Reduction of Laccase Activity and Other Hypovirulence-Associated Traits in dsRNA-Containing Strains of *Diaporthe ambigua*

W. A. Smit, B. D. Wingfield, and M. J. Wingfield

First author: Department of Plant Biotechnology and Pathology, Stellenbosch Institute for Fruit Technology (INFRUITEC), Private Bag X5013, Stellenbosch 7599, South Africa; and second and third authors: Department of Microbiology & Biochemistry, University of the Orange Free State, Bloemfontein 9300, South Africa.
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ABSTRACT

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A single double-stranded RNA (dsRNA) segment was detected in hypovirulent but not in virulent strains of *Diaporthe ambigua* isolated from apple rootstocks in South Africa. To test for phenol oxidase activity and gallic acid oxidation (Bavendamm's tests), the strains were grown on malt extract agar containing tannic and gallic acid, respectively. Laccase and peroxidase activities were determined with 2,6-dimethoxyphenol as substrate. Oxalic acid production in virulent and hypovirulent strains was determined by ultraviolet spectrophotometric analysis of NADH. Conversion of virulent strains was achieved by pairing hypovirulent and virulent strains on dialysis membrane on the surface of Czapek-Dox agar. Pathogenicity tests were conducted on 3-year-old M793 and M25 apple rootstock cultivars. In both Bavendamm's tests, virulent strains produced a strong color reaction, whereas hypovirulent strains showed weak or no activity. The enzyme responsible for the color reaction on Bavendamm's medium was identified as phenol oxidase of the laccase type. dsRNA could be transmitted to strains of the same vegetative compatibility group by hyphal anastomosis. Converted strains lost virulence and showed loss of phenol oxidase activity, reduced gallic acid oxidation, diminished oxalic acid accumulation, and suppressed sporulation. From these studies, we conclude that dsRNA was transferred to virulent strains via hyphal anastomosis. This resulted in hypovirulence as tested in the field, as well as reduction of laccase activity and other hypovirulence-associated traits.

Additional keyword: fungal virus.

Many important agricultural crops are affected by Diaporthe canker worldwide (19,23,29,41). *Diaporthe ambigua* Nitschke is causing a newly recognized disease of apple, pear, and plum rootstocks in South Africa. Differences in pathogenicity among strains of *D. ambigua* have been observed, and different morphological variants of this fungus have been seen in culture (W. A. Smit, *unpublished data*).

The presence of endogenous virus-like double-stranded RNA (dsRNA) genetic elements has been correlated with altered virulence in numerous plant-pathogenic fungi (44). dsRNAs serve as biocontrol agents by virtue of their ability to convert compatible virulent, virus-free strains of *Cryphonectria parasitica* to hypovirulence (reduced virulence) after hyphal anastomosis (3). DNA-mediated–transformation studies provided the first direct evidence that viral RNA is responsible for the hypovirulence phenotype (14,15). A specific viral coding domain that is responsible for several hypovirulence-associated traits also has been identified (15).

Laccase, a copper-containing phenol oxidase (34), is an enzyme that oxidizes a large variety of organic substrates (9,11,12). The characteristics of purified laccases differ in various aspects, and multiple forms (isoenzymes) of laccases are often produced by a single species of a fungus (9). This enzyme is widely distributed in fungi (6,21,30,38,40).

The laccase of *C. parasitica* recently has attracted interest because it is one of a number of fungal gene products (including sporulation, pigmentation, oxalate accumulation, cellulase, and laccase activity) that is specifically suppressed in the presence of a dsRNA virus (1,2,18,25,46). Although the mechanism by which a hypovirulence-associated virus can mediate a diverse array of suppressive activities remains unclear (48), some progress has been made in understanding the basis of dsRNA-mediated suppression of laccase accumulation (13,45,47). Larson and Nuss (32) provided evidence suggesting L-dsRNA (the large viral dsRNA isolated from hypovirulent *C. parasitica* strain EP713) mediates suppression of laccase accumulation at the transcription level by interfering with an intracellular inositol triphosphate/calcium-dependent signal transduction pathway. Because laccase gene transcript levels are suppressed by the hypovirulence-associated virus carried by strain EP713, the attenuation of laccase gene expression may serve as a model system for understanding the origin of many hypovirulence-associated traits (31).

In this study, we report on the detection of dsRNA in hypovirulent strains of *D. ambigua* isolated from apple rootstocks in South Africa. We also present evidence that the dsRNA affects laccase activity, the oxidation of gallic acid, the accumulation of oxalic acid, and cultural characteristics, including sporulation and pathogenicity.

MATERIALS AND METHODS

Fungal strains and growth conditions. Virulent and hypovirulent strains of *D. ambigua* were isolated from naturally occurring cankers on M793 apple (*Malus domestica* Borkh.) rootstocks in the Simondium area of Cape Province, South Africa. Strains were isolated and routinely grown on Difco (Detroit) potato dextrose agar (PDA). All strains were sporulated on sterile twigs of M793 and M25 apple cultivars placed on the surface of 2% water agar in petri dishes, sealed with Parafilm, and incubated for 7 days at 25°C in the dark. The cultures were subsequently incubated at 20°C,

Corresponding author: W. A. Smit; E-mail address: adriaan@infruit.agric.za

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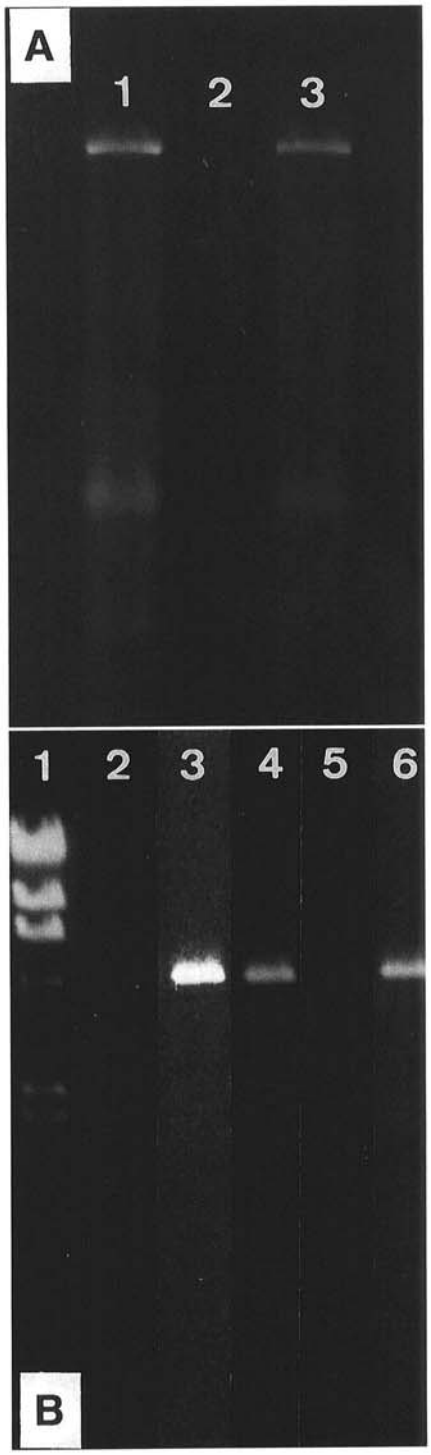

Fig. 1. Banding patterns and nuclease analysis of double-stranded (ds) RNA from *Diaporthe ambigua*. **A,** Agarose gel (1%) electrophoresis of dsRNA extracted from donor hypovirulent strain GR20 and recipient virulent strain GR231 of *D. ambigua* before and after hyphal anastomosis. Lane 1, dsRNA recovered from donor hypovirulent strain GR20; lane 2, absence of detectable dsRNA in recipient virulent strain GR231 before hyphal anastomosis with strain GR20; and lane 3, dsRNA in recipient strain GR231[GR20] after hyphal anastomosis with strain GR20. **B,** RNase A and DNase I treatments of dsRNA extracted from strain GR20 of *D. ambigua*. Lane 1, λ DNA cut with *Hin*dIII as molecular weight markers; lane 2, λ DNA digested with DNase I in 0.005 M $MgCl_2$ as a control treatment; lane 3, untreated dsRNA from strain GR20; lane 4, dsRNA from strain GR20 digested with RNase A in 0.3 M NaCl; lane 5, dsRNA from strain GR20 digested with RNase A in 0.03 M NaCl; and lane 6, dsRNA from strain GR20 digested with DNase I in 0.005 M $MgCl_2$. Samples were electrophoresed through 1% agarose gels and stained with ethidium bromide.

exposed to 8 h of illumination with mixed cool-white fluorescent and near-ultraviolet lights held 400 mm above the plates per day, and observed at regular intervals for up to 21 weeks. *C. parasitica* strains EP713 (ATCC 52571, hypovirulent) and EP155 (ATCC 38755, virulent), obtained from the American Type Culture Collection, Rockville, MD, were used as controls in all tests, except for pathogenicity. All strains were lyophilized and stored at –20°C or maintained on PDA at –80°C as well as at 20°C in the dark.

Extraction, purification, and analysis of dsRNA. The initial screening for hypovirulence was achieved by growing strains on Difco Czapek-Dox agar (CDA). Hypovirulent strains were grown in liquid culture in Difco potato dextrose broth (PDB), either as static cultures at 20°C or on a rotary shaker at 25°C for 6 weeks in the dark. The dsRNA was extracted and purified by the methods of Morris and Dodds (42) and Valverde et al. (51) with minor modifications. Columns were made as described by Valverde and Fontenot (50), except that glass-wool–plugged, 50-ml plastic syringes were used. The dsRNA was concentrated by ethanol precipitation and electrophoresed through 1% agarose gel in 1× TBE (0.089 M

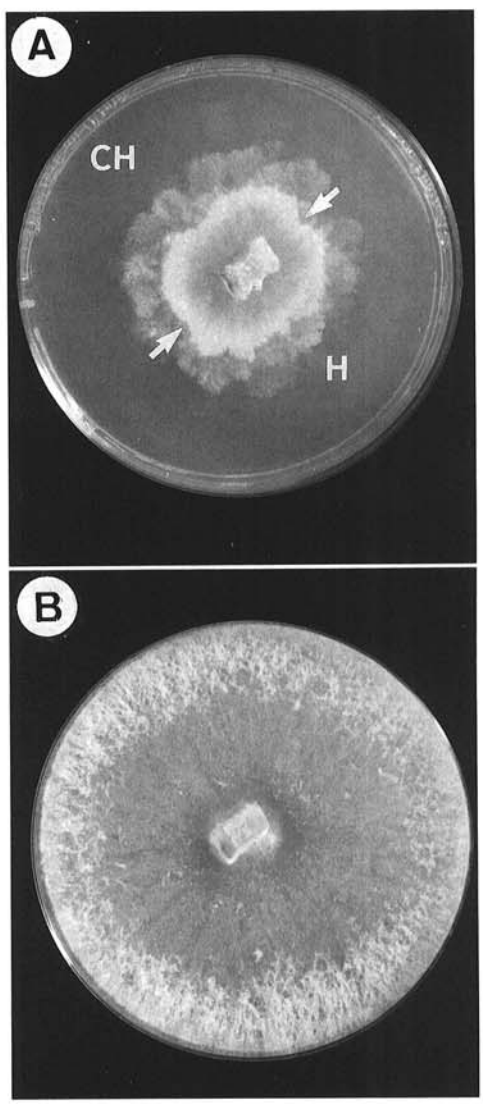

Fig. 2. Comparative morphology of virulent *Diaporthe ambigua* strain GR216 before and after anastomosis with *D. ambigua* hypovirulent strain GR20. **A,** H = hypovirulent, double-stranded (ds) RNA-containing strain GR20; CH = virulent, dsRNA-free strain GR216 after anastomosis with hypovirulent, dsRNA-containing strain GR20 (GR216[GR20]). **B,** Virulent, dsRNA-free strain GR216. All cultures were grown for 7 days on dialysis membrane on the surface of Czapek-Dox agar.

Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8) buffer at 40 V for 3 h. The gels were stained with ethidium bromide (0.17 µg/ml) and photographed in transmitted UV light.

All samples were treated with deoxyribonuclease I, ribonuclease-free (DNase I, RNase-free) (Boehringer, Mannheim, Germany) and ribonuclease A (RNase A) (Boehringer Mannheim) to remove contaminating DNA or single-stranded RNA, respectively. The sensitivity of dsRNA to nucleases was tested before and after gel electrophoresis. Before electrophoresis, sample aliquots were incubated for 30 min at 30°C with 10 µg of DNase I, RNase-free per ml in 1× STE (0.1 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, pH 6.8) buffer with 5 mM $MgCl_2$. RNase sensitivity of the dsRNA was tested by incubation for 30 min at 25°C with 0.5 µg of RNase A per ml at high (0.3 M NaCl) and low salt (0.03 M NaCl) concentrations. The samples were incubated with 2.5 µg of proteinase K per ml (Boehringer Mannheim) for 2 h at room temperature to destroy the RNase before electrophoresis. After electrophoresis, the gels were soaked with RNase A (50 µg/ml) prepared in 0.3 and 0.03 M NaCl, respectively, for 4 h at 37°C to determine the nature of the RNA.

Conversion of virulent strains. Wettable dialysis membrane was cut into round disks, boiled and autoclaved in distilled water, and placed on the surface of CDA in sterile petri dishes. All pairings were made on the surface of dialysis membrane to make conversions more easily visible and to facilitate recovery of converted mycelium. Pairings were made as described by Anagnostakis and Day (4). All pairings were made on the surface of dialysis membrane placed on the surface of CDA in sterile petri dishes (4). Two dsRNA-containing strains (GR7 and GR20) representing one vegetative compatibility group (VCG) were used to convert three dsRNA-free strains (GR216, GR228, and GR231) of the same VCG. Two dsRNA-containing strains from a different VCG (ORR7 and ORR17) were concurrently used to convert three dsRNA-free strains (ORR26, ORR27, and ORR41). Each of the strains, before and after conversion, was tested for the presence of dsRNA, phenol oxidase reaction, laccase activity, gallic acid oxidation, and oxalic acid production. The strains also were tested for sporulation and their ability to induce cankers.

Pathogenicity tests. Three-year-old apple rootstocks of M793 and M25 were used to determine pathogenicity of various dsRNA-free and original and converted dsRNA-containing *D. ambigua* strains under orchard conditions. Holes, 2.8 mm in diameter, were drilled into the stems of trees, wooden sticks colonized by the test mycelia were forced into the holes, and the protruding ends were broken off. Each strain was inoculated into 10 rootstocks of each cultivar at the end of March 1993. Canker length was measured after 12 weeks, and means were compared with the aid of Student's *t* LSD (49).

Bavendamm's phenol oxidase test. To test for phenol oxidase activity, strains were grown on Bavendamm's medium, containing 0.5% tannic acid adjusted with NaOH to pH 4.5, 1.5% Difco malt extract, and 2% Difco Bacto agar. Fungal strains were inoculated as plugs, 5 mm in diameter, cut from the growing edge of actively growing cultures on PDA. Plates were incubated at 25°C in the dark. The color of the agar medium was used as an indication of phenol oxidase activity (7,8).

For a crude enzyme preparation, Bavendamm's medium together with the mycelial mat of each of the test strains growing on this medium was cut into small pieces, extracted with sterile double-distilled water at 4°C for 30 min, and centrifuged for 3 min at 10,000 × *g*. The supernatants were assayed for enzyme activity (46).

Laccase and peroxidase activities were determined with 2,6-dimethoxyphenol (DMOP) as substrate (12). Enzyme extract (0.2 ml) was added to 0.8 ml of 2.5 mM DMOP in 100 mM phosphate

TABLE 1. Phenol oxidase reaction, laccase activity, gallic acid oxidation, oxalic acid production, sporulation, and mean canker length of strains of *Diaporthe ambigua* with and without double-stranded (ds) RNA

Strain[r]	dsRNA[s]	Phenol oxidase reaction[t]	Laccase activity (ΔA468/5 min)[u]	Gallic acid oxidation[v]	Oxalic acid production[w] (mg/liter)	Sporulation[x]	Canker length (mm)[y]	
							M793	M25
GR216	–	+	0.171	+	5.60	+	90.4 a	79.0 a
GR228	–	+	0.159	+	4.48	+	91.1 a	68.4 c
GR231	–	+	0.115	+	5.76	+	70.2 d	73.0 b
ORR26	–	+	0.138	+	5.52	+	85.3 b	69.0 c
ORR27	–	+	0.147	+	5.44	+	77.4 c	67.4 c
ORR41	–	+	0.098	+	4.96	+	89.2 ab	72.4 b
GR7	+	–	0.009	–	1.68	–	10.1 ef	4.8 f–h
GR216[GR7][z]	+	–	0.036	–	1.84	–	7.9 ef	5.7 e–h
GR228[GR7]	+	–	0.019	–	1.60	–	8.9 ef	5.2 e–h
GR231[GR7]	+	–	0.022	–	1.60	–	10.8 e	5.1 e–h
GR20	+	–	0.011	–	1.52	–	8.0 ef	6.3 d–h
GR216[GR20]	+	–	0.023	–	1.76	–	9.5 ef	6.5 d–g
GR228[GR20]	+	–	0.012	–	1.84	–	5.9 f	8.1 de
GR231[GR20]	+	–	0.010	–	1.84	–	7.3 ef	8.8 d
ORR7	+	–	0.014	–	1.92	–	9.4 ef	4.4 f–h
ORR26[ORR7]	+	–	0.021	–	2.00	–	8.4 ef	6.8 d–f
ORR27[ORR7]	+	–	0.020	–	2.16	–	8.3 ef	3.3 h
ORR41[ORR7]	+	–	0.019	–	2.08	–	6.3 f	4.6 f–h
ORR17	+	–	0.010	–	1.60	–	6.7 ef	3.6 gh
ORR26[ORR17]	+	–	0.015	–	1.68	–	6.3 f	5.6 e–h
ORR27[ORR17]	+	–	0.019	–	1.52	–	6.6 ef	5.6 e–h
ORR41[ORR17]	+	–	0.011	–	1.60	–	8.8 ef	5.5 e–h
LSD ($P = 0.05$)							4.39	3.01

[r] All strains were isolated from M793 apple rootstocks in one orchard.
[s] + = dsRNA detected; – = dsRNA not detected.
[t] Color reaction on Bavendamm's medium for phenol oxidase activity: + = reaction; – = vague or no reaction.
[u] Laccase activity at the edge of agar cultures grown on Bavendamm's medium. Relative activity per sample.
[v] + = color reaction; – = vague or no color reaction.
[w] NADH is determined at 340 nm; the amount of NADH formed is stoichiometric with the amount of oxalic acid.
[x] M793 and M25 apple twigs were exposed to 8 h of illumination with mixed cool-white fluorescent and near-ultraviolet light per day; + = sporulation; – = no sporulation.
[y] Mean canker length 12 weeks after inoculation in 3-year-old orchard-grown apple (*Malus domestica*) rootstocks, $n = 10$.
[z] Brackets designate a converted strain, e.g., strain GR216 is converted by strain GR7, etc.

buffer, pH 6.9, at 37°C. The increase in absorbance at 468 nm was measured continuously at 25°C. Enzyme activity was calculated as absorbance change per 5 min per sample.

Bavendamm's gallic acid test. Test strains were grown on malt extract agar containing 0.5% gallic acid adjusted to pH 4.5. The coloring of the agar media indicated oxidation of gallic acid (7,8).

Oxalic acid (oxalate) production. Strains were grown on PDA for 3 days. Agar disks, 5 mm in diameter, were cut from the cultures and placed in the center of empty 100-mm petri dishes. A standardized volume of PDB was added, and the culture was incubated at 25°C in the dark. Aliquots of culture fluid were removed 7 days after inoculation, and the oxalate content was measured. The UV method for the determination of oxalate was used (25). The test is based on the principle that oxalate is cleaved to formic acid (formate) and CO_2 at pH 5 in the presence of oxalate decarboxylase. The formate formed is quantitatively oxidized to bicarbonate by NAD at pH 7.5 in the presence of the enzyme formate dehydrogenase. The amount of NADH formed during the second reaction is stoichiometric with the amount of oxalate. The production of NADH, thus, was determined based on its absorbance at 340 nm with a Beckman DU 7500 spectrophotometer (Beckman Instruments, Fullerton, CA).

RESULTS

Extraction, purification, and analysis of dsRNA. All hypovirulent strains examined contained a single high molecular weight dsRNA with identical mobility in agarose gels (Fig. 1). The *D. ambigua* dsRNA comigrated with the 4.3-kb position on the λ DNA (*Hin*dIII digest) ladder. The exact size of the *D. ambigua* dsRNA has not yet been determined with RNA standards. The nature of the dsRNA was confirmed by its resistance to DNase I, resistance to RNase A at high (0.3 M) salt concentration, and susceptibility to RNase A at low (0.03 M) salt concentration (Fig. 1).

Conversion of virulent strains. dsRNA-containing strains of *D. ambigua* (GR7, GR20, ORR7, and ORR17) converted dsRNA-free strains of the same VCG to hypovirulence (Fig. 2; Table 1). dsRNA-free strains GR216, GR228, and GR231 were converted by dsRNA-containing strains GR7 and GR20, whereas dsRNA-free strains ORR26, ORR27, and ORR41 were converted by dsRNA-containing strains ORR7 and ORR17.

Pathogenicity and growth characteristics. *D. ambigua* strains were tested for pathogenicity under orchard conditions (Fig. 3; Table 1). The strains showing high virulence (GR216, GR228, GR231, ORR26, ORR27, and ORR41) contained no detectable dsRNA. These strains had normal cultural characteristics on CDA and sporulated readily on apple twigs. Furthermore, all virulent strains produced fertile perithecia and pycnidia, whereas hypovirulent and converted strains were incapable of sporulation and did not produce ascospores or conidia. The dsRNA-free strains were significantly ($P = 0.05$) more virulent than the nonconverted dsRNA-containing strains (GR7, GR20, ORR7, and ORR17) or dsRNA-recipient strains (GR216[GR7], GR228[GR7], GR231[GR7], GR216[GR20], GR228[GR20], GR231[GR20], ORR26[ORR7], ORR27[ORR7], ORR41[ORR7], ORR26[ORR17], ORR27[ORR17], and ORR41[ORR17]). The dsRNA-containing strains (original and converted) were consistently associated with hypovirulence, showed abnormal characteristics on CDA, and did not sporulate in vitro. The growth rate of dsRNA-containing strains on PDA and CDA was comparable to the growth rate of the dsRNA-free strains.

Phenol oxidase reaction/gallic acid oxidation. In both Bavendamm's tests, virulent strains produced a strong color reaction, whereas hypovirulent strains (original and converted) showed a slight or no reaction (Fig. 4; Table 1). The growth of the hypovirulent strains on Bavendamm's medium for phenol oxidase activity and Bavendamm's medium for gallic acid determination was comparable to the growth of the virulent strains.

Laccase activity. The enzyme responsible for the color reaction on Bavendamm's medium for phenol oxidase activity was identified as phenol oxidase of the laccase type. Because laccase activity has been correlated with the Bavendamm reaction in other fungi, DMOP, a substrate for laccase and peroxidase but not for tyrosinase, was used for the quantitative assay. Laccase activity was found beyond and at the advancing edge of the colony but not under the older mycelium. Because it was found at the advancing

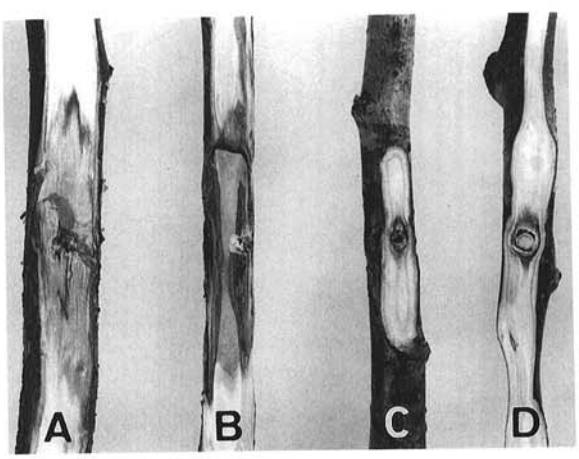

Fig. 3. Assessment of virulence and transmission of hypovirulence 12 weeks after inoculation of 3-year-old M793 and M25 apple rootstocks with isogenic strains of *Diaporthe ambigua*. **A,** M793 infected by double-stranded (ds) RNA-free strain ORR26. **B,** M25 infected by dsRNA-free strain ORR26. **C,** M793 infected by dsRNA-recipient (revertant) strain ORR26[ORR7]. **D,** M793 infected by dsRNA-containing strain ORR7.

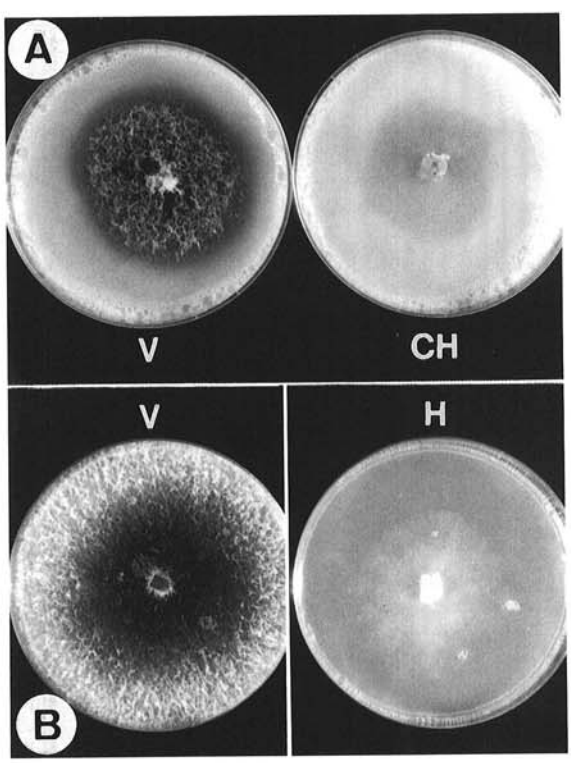

Fig. 4. Bavendamm's reactions of strains of *Diaporthe ambigua* after 7 days at 25°C in the dark. **A,** Phenol oxidase reaction of a virulent and converted strain of *D. ambigua* on Bavendamm's medium containing tannic acid. V = original double-stranded (ds) RNA-free, virulent strain GR216; CH = convertant strain GR216[GR20]. **B,** Gallic acid oxidation of a virulent and hypovirulent strain of *D. ambigua* on Bavendamm's medium containing gallic acid. V = original dsRNA-free, virulent strain GR216; H = dsRNA-containing, hypovirulent strain GR20.

edge of the colony and easily extractable from the agar, it was concluded that the laccase activity is extracellular (45).

To test the possibility that peroxidases were responsible for the phenol oxidase activity, catalase (90 units/ml) was added in the DMOP assay to destroy the peroxides (45). No decrease in activity was observed. No activity was detected with tyrosine as substrate. Virulent strains showed laccase activity (0.098 to 0.171) and were positive for gallic acid oxidation, whereas hypovirulent strains showed low levels of laccase activity (0.009 to 0.036) and tested negative for gallic acid oxidation (Table 1). Neither virulent nor hypovirulent strains showed peroxidase or tyrosinase activity.

Oxalate production. The virulent strains produced oxalate in the range of 4.48 to 5.76 mg/liter. Hypovirulent strains, as opposed to virulent strains, showed a reduction of at least 58% in the amount of oxalate produced (Table 1).

DISCUSSION

In this study, we demonstrated for the first time the occurrence of hypovirulence in *D. ambigua*. Hypovirulence was associated with the presence of a single dsRNA segment. In addition to reduced virulence, dsRNA-containing strains of *D. ambigua* also exhibited a number of hypovirulence-associated traits, including reduced phenol oxidase activity, reduced gallic acid oxidation, diminished oxalate accumulation, and suppressed sporulation. These distinguishing characteristics are consistent with data previously published for the related *C. parasitica* (1,2,17,18,24,25,46). However, the presence of dsRNA in fungi is not always associated with reduced virulence and hypovirulence-associated traits (44).

dsRNA-containing hypovirulent strains of *D. ambigua* were able to convert compatible virulent, virus-free strains of the same VCG to hypovirulence after anastomosis. Moreover, dsRNA-free strains of *D. ambigua* that were converted to hypovirulence exhibited the same composition of hypovirulence-associated traits as was displayed by the original dsRNA-containing hypovirulent strains. These results provide clear evidence that the dsRNA confers hypovirulence to strains of *D. ambigua* in South Africa. Furthermore, the characteristics of this hypovirulence are similar to those found in *C. parasitica* (1,4,35,43,52,53). dsRNA associated with the hypovirulence phenotype also have been reported for a number of other plant-pathogenic fungi, such as *Leucostoma persoonii* (22), *Ophiostoma ulmi* (reviewed by Nuss and Koltin [44]), and *Sclerotinia sclerotiorum* (10). Conversion of dsRNA-free strains to the hypovirulence phenotype is coincident with transmission of dsRNAs during anastomosis with compatible hypovirulent strains, providing the basis for biological disease control in *D. ambigua*.

Using Bavendamm's tests for phenol oxidase reaction and gallic acid oxidation, we found clear differences in oxidation between dsRNA-containing and dsRNA-free strains of *D. ambigua*. Bavendamm (7,8) pointed out differences between fungi with respect to their oxidative enzymes. According to Davidson et al. (16), most fungi that oxidize tannic acid also oxidize gallic acid. Although Bavendamm's tests have been applied mainly in Basidiomycete fungi (26), they were recently used to determine phenol oxidation in dsRNA-free and dsRNA-containing strains of *C. parasitica* (46) and evidently are applicable also in the case of *D. ambigua*.

The enzyme responsible for the color reaction in Bavendamm's test for phenol oxidase in this study was identified as phenol oxidase of the laccase type. In fungi, laccase activity has been suggested to be involved in degradation of lignin (5,28), pathogenesis (20,36,39), and sporulation (33). Therefore, it was not surprising that we found a reduction of laccase activity in hypovirulent dsRNA-containing strains of *D. ambigua*. Similar results have been found in the related fungus *C. parasitica* (13,25,46,47).

Laccase activity in dsRNA-free strains of *D. ambigua* was found at the advancing edges of colonies. This is an important requirement for an enzyme suggested to play a role in the infection process (46). In the related fungus *C. parasitica*, however, laccase activity is extracellular as well as intracellular, as shown by Rigling and Van Alfen (48). According to these authors, the extracellular laccase activity in *C. parasitica* temporally precedes the intracellular laccase, and the two activities are encoded by different genes. They also observed both laccases to be down-regulated by the dsRNA in a hypovirulent strain of *C. parasitica*, suggesting common regulatory factors for the separate laccase genes (47,48). Moreover, a third type of laccase recently has been discovered in *C. parasitica* (27). In *D. ambigua*, however, there is still much to learn concerning the basis of dsRNA-mediated regulation of the laccase gene(s) involved.

In this study we also found a reduction of oxalate accumulation in dsRNA-containing strains of *D. ambigua* compared to dsRNA-free strains of this fungus. Oxalate accumulation, which is potentially involved in pathogenesis, is suppressed in hypovirulent strains of *C. parasitica* (24,25,46). Variations in pathogenicity also are associated with oxalate in other fungi (37).

The combined results obtained from dsRNA-containing strains of *D. ambigua* suggest a specific viral coding domain that controls several hypovirulence-associated traits, as is found in *C. parasitica*. To understand the mechanism by which dsRNA perturbs fungal gene expression, knowledge of the host genes affected and their roles in fungal development and virulence is important. In addition, information concerning the molecular structure and coding potential of the dsRNA itself is required. Furthermore, the relatedness of dsRNAs associated with hypovirulence in North American and European strains of *C. parasitica* and *D. ambigua* strains of South African origin need to be examined at the molecular level. From such studies we would expect to obtain a better understanding of the traits originating from the presence of the hypovirulence-associated dsRNA in *D. ambigua*, as well as the mechanism underlying hypovirulence itself.

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