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Vegetative incompatibility in *Diaporthe ambigua*

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Vegetative compatibility of strains of *Diaporthe ambigua* has not previously been examined. Single ascospore and single ascus strains, originating from individual apple, pear and plum rootstocks, were paired on freshly prepared oatmeal agar to determine if vegetative incompatibility could be detected in *D. ambigua*. Barrage reactions were evident as black lines along the zone of mycelial contact between expanding colonies (vegetative incompatibility reaction). Strains from cankers within an area were of numerous vegetative compatibility groups (VCGs). Strains from adjacent rootstocks usually differed in VCG. *D. ambigua* has the ability to outcross, and does so, despite its apparent homothallic nature. DsRNA-containing strains of *D. ambigua* developed a broad, clear zone when paired with a dsRNA-containing strain from a different VCG.

INTRODUCTION

Diaporthe canker of apple, pear and plum rootstocks is an important disease affecting the longevity and productivity of orchards in the Cape Province of South Africa (Smit *et al*., 1994a). Symptoms of the disease include pointed lesions that are depressed and separated from the bark by longitudinal cracks along their margins. Die-back occurs as the fungus girdles the shoots. Tree death follows under conducive environmental conditions. The filamentous Ascomycete *Diaporthe ambigua* Nits., anamorphs *Phomopsis ambigua* (Sacc.) Trav. (Saccardo, 1915; Grove, 1917; Petrak & Sydow, 1936) and *Phomopsis mali* (Schulzer & Sacc.) Died. (Uecker, 1988), is known as a canker pathogen of apple and pear trees (Uecker, 1988). Other researchers have reported *Diaporthe perniciosa* Marchal (anamorph *Phomopsis mali* Roberts) in association with die-back diseases of apple, pear, plum and peach (Cayley, 1923; Nawawi Bin Hoji Ayub & Swinburne, 1970; Harris, 1988). Wehmeyer (1933), however, considered *D. ambigua* (described in 1867) and *D. perniciosa* (described in 1921) as synonyms of *Diaporthe eres* Nits. (described in 1867). The taxonomy of this fungus is currently uncertain, and under investigation using molecular techniques (Rehner & Uecker, 1994). For the purpose of this study we will refer to the causal agent of *Diaporthe* canker on pome and stone fruit trees as *D. ambigua*.

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Vegetative or heterokaryon incompatibility is known to limit heterokaryosis in filamentous fungi. Cayley (1923) discussed the phenomenon of mutual aversion between monospore mycelia of *D. perniciosa*. The role of heterokaryosis in the biology of fungi, however, is still in question (Glass & Kuldau, 1992). In most cases, hyphae fuse but the resulting heterokaryotic cells are rapidly destroyed through a degenerative and lytic reaction. This incompatibility results from genetic differences at specific loci (Leslie, 1993).

The presence of endogenous virus-like double-stranded RNA (dsRNA) genetic elements has been correlated with altered virulence in numerous plant pathogenic fungi, as recently reviewed by Nuss & Koltin (1990). Some strains of *D. ambigua* have been found to contain a single dsRNA segment (Smit *et al*., 1994b), and to be hypovirulent. The potential for biological control of *D. ambigua* canker through hypovirulence may be dependent on transmission of the dsRNA to virulent strains of *D. ambigua* through hyphal anastomosis. Separating strains of *D. ambigua* into vegetative compatibility groups (VCGs) (Anagnostakis, 1984; Rayner *et al*., 1984; Glass & Kuldau, 1992; Leslie, 1993) would aid studies of transmission of dsRNA between strains. Identifying the frequency of different VCGs in nature would enable us to

evaluate the potential for spread of the dsRNA within the population of the pathogen.

The efficacy of a potential biological control strategy against *Diaporthe* canker is largely dependent on the phenomenon of vegetative compatibility as shown in various ascomycetous fungi (Anagnostakis, 1977; Adams *et al*., 1990). In spite of the recent importance of *Diaporthe* canker, little is known about the biology of the pathogen. This paper deals with the determination of vegetative compatibility among strains of *D. ambigua* in individual rootstocks and orchards.

MATERIALS AND METHODS

Source of strains

Single ascospore and single ascus strains of *D. ambigua* were obtained from cankers on 2- to 3-year-old apple (M793; M25; Granny Smith), pear (Winter Nelis; BP-1; BP-3) and plum (Marianna) rootstocks collected in separate orchards from a nursery in Simondium, from a canker on a 4-year-old BP-3 pear rootstock in Ceres, and from a canker on a 5-year-old Marianna plum rootstock in Villiersdorp, western Cape Province of South Africa.

Mature ascospores and asci were isolated from individual perithecia that were horizontally dissected, and the centrum macerated in sterile distilled water (Luttrell, 1951; Jensen, 1983). A series of dilute spore suspensions were washed onto the surface of 2% water agar (WA) plates or WA acidified to pH 4·5 with 85% lactic acid. Excess water on the surface of the plates was decanted, leaving only a film holding the spores. Prior to incubation, the plates were shaken to remove accumulated moisture and incubated in an inclined position for 24–36 h at 25°C. With the aid of a dissecting microscope, single germinated asci and ascospores were isolated by cutting the agar surface around them. Small agar squares containing a single germinating ascospore were lifted with a sterile needle and transferred to individual Petri dishes containing Difco (Difco Laboratories, Detroit, MI, USA) potato-dextrose agar (PDA). Small agar squares containing a single germinating ascus were transferred to individual Petri dishes containing freshly prepared oatmeal agar (Sonoda *et al*., 1982). Single asci representative of more than one VCG were excluded from further studies. Single ascus (with all eight ascospores in one compatibility group) and single ascospore strains were designated as originating from a specific canker, stroma and perithecium.

Initial dsRNA screening

Strains of *D. ambigua* originating from apple, pear and plum cultivars were grown on Difco Czapek Dox agar. Strains showing abnormal cultural characteristics such as lobular edges were screened for the presence of dsRNA. Extraction of nucleic acids from fungal mycelium and purification of the dsRNA fraction by cellulose chromatography was performed as described by Morris & Dodds (1979) and Valverde *et al*. (1990). Columns were prepared as described by Valverde & Fontenot (1991). Two cycles of cellulose (CF-11 Whatman, Maidstone, UK) column chromatography were used to purify the dsRNA.

Determination of vegetative incompatibility

Cultures were grown for 7 days on PDA in the dark at 25°C. Squares (3 mm) from colony margins of the cultures were paired on PDA, WA, Difco oatmeal agar, freshly prepared oatmeal agar and freshly prepared clarified oatmeal agar (Adams *et al*., 1990) to determine the best medium for detecting the black barrage reaction lines along the zone of mycelial contact between expanding colonies (vegetative incompatibility reaction). Subsequent studies were conducted on freshly prepared oatmeal agar, which was shown to display the best vegetative incompatibility reactions. Plugs of colonized agar were plated 1 cm apart from each other in round 90 × 12 mm Petri dishes (Promex) or square 243 × 18 mm bio-assay dishes (Nunc, Kamstrup, Denmark) such that 42 and 400 plugs, respectively, were on each plate. Vegetative pairing tests were based on the grid layout described by Ploetz & Shokes (1986) and Brayford (1990). A strain in a Petri dish was paired with itself and with 21 other strains. In a Bio-assay dish, a strain was paired with itself and with 200 other strains. In a set of pairings, however, each strain was paired with itself and with each of the other strains. Petri and Bio-assay dishes with paired strains were incubated in the dark at 25°C for 10 days and then, based on the absence or presence of black reaction lines between strains, rated as compatible or incompatible, respectively. All incompatibility reactions and each treatment plate in a test were replicated at least twice and in many cases up to four times.

Genetic variability of strains per orchard

One hundred and twenty M793 apple rootstocks from eight rows (15 rootstocks per row, three rootstocks per section of each row) were sampled in orchard B11 (Table 1). An additional 50 adjacent

Table 1 Genetic variability of strains of *Diaporthe ambigua* collected from various apple, pear and plum orchards[a]

Rootstock	Orchard number	Number of rootstocks	Number of perithecia studied	Number of VCGs/ orchard	Number of perithecia with >1 VCG
Apple					
M793	B11	170	170	189	27
M25	C9	30	30	27	0
Granny Smith	C18	30	30	41	13
Pear					
Winter Nelis	B13	30	30	29	0
BP-1	RB15	30	30	30	0
BP-3	RB18	30	30	29	0
Plum					
Marianna	NR1	30	30	30	0

[a] Vegetative compatibility groups (VCGs) were determined on freshly prepared oatmeal agar.

rootstocks were sampled from the first section of the eighth row in orchard B11. Thirty M25 apple rootstocks (orchard C9), 30 Granny Smith apple rootstocks (orchard C18), 30 Winter Nelis pear rootstocks (orchard B13), 30 BP-1 pear rootstocks (orchard RB15), 30 BP-3 pear rootstocks (orchard RB18) and 30 Marianna plum rootstocks (orchard NR1) were randomly sampled in the same nursery. Two single ascospores and one single ascus of *D. ambigua* were isolated from one perithecium from each sampled rootstock.

Genetic variability of strains per rootstock

Cultures from 10 single ascospores and five single asci (total of 15 cultures) from each of three perithecia (one perithecium per stroma), isolated from individual rootstocks, were used to determine the genetic variability of *D. ambigua* per rootstock. In orchard B11, the asci and ascospores were isolated from each of 40 M793 apple rootstocks (five rootstocks per row, one rootstock per section of each row) (Table 2). Asci and ascospores were also isolated from each of 10 of the following,

Table 2 Genetic variability of *Diaporthe ambigua* strains on specific apple, pear and plum rootstocks[a]

Cultivar	Origin	Number of perithecia studied	Number of rootstocks with 1 VCG	Number of rootstocks with >1 VCG
Apple				
M793	Simondium	120	6	34
M25	Simondium	30	10	0
Granny Smith	Simondium	30	1	9
Pear				
Winter Nelis	Simondium	30	10	0
BP-1	Simondium	30	10	0
BP-3	Simondium	30	10	0
BP-3	Ceres	3	1	0
Plum				
Marianna	Simondium	30	10	0
Marianna	Villiersdorp	3	1	0

[a] Vegetative compatibility groups (VCGs) were determined on freshly prepared oatmeal agar.

randomly sampled rootstocks: M25 (apple orchard C9), Granny Smith (apple orchard C18), Winter Nelis (pear orchard B13), BP-1 (pear orchard RB15), BP-3 (pear orchard RB18), and Marianna (plum orchard NR1). Ten single ascospore and five single ascus strains from each of three perithecia were isolated from a *D. ambigua* canker (BP-3 pear rootstock) originating from Ceres, as well as a *D. ambigua* canker (Marianna plum rootstock) originating from Villiersdorp.

Sexual preference

Strains from 25 single ascospores representing each of the apple, pear and plum rootstock cultivars were used to determine the sexual preference of *D. ambigua*. Strains originating from a specific cultivar were tested against each other in all combinations. Young, healthy twigs (4–7 mm in diameter) of apple, pear or plum rootstocks were freshly collected, cut into 20–30 mm lengths, thoroughly rinsed in several changes of distilled water, and sterilized by autoclaving for 70 min at 121°C. Twig pieces were aseptically placed onto the surface of WA plates and mycelial agar plug inocula originating from individual single ascospores applied, either between two parallel twigs in the case of single-strain inoculations, or one plug at each end in the case of paired strains. Petri dishes were sealed with Parafilm and incubated for 7 days at 25°C in the dark. Cultures were then incubated for a further 90 days at 20°C, while exposed to 8 h day^{-1} illumination with mixed cool-white fluorescent and near-ultraviolet lights held 400 mm above the plates, and observed for perithecium formation at intervals of up to 5 months after inoculation. All resulting perithecia were dissected and checked for the presence and germination of ascospores before recording them as fertile.

RESULTS

Isolation and testing procedures

Strains of *D. ambigua* grew uniformly from the mycelial plugs on freshly prepared oatmeal agar and a black reaction line characteristic of the incompatible response was evident after approximately 10 days (Fig. 1). Self-pairings used as controls were always compatible and in these cases the mycelia merged completely. Growth and interactions on freshly prepared oatmeal agar were comparable to those on freshly prepared clarified oatmeal agar. The nonclarified medium was preferred for testing compatibility as it was easier to recognize the barrage reaction. Difco oatmeal agar, PDA and WA gave inconsistent results and were not used after initial testing.

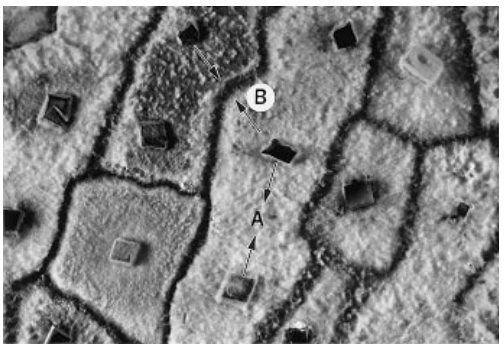

Fig. 1 Determination of vegetative compatibility among dsRNA-free, single ascospore strains of *Diaporthe ambigua* on freshly prepared oatmeal agar: (A) compatible strains merge along the line of contact; (B) black barrage reaction line formation along the zone of contact of incompatible strains.

Genetic variability of strains per orchard

The vegetative compatibility/incompatibility reactions among *D. ambigua* strains, expressed as VCGs representative of individual apple, pear and plum orchards, are summarized in Table 1.

Apple orchards

In orchard B11, 189 single discrete VCGs were identified from 340 single ascospore and 170 single ascus strains sampled from 170 M793 rootstocks in eight rows. Twenty-seven of the 170 perithecia yielded strains in more than one VCG. Fifty adjacent rootstocks in the first section of the eighth row in orchard B11 were not infected with strains of the same VCG. However, one single ascus strain in orchard B11 was vegetatively compatible with a single ascospore strain from orchard B13.

In orchard C9, 27 VCGs were identified from 60 single ascospore and 30 single ascus strains from 30 randomly sampled M25 rootstocks. None of the 30 perithecia yielded strains in more than one VCG. In orchard C18, 41 VCGs were identified among 60 single ascospore and 30 single ascus strains from 30 randomly sampled Granny Smith rootstocks. Thirteen of the 30 perithecia yielded strains in more than one VCG.

dsRNA-containing strains of *D. ambigua*, originating from individual single ascospores and single asci in orchard B11, did not form vegetative

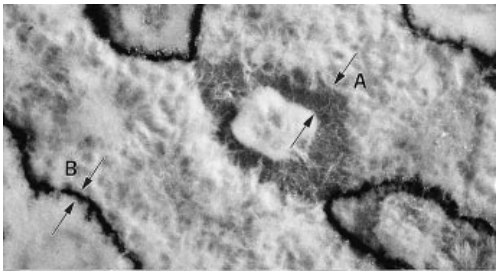

Fig. 2 Vegetative compatibility reactions among dsRNA-containing and dsRNA-free, single ascospore strains of *Diaporthe ambigua* on freshly prepared oatmeal agar: (A) broad, clear zone formation between two dsRNA-containing strains of different origins; (B) black barrage line formation between a dsRNA-containing and dsRNA-free strain.

compatibility reactions when paired with themselves ('self' reaction), but did form vegetative compatibility reactions when paired with individual dsRNA-free strains representative of different VCGs. dsRNA-containing strains of *D. ambigua* did not develop a pigmented reaction when paired with dsRNA-containing strains representative of different VCGs. Instead, a broad, clear zone (4–5 mm) developed where the colonies had previously merged, with dense hyphal knots on both sides of the cleared zone (Fig. 2).

Pear and plum orchards

Twenty-nine (orchard B13), 30 (orchard RB15) and 29 (orchard RB18) VCGs were identified among 60 single ascospore and 30 single ascus strains from 30 randomly sampled Winter Nelis, BP-1 and BP-3 pear rootstocks, respectively. None of the strains from 30 perithecia exhibited more than one VCG. In plum orchard NR1, 30 VCGs were identified among 60 single ascospore and 30 single ascus strains sampled from 30 randomly sampled Marianna rootstocks. No strains originating from 30 single perithecia were in more than one VCG.

Genetic variability of strains per rootstock

In orchards B11 and C18, strains from single rootstocks tended to be of more than one VCG (Table 2). For example, two to seven VCGs per rootstock were identified among 34 of the 40 M793 and nine of the 10 Granny Smith apple rootstocks. In contrast, the 10 randomly sampled M25 rootstocks (apple orchard C9), the BP-3 pear rootstock of Ceres origin and all randomly sampled Winter Nelis (orchard B13), BP-1 (orchard RB15) and BP-3 (orchard RB18) pear rootstocks, as well as the Marianna plum rootstock of Villiersdorp origin and all randomly sampled Marianna rootstocks (orchard NR1), had only one VCG per rootstock.

Sexual preference

Fertile perithecia developed when single or paired ascospores of *D. ambigua* were grown for 2–3 months on apple, pear and plum twigs, as well as on agar media tested (freshly prepared oatmeal agar; PDA). The perithecia formed fully or partially embedded in the twig xylem, each with a single neck erumpent through the periderm. All ascospores derived from these perithecia germinated within 36 h when placed on WA. The morphology of the teleomorph state in culture corresponded with that observed in nature.

DISCUSSION

Results of this study have shown that the population of *D. ambigua* in apple, pear and plum orchards in South Africa contains strains belonging to a large number of VCGs. Given the ubiquitous presence of perithecia throughout the area of disease occurrence, this is perhaps not surprising. Strains from adjacent trees usually differed in VCG. One interpretation for the large number of VCGs in close proximity to one another suggests that ascospores are effective primary propagules. In general, a sexually reproducing population would be expected to have a high level of VCG diversity (Leslie, 1993). It is possible, however, that nursery seedlings possess latent infections caused by many VCGs of *D. ambigua* which then serve as foci of conidial infection. Similar conclusions were made in a study of Cytospora canker of peach caused by *Leucostoma persoonii* (Adams *et al*., 1990).

Analysis of VCGs 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 (Glass & Kuldau, 1992). In the case of *D. ambigua*, it would be difficult to draw such conclusions without knowledge regarding the regulation of the sexual cycle. The formation of the sexual state of *D. ambigua* by individual single ascospores in culture is indicative of homothallism. Furthermore, fertile perithecia occurred abundantly on each of the sampled rootstocks. The presence of more than one VCG in progeny from individual perithecia collected from various M793 and Granny

Smith apple rootstocks, however, is indicative of outcrossing. Apparently *D. ambigua* is a homothallic fungus that has the capacity to outcross, as in the case of *Cryphonectria parasitica* (Anagnostakis, 1977). The basis of sexuality in *D. ambigua* clearly needs further study. At this stage we suspect, however, that *D. ambigua* on fruit trees is native in the country and that it probably originated from native woody plants such as rooibos tea (*Aspalathus linearis*) (Smit *et al*., 1994a).

Strains from different orchards generally were from different VCGs. Our samples originated mainly from seven newly established nursery orchards, each with a different source (and sometimes mixed sources) of seedling and budding material. Most studies of VCGs in other Ascomycetes have been conducted in well established orchards (Proffer & Jones, 1989; Adams *et al*., 1990). In such orchards, a strain of a specific VCG could have spread the disease to eventually produce an aggregation of trees with cankers representative of a common VCG. However, a clustered pattern of VCGs within a well established orchard could have originated from nursery seedlings possessing latent infections caused by many VCGs.

The predominance of one or a few VCGs among *D. ambigua* strains recovered from the intensively sampled orchard B11 was not observed. This is in contrast to *Diaporthe phaseolorum* where one VCG dominates in strains collected from small areas (Ploetz & Shokes, 1986). Our data are consistent with the hypothesis that VCG and fitness are not correlated.

If no VCG dominates the population, then as the number of VCGs increases the efficacy of dsRNA transfer and thus the success of biological control appears to be reduced. The proliferation of VCGs probably limits the use of dsRNAs as a biological control of *C. parasitica* in North America (Anagnostakis, 1982b; Anagnostakis *et al*., 1986; Anagnostakis & Kranz, 1987; MacDonald & Fulbright, 1991). This proliferation is in contrast to the situation in Europe, where biological control through hypovirulence is successful and where there is less diversity in the pathogen population (Anagnostakis *et al*., 1986; Heiniger & Rigling, 1994). Interaction between vegetative incompatibility genes affects horizontal (intermycelial) dsRNA transmission in *C. parasitica* (Huber & Fulbright, 1994). However, dsRNA in *C. parasitica* stops reassortment of vegetative compatibility genes by suppressing mating-type-specific gene expression (Zhang & Van Alfen, 1994).

All sampled dsRNA-containing strains of *D. ambigua* in this study originated from sexual spores. Polashock & Hillman (1994a,b) reported a small dsRNA element associated with the mitochondria of a *C. parasitica* strain. This small mitochondrially associated element can be transmitted into compatible strains by hyphal anastomosis, as in the case of the more common larger size dsRNA viruses of *C. parasitica*, which are members of the Hypoviridae. Transmission of virus-like elements to ascospore progeny appears to be blocked in the Hypoviridae (Anagnostakis 1982a,b). In contrast to the Hypoviridae, however, the small mitochondrially associated element can be transferred to sexual progeny (Polashock & Hillman, 1994b). Since it is present in ascospores, it is possible that the relatively small dsRNA element in *D. ambigua* (Smit *et al*., 1994b) is mitochondrially associated. All dsRNA-containing strains of *D. ambigua* need to be analysed in the future to determine whether transfer of dsRNA by anastomosis is accompanied by mitochondrial recombination in a recipient strain.

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