A New Canker Disease of Apple, Pear, and Plum Rootstocks Caused by *Diaporthe ambigua* in South Africa

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ABSTRACT

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Diaporthe ambigua was found to be the cause of a newly recognized disease of apple, pear, and plum rootstocks in South Africa. The fungus was isolated from margins of cankers on rootstocks and branches of diseased trees, and from spores taken from perithecia and pycnidia imbedded in cankers. Characteristic symptoms included sunken, pointed lesions with marginal longitudinal cracks. Key identifying characters were perithecia, separate or in groups, with elongated necks protruding from the bark under moist conditions, and stromata delimited at the outer margins by broad, blackened zones. Pathogenicity tests were conducted on 3-year-old apple, pear, and plum rootstocks. Vegetative compatibility (VC) groups were identified by pairing isolates on oatmeal agar, and the sexual system was studied by inoculating single ascospores onto sterile apple twigs on water agar medium. *D. ambigua* was consistently associated with cankers on apple, pear, and plum rootstocks, and testing of Koch's postulates demonstrated its pathogenicity conclusively. The fungus was found to be homothallic. In addition, isolates from one rootstock tended to be of the same VC group, whereas those from adjacent rootstocks usually represented different genetic entities.

Additional keywords: *Malus domestica, Prunus salicina, Pyrus communis*

The South African apple, pear, and plum industry earned more than R800 million ($280 million) during the 1992 to 1993 season (1). Most of the South African deciduous fruit crop is marketed outside Africa. Fruit-growing is a long-term process and fruit growers cannot afford the large, direct-yield losses that result from having to replace orchards lost to diseases. Deciduous pome and stone fruit crops grown in South Africa are attacked by canker diseases caused by bacterial as well as fungal pathogens. Bacterial canker is one of the most important diseases in the deciduous fruit industry (6). Canker diseases caused by fungi have been considered less important in the past.

In 1989, a die-back of pear and plum rootstocks associated with cankers was noted by growers in several commercial pear (*Pyrus communis* L.) and plum (*Prunus salicina* Lindl.) orchards in the towns of Ceres and Villiersdorp in western Cape Province, South Africa. Perithecial stromata were present on the cankers and single-ascospore isolates were identical in colony morphology to isolates obtained from infected host tissue at the canker margins. The first infected apple (*Malus domestica* Borkh.) rootstocks were found in a Simondium nursery in 1990. It was tentatively determined that the canker disease of apple, pear, and plum rootstocks was caused by a species of *Diaporthe*.

The aim of the study was primarily to determine the cause of the disease. In addition, the virulence of a wide range of isolates on apple, pear, and plum was compared. *Diaporthe* isolates from diseased rooibos tea (*Aspalathus linearis* (Burm. f.) R. Dahlgren), a native plant used for production of a herbal tea, were included to establish their role in the etiology of the disease.

MATERIALS AND METHODS

Orchard canker sample collection. In the winter and spring of 1989 and 1990, pome (apple; pear) and stone (plum) fruit orchards and rooibos tea plantations were visited, and rootstocks with cankers were collected and stored at 4°C in a cold room for 1 to 3 months. Fungal fruiting bodies present on the cankers were sectioned with a surgical blade, mounted in lactophenol or water, and examined with a light microscope. Identifications were based on fruiting body and spore characteristics.

Isolation of fungi. Cankers were surface disinfested with 90% ethanol and flamed, and the bark was removed to expose the underlying cambial and cortical tissues. Segments (2 × 2 mm) were cut from the margin of each canker and plated in petri dishes on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI). All isolates were cultured on PDA at 25°C in the dark. Single-spore isolates of *Diaporthe* (Table 1) were induced to sporulate by placing them on sterile twigs of apple rootstocks placed on the surface of 2% water agar (WA) in petri dishes, sealing with Parafilm, and incubating for 7 days at 25°C in the dark. The cultures were subsequently incubated at 20°C while exposed to illumination (8 h per day) with mixed cool-white fluorescent and near-UV lights held 400 mm above the plates, and observed at regular intervals for up to 21 weeks after inoculation. Vegetative compatibility (VC) groups were identified by pairing isolates on freshly prepared oatmeal agar (17).

For isolation of mature ascospores, individual perithecia were horizontally dissected, and the inner centrum macerated in sterile, distilled water. Dilute spore suspensions were washed onto the surface of WA plates or WA acidified to pH 4.5 with 85% lactic acid. Prior to incubation, the plates were shaken to remove accumulated moisture and incubated in an inclined position for 24 to 36 h at 25°C. Small agar squares containing a single germinating ascospore were lifted with a sterile needle and transferred to individual petri dishes containing PDA. Three single-ascospore isolates (Table 1) were used in both the preliminary and pathogenicity trials. Five additional single-ascospore isolates were used in the pathogenicity trial. A single-conidium isolate (GR214(S) from a pycnidial cirrus was also used in the pathogenicity trial.

Inoculum production. For inoculation of plant material, wooden cocktail sticks were boiled three times for 20 min each in changes of distilled water, blotted dry, and autoclaved for 20 min at 121°C. The wooden sticks were then placed on the surface of PDA in petri

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dishes, and inoculated with single-spore isolates of *Diaporthe*. Plates were sealed with Parafilm and incubated for 10 days at 25°C in the dark. The cultures were subsequently incubated at 20°C, while exposed to illumination (12 h per day) with mixed cool-white fluorescent and near-UV lights held 400 mm above the plates.

Pathogen identification. To identify the *Diaporthe* sp. associated with cankers on apple, pear, plum, and rooibos tea rootstocks, we compared the morphology of specimens with the descriptions of Wehmeyer (21) and Uecker (19). Perithecial stromata of the different isolates (originating from apple, pear, plum, and tea rootstocks) were hand sectioned and 100 asci and ascospores per isolate were measured.

Isolates were also compared by random amplified polymorphic DNA (RAPD) analysis (22). This involves the enzymatic amplification of genomic DNA, directed by a single primer of arbitrary sequence, under low-stringency polymerase chain reaction conditions. Cultures were grown on sterile cellophane disks on malt extract agar (20 g/liter). The disks, covered with mycelia, were removed from the plates and lyophilized. DNA was extracted from the dried mycelium by the method described by Viljoen et al. (20). The study included duplicate DNA preparations from different batch cultures of the same isolate to ensure the reproducibility of the results. Amplification reactions were performed as described by Williams et al. (22). The optimum $MgCl_2$ concentration was found to be 2.5 mM and this was used for all DNA amplifications. Amplification reactions were done with a Hybaid Thermal Cycler (Hybaid Limited, Middlesex, UK). An initial denaturation was done at 96°C for 5 min, followed by 35 cycles of 92°C denaturation for 15 s, and 34°C annealing for 1 min; thereafter, the temperature was increased at a rate of 1°C per 1.5 s to 72°C. The reaction was then allowed to proceed for 2 min. The reaction was completed with a 72°C chain elongation for 5 min. Ten 10-mer oligonucleotides of random sequence, with a G+C content of 60 to 70% (Operon Technologies, Alameda, CA) were tested. From these, two primers (OPA9, sequence 5′ GGGTAACGCC 3′; OPA10, sequence 5′ GTGATCGCAG 3′) were selected for comparison of *Diaporthe* reference isolates (Table 2) and South African isolates of *Diaporthe* (Table 1). A negative control sample, to which no DNA was added, was set up in parallel with every batch of samples that were amplified to monitor the possibility of contamination.

The DNA amplification products were separated on 5% polyacrylamide gels and silver stained (14) to visualize the DNA. The images of the gels were scanned with the Gel Manager program (BioSystematica, Prague, Czechoslovakia).

Pathogenicity tests: Preliminary trial. Canker initiation and symptom expression due to infection by *Diaporthe* isolates were tested on 5-year-old potted, pruned M793 apple, BP-1 pear, and Marianna plum rootstocks, respectively. Inoculations were conducted on the property of INFRUITEC, Stellenbosch, during September (spring) of 1991. Forty rootstocks of each cultivar (10 rootstocks per isolate) were selected randomly and wounded on the main branch between the two lowest lateral branches. Holes, 2.8 mm in diameter, were drilled into the individual main branches (trunk diameter about 30 to 50 mm) and wooden sticks colonized by the test fungi were forced into the holes and the protruding ends were broken off. Sterile wooden sticks were forced into the holes drilled into the individual shoots of 10 rootstocks of each cultivar. The rootstocks were grown under normal greenhouse conditions. Canker initiation and symptom expression were recorded after 24 months.

Pathogenicity tests: Field trial. Inoculations were conducted in a Simondium nursery in the southwestern Cape Province of South Africa at the end of March (fall) of 1993. The experiment was done in an unpruned stand of 3-year-old apple (M793; M25), pear (BP-1; BP-3), and plum (Marianna) rootstocks planted 20 cm apart. One hundred rootstocks of each cultivar were selected randomly and wounded 100 mm above soil level on the main stem. Holes, 2.8 mm in diameter, were drilled into the individual stems and wooden sticks colonized by the test isolate were forced into the holes and the protruding ends broken off. Each of nine isolates of *Diaporthe* was inoculated into 10 rootstocks of each cultivar (50 wounds per isolate). Sterile wooden sticks were forced into the holes drilled into the individual shoots of 10 rootstocks of each cultivar. After 3 months, bark and phloem surrounding inoculation points were removed with a scalpel and the length of lesions surrounding the inoculation points was measured. Isolations were made from discolored tissue surrounding all inoculation points to test for the presence of the inoculated fungus.

The field pathogenicity trial was arranged as a completely randomized design

Table 1. South African isolates of *Diaporthe*, and mean length of cankers on BP-1 and BP-3 pear, M25 and M793 apple, and Marianna plum rootstocks 3 months after inoculation with nine isolates of *Diaporthe ambigua* from fruit trees and tea

		Mean canker length (mm)[a]				
		Apple		Pear		Plum
Diaporthe isolate	Cultivar of origin	M793	M25	BP-1	BP-3	Marianna
	Apple					
GR214(S)[b]	M793	88.2	79.3	67.7	72.5	91.1
GR214(A)[c]	M793	90.6	82.5	75.6	74.3	91.8
OR8[c]	M793	...	...	...	...	...
OR15[c]	M793	...[d]	...	...	...	...
MMC9[c]	M25	71.3	66.2	59.8	64.6	88.4
GRX8[c]	Granny Smith	92.5	76.7	73.6	71.8	96.7
GRX9[c]	Granny Smith	...	...	...	...	...
GRX11[c]	Granny Smith	...	...	...	...	...
	Pear					
EFB11[c]	BP-1	70.3	60.5	67.8	59.9	75.7
FBC2[c]	BP-3	75.0	79.7	62.0	72.2	86.1
GRX18[c]	Winter Nelis	83.7	85.0	65.8	66.9	82.3
	Plum					
HP103[c]	Marianna	79.1	72.3	78.9	71.7	92.9
	Tea					
RTC5[c]	Nortier	80.2	71.6	75.9	87.2	81.4
RTC14[c]	Nortier	...	...	...	...	...
RTC19[c]	Nortier	...	...	...	...	...
Control		0.3	0.3	0.3	0.2	0.2

[a] Least significant difference (LSD) ($P = 0.01$) = 10.06; LSD ($P = 0.05$) = 7.65. Mean square error (MSE) = 76.07235 with 405 df.
[b] Single-conidium isolate.
[c] Single-ascospore isolate.
[d] Inoculations not made.

Table 2. *Diaporthe* reference cultures used in random amplified polymorphic DNA analysis

Organism	Source of isolate[a]
D. ambigua Nitschke	CBS 134.42
D. batatatis Harter & Field	CBS 122.21
D. conorum (Desmazières) Niessl	CBS 186.37
D. eres Nitschke	CBS 585.69
D. medusaea Nitschke	CBS 102.81
D. perniciosa Marchal f. sp. *pruni* Cayley	CBS 111.23
D. phaseolorum (Cooke & Ellis) Sacc. var. *caulivora* Athow & Caldwell	CBS 178.55
D. phaseolorum (Cooke & Ellis) Sacc. var. *sojae* (Lehman) Wehmeyer	CBS 180.55

[a] CBS refers to the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

with 50 treatment combinations; each combination had 10 replications. The treatment combinations consisted of a 10 × 5 factorial design arising from the combination of nine *Diaporthe* isolates plus a control and five rootstocks. An experimental unit consisted of a cutting with a single stem.

Standard 9 × 5 factorial (without the control treatment) analysis of variance was done on canker lengths by SAS 6.08 statistical software. Student's *t* (least significant difference) test was used to compare treatment means (16).

RESULTS

Identification. The morphologic features of the *Diaporthe* sp. associated with cankers on apple, pear, and plum were consistent with those described for *Diaporthe ambigua* Nitschke, *Diaporthe perniciosa* Marchal f. sp. *pruni* Cayley, and *Diaporthe eres* Nitschke (21). Asci were clavate, eight-spored, 35 to 44 × 5 to 7.5 µm in size, detached, and free floating in the centrum at maturity. Ascospores were 10.5 to 13 × 2.5 to 4 µm (average 12 × 3.5 µm) in size, hyaline, biseriate, ellipsoidal, slightly or not constricted at the single septum, and pointed at the ends (Fig. 1). The fungus was homothallic and perithecia formed on rootstocks inoculated with single ascospore isolates. Isolates from one rootstock tended to be of the same group.

The results of the RAPD analysis with the two selected primers, OPA9 (Fig. 2A) and OPA10 (Fig. 2B), clearly showed that the *Diaporthe* isolates isolated from apple (GR214(A); OR8; OR15; MMC9; GRX8; GRX9; GRX11), pear (EFB11; FBC2; GRX18), plum (HP103), and tea (RTC5; RTC14; RTC19) cultivars in South Africa (Table 1) grouped together with reference isolates of *D. ambigua*, *D. perniciosa* and *D. eres* (Table 2). The *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. var. *caulivora* Athow & Caldwell, *D. phaseolorum* (Cooke & Ellis) Sacc. var. *sojae* (Lehman) Wehmeyer, and *Diaporthe batatatis* Harter & Field reference isolates grouped together, and were clearly different from the first group. The *Diaporthe conorum*

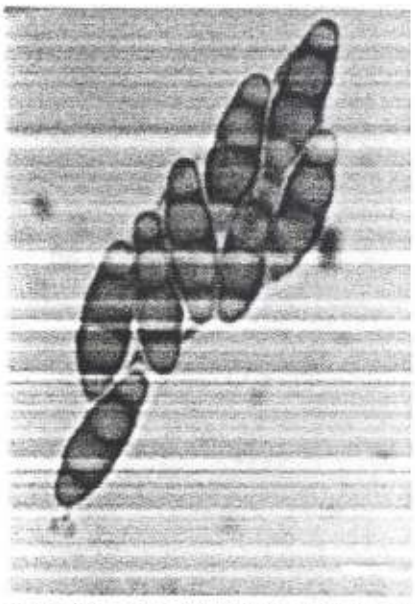

Fig. 1. Guttulate ascospores from mature perithecia of *Diaporthe ambigua* isolate EFB11 (bar = 3 µm).

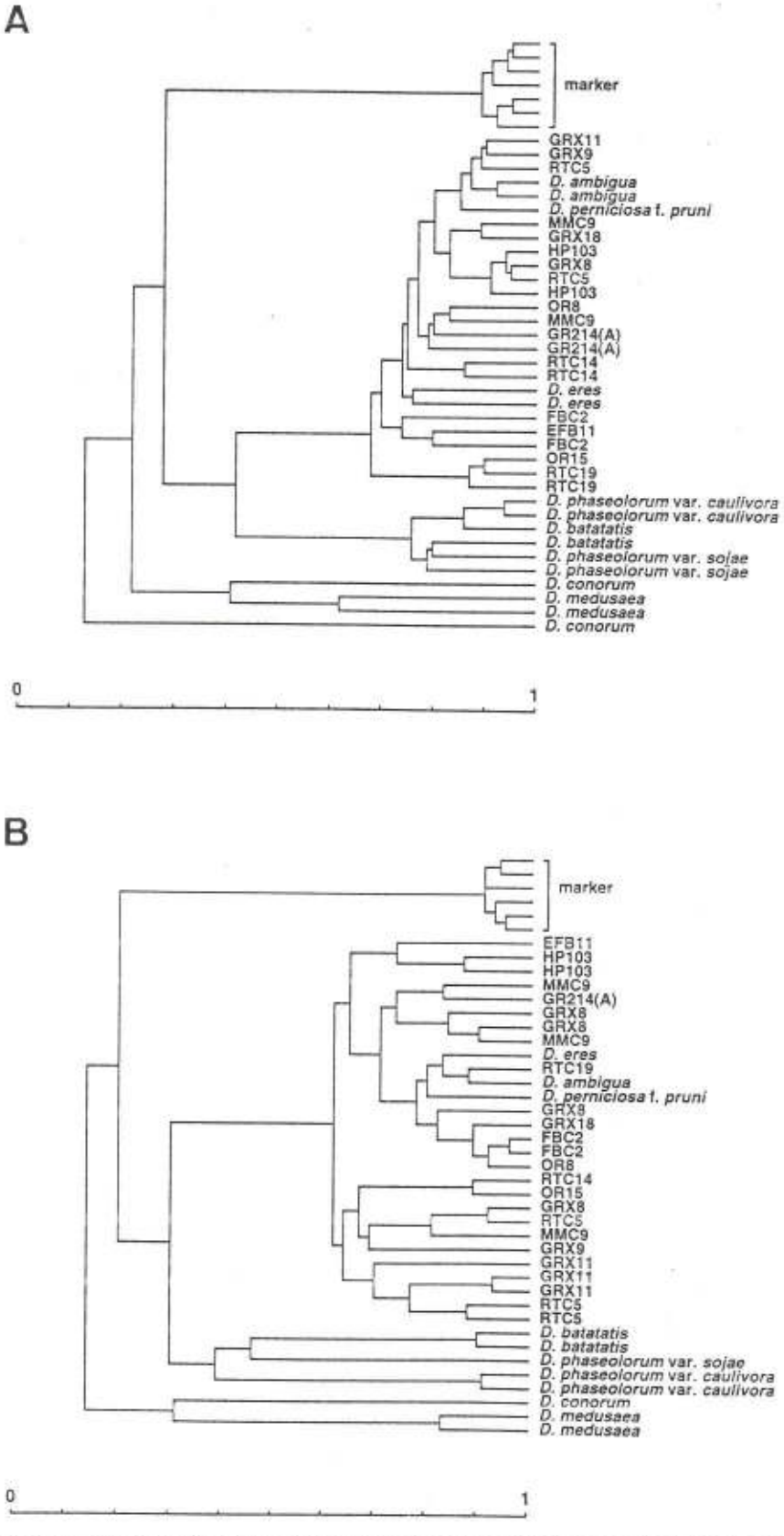

Fig. 2. (**A** and **B**) Analysis of the random amplified polymorphic DNA data generated with two primers, (**A**) OPA9 and (**B**) OPA10, for amplification of South African isolates of *Diaporthe ambigua* and *Diaporthe* reference isolates described in Tables 1 and 2.

(Desmazières) Niessl and *Diaporthe medusaea* Nitschke reference isolates, however, formed a group distinct from the first two, which were more similar to each other than to the third.

Pathogenicity: Preliminary and field trials. The symptoms observed on nursery-infected rootstocks and those previously observed on mature, orchard-grown rootstocks were similar. However, the nursery-grown rootstocks were attacked in a manner that differed from that of mature rootstocks. *Diaporthe* firstly infected the crown of nursery-grown rootstocks, then spread to the upper stem as well as the root system. Nursery-grown rootstocks infected by *Diaporthe* were readily killed, as opposed to mature, orchard-grown rootstocks, which normally were killed over an extended period.

Characteristic symptoms on 5-year-old apple, pear, and plum rootstocks included sunken, pointed lesions with marginal longitudinal cracks (Fig. 3A,B). Perithecia developed in dead wood. The control treatments resulted in no canker development.

A significant ($P < 0.001$) isolate × rootstock interaction was found. Interpretation of the main effects would therefore be inappropriate (11). The interaction means of canker length indicated that isolates of *D. ambigua* reacted differently on the various 3-year-old rootstocks (Table 1; Fig. 4). The control treatment resulted in no canker development, and was excluded in further analyses. There was no evidence against normality ($P = 0.3529$), and therefore no need for transformation.

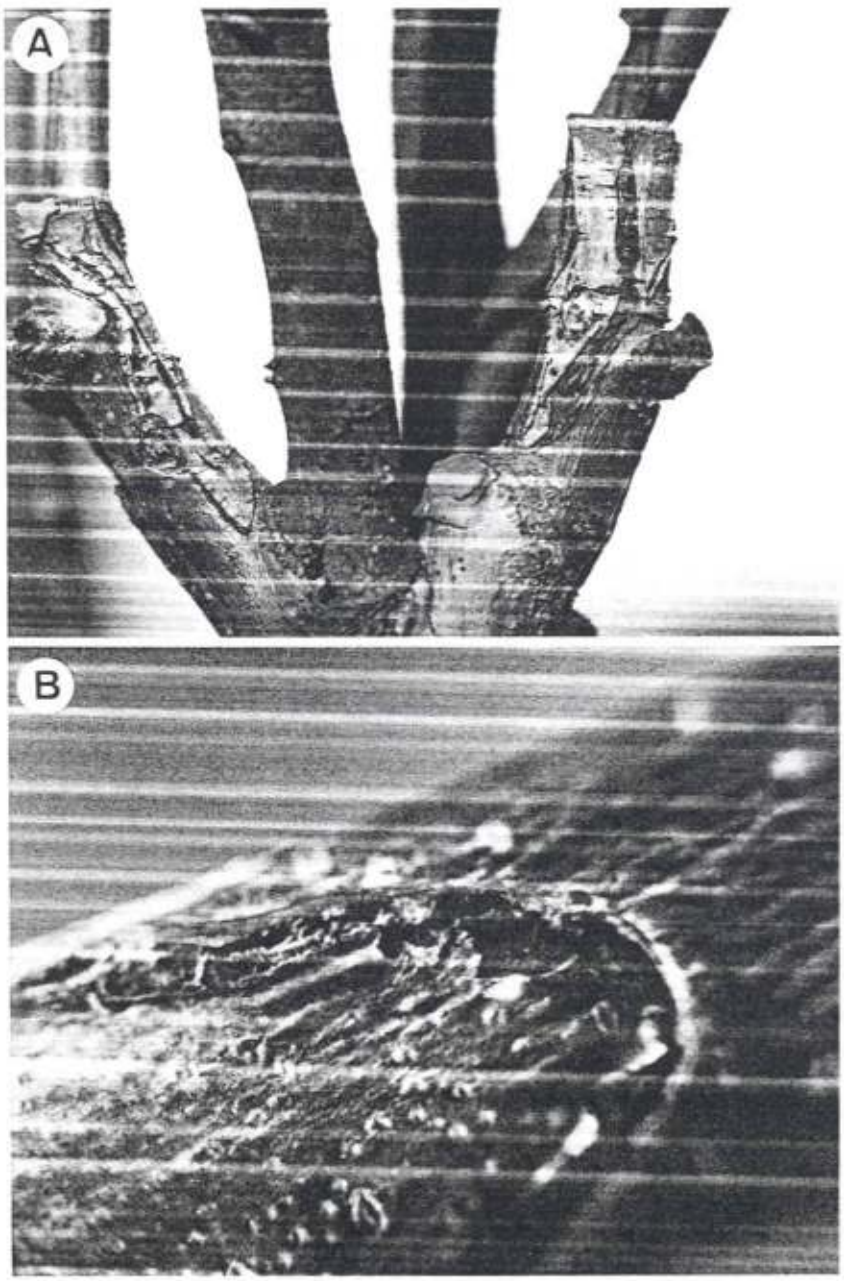

Fig. 3. (A and B) Canker of pear rootstocks 24 months after inoculation with isolate EFB11 of *Diaporthe ambigua*. Characteristic symptoms included sunken, pointed lesions with marginal longitudinal cracks.

DISCUSSION

This study has shown that the causal agent of the canker disease of apple, pear, and plum rootstocks in South Africa is a species of *Diaporthe*. Wehmeyer (21) considered *D. ambigua* (described in 1867) and *D. perniciosa* (described in 1921) synonymous with *D. eres* (described in 1867). However, several researchers have reported *D. perniciosa* in association with die-back diseases of apple, pear, plum, and peach (2,5,10). The RAPD analysis of *Diaporthe* isolates, with different primers of arbitrary sequence, is supportive of Wehmeyer's grouping of *D. ambigua*, *D. perniciosa*, and *D. eres*. The taxonomy of the *Diaporthe* sp. on pome and stone fruit trees in South Africa needs further investigation, but this must await a thorough treatment of the genus *Diaporthe*. Over-reliance on host association has led to the establishment of a plethora of *Diaporthe* spp. (12). We foresee that sequencing studies will make synonymous many of the various *Diaporthe* spp. described on different hosts worldwide. We believe that the most appropriate name to use for the fungus is *D. ambigua*. The identity of the fungus is supported by morphological data of South African isolates of *Diaporthe*.

All *D. ambigua* isolates were found to be homothallic and fertile perithecia occurred abundantly on previously sterilized rootstocks inoculated with single-ascospore isolates. In addition, isolates from one rootstock tended to be of the same VC group, whereas those from adjacent rootstocks usually represented different genetic entities. Analysis of VC groups in populations of filamentous Ascomycetes has been used to assess whether a pathogen has been recently introduced into an area or whether it has been present for an extended period of time (4). In the case of *D. ambigua*, it would be difficult to draw such conclusions without knowledge regarding the regulation of the sexual cycle. The basis of sexuality in *D. ambigua* clearly needs further study.

Nursery rootstocks infected by *D. ambigua* were readily killed. This is in contrast to mature rootstocks, which normally are killed over an extended period. Although they did not monitor nursery infections, Nakatani et al. (8) and Fujita et al. (3) reported similar results for mature apple and pear trees inoculated with *Diaporthe tanakae* Kobayashi & Sakuma in Japan. Their results indicated that typical lesions generally appeared only 2 years after infection, explaining the absence of symptoms on current and biennial shoots in the field.

Characteristic symptoms of *D. ambigua* infections included sunken, pointed lesions with marginal longitudinal cracks. Similar symptom expression was reported for ap-

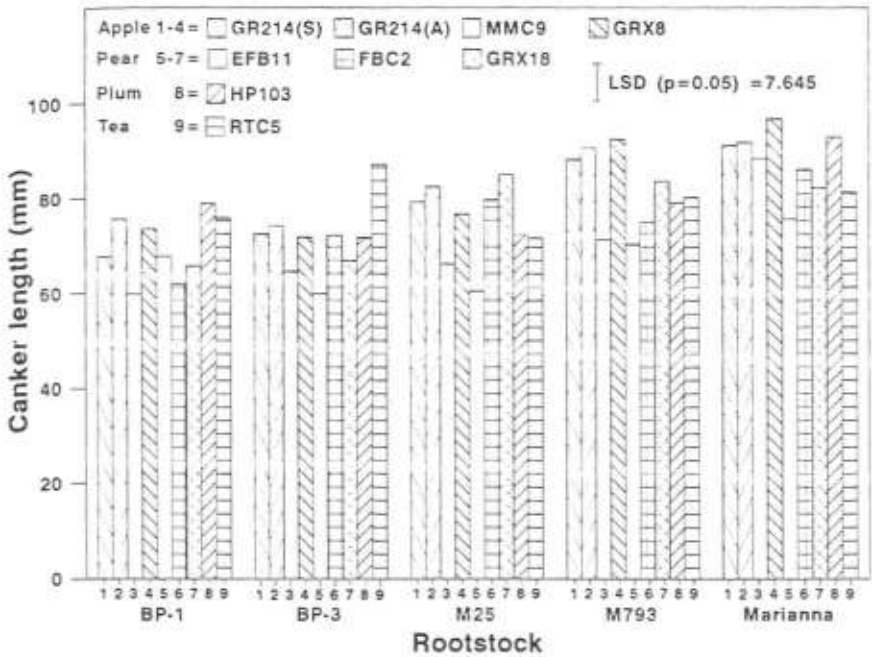

Fig. 4. Interaction means of canker length 3 months after wound inoculation with nine isolates of *Diaporthe ambigua*.

ple and pear cultivars infected by *D. tanakae* in Japan (3,7,9,13,18), and plum cultivars infected by *D. perniciosa* in Britain (2,5).

A significant ($P < 0.001$) isolate × rootstock interaction was found, indicating that isolates of *D. ambigua* reacted differently on various rootstocks. However, all deciduous fruit cultivars tested were susceptible to *D. ambigua* infection. This is in contrast to results of Fujita et al. (3), in which *D. tanakae* isolates from apple infected cuttings of both apple and European pear cultivars through wounds, whereas their isolates from pear cultivars infected only European pear shoots. Furthermore, their inoculations of unwounded shoots in the field indicated that *D. tanakae* isolates from apple infect apple cultivars, and those from European pear infect pear cultivars.

The canker pathogen on rooibos tea, previously identified as *D. phaseolorum* (15), is morphologically identical to the *Diaporthe* sp. isolated from pome and stone fruit rootstocks in this study. Furthermore, all isolates, including those from rooibos tea, clustered together in RAPD analyses. The *Diaporthe* sp. from rooibos tea was also pathogenic to all apple, pear, and plum cultivars tested. Rooibos tea is indigenous to South Africa, and geographically separated from pome and stone fruit cultivation. We consider the *Diaporthe* sp. on rooibos tea to be *D. ambigua*. We also believe the *D. ambigua* that infects fruit trees in South Africa is native in the country and that it probably originated from native woody plants such as rooibos tea. Population studies, particularly at the molecular level, are required to clarify this hypothesis.

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