rust infections, indicating that the rust fungus found elsewhere on this host may be different from *P. nishidana* which infects edible fig plants. *Phakospora nishidana* is known as a pathogen only of several species of *Ficus* (Farr and Rossman, 2021), whereas *C. fici* is recorded from species of *Broussonetia*, *Ficus*, *Maclura* and *Morus* (Moraceae) (McKenzie, 1986;

Farr and Rossman, 2021). Inhibition of colony establishment at an early stage, as seen in both mulberries, was classified as abortive penetration according to Kochman and Brown (1975). In *F. carica*, infection occurs after formation of appressoria over stomata on abaxial leaf surfaces of the hypostomatic leaves. Directional germ tube growth was not observed for *P. nishidana*, but it was evident that trichomes on lower leaf surfaces interfered with germ tube extension. Infection of lower leaf surfaces, and thick upper epidermis with underlying leaf anatomical structures, may explain the predominant colonization and sporulation on the abaxial surfaces.

Selection of cultivars tested in the present study was influenced by the availability of trees at local nurseries. Although none of the cultivars was immune to rust infections, differences were observed in the severity of infections, which may impact disease development and economic losses. Parisian, the dominant purple fig cultivar grown in SA, which accounts for about 75% of fresh fig production, and Ronde de Bordeaux, were intermediate in their rust responses. Deanna is a yellow peel-coloured cultivar grown especially in the North West Province. It is early and productive, with large fruit for the local market, and was less susceptible to rust in the present study. Mean rust severity on Black Genoa, which is grown in many countries under names such as San Piero, California Brown Turkey, Roxo de Valinhos, Black Jack, and Negro Largo, was significantly less than on most other cultivars. In the present greenhouse study, cultivar responses were assessed as the number of pustules per unit leaf area. However, the severity scale developed by Da Silva *et al.* (2019) provided a useful resource should rust assessments be expanded to field trials. The present results have shown that routine methodology to study rust pathosystems of field crops is equally applicable for fig rust research, and has potential for further application in horticultural systems. Rogovski Czaja *et al.* (2021) also obtained successful *C. fici* infection using a fig leaf disc technique as well as spray-inoculation of 1-year-old potted fig plants.

Control of diseases on figs is hampered by the lack of coordinated research including data on the occurrence and economic importance of a disease such as rust. Furthermore, fresh figs produced for export must adhere to pesticide residue limits which are strictly imposed by importing countries. At present, SA is excluded from exporting to the biggest markets of the USA and China until regulatory protocols have been established, due to the phytosanitary requirements for these countries. These regulations and the relatively small area planted with commercial figs in SA have contributed to the scarcity of relevant published information on pests and pathogens on fig, as well as almost non-existence of chemicals registered to for their control. Advice on chemical control of fig rust to commercial producers and residential fig growers is currently limited due to a lack of registered products in SA. Kanguard 940™, labelled as a contact fungicide and containing plant organic acids as active ingredients, is the only disease control product registered on figs in SA (https://www.agri-intel.com; http://www.kannar.co.za; accessed 26 October 2020). Spray programmes involving this product require preventative application at 5 d intervals and good coverage of plants. However, the efficacy of this product has not been confirmed against *P. nishidana* and requires further evaluation along with other fig rust management options.

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