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Characterisation of *Fusarium oxysporum* isolated from carnation in Australia based on pathogenicity, vegetative compatibility and random amplified polymorphic DNA (RAPD) assay

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Abstract

Isolates of *F. oxysporum* collected from symptomless carnation cuttings from Australian carnation growers properties, together with isolates from national collections, were screened for pathogenicity and grouped according to vegetative compatibility and random amplified polymorphic DNA (RAPD) patterns. The collection of 82 Australian isolates sorted into 23 different vegetative compatibility groups (VCGs). Of 69 isolates tested for pathogenicity, 24 were pathogenic to carnations, while the remaining 45 were non-pathogenic. All pathogenic isolates were within two VCGs, one of which was also compatible with an isolate obtained from an international culture collection, and which is known to represent VCG 0021 and race 2. Race status of the two pathogenic VCGs remains unknown. The RAPD assay revealed distinct DNA banding patterns which could distinguish pathogenic from non-pathogenic isolates as well as differentiate between isolates from the two pathogenic VCGs.

Fusarium oxysporum Schlecht. causes vascular wilt in a broad range of host plants including carnations (*Dianthus caryophyllus* L.). This species of *Fusarium* displays extensive variability regarding pathogenicity and at a sub-specific level isolates have been classified into formae speciales and races according to host and cultivar range respectively (Garibaldi, 1977; Armstrong and Armstrong, 1981). These sub-specific taxa are morphologically indistinguishable (Jacobson and Gordon, 1991; Puhalla, 1981) and their validity as taxonomic units is unclear (Armstrong and Armstrong, 1975; Windels, 1991). Eight physiologic races of *F. oxysporum* f. sp. *dianthi* (Prill & Del) Snyd. & Hans. (*Fod*) have been identified to date and race 2 is the most common, having worldwide distribution (Garibaldi, 1977). To date, no information exists on the diversity of *Fod* in Australia, nor on races present.

Attempts to assess genetic variability and to study the phylogeny of *Fusarium* species have been made using a number of techniques, including vegetative compatibility group (VCG) testing (Puhalla, 1985). In the absence of a sexual stage, it was proposed that exchange of genetic material would be limited to compatible isolates within a VCG and so each VCG represents a genetically isolated population. This method has since been used to examine the relationships between *F. oxysporum* isolates from a wide range of hosts (Correll, 1991) including carnation (Baayen and Kleijn, 1989; Katan et al., 1989; Manicom et al., 1990; Aloi and Baayen, 1993).

In recent years molecular biology techniques have been introduced for classification of fungi and examination of evolutionary relationships. In the case of *F. oxysporum*, restriction fragment length polymorphisms (RFLPs) have shown some potential for measuring genetic variability by direct examination at the nucleic acid level (Manicom et al., 1987). In particular, the grouping of isolates on the basis of RFLP pattern has been shown to correlate with their vegetative compatibility grouping (Manicom et al., 1990; Manicom and Baayen, 1993).

Table 1. Vegetative compatibility groups (VCGs) of 82 Australian isolates of *Fusarium oxysporum* and 4 *Fod* tester isolates

VCG	Isolate(s)				
A	21[a]	24[a]	36[a]	75[a]	123[a]
	6/1W[a]	1/1E[a]	5/1W[a]	12/1E[a]	B9D223/1[b]
	B9D242/1[b]	B9D242/2[b]	B9D243/1[b]	B9D243/4[b]	H8BC12[a]
B	68[b]	68II[b]	70[b]	70II[b]	121[b]
	B6D214/2[b]	B8D291/1[b]	B9D202/1[b]	B9D221/1[b]	B9D221/2[b]
	B9D221/3[b]	B9D221/4[b]	B9D221/5[b]	H946/4[a]	H9BC16[a]
	C 2836[c]				
C	47[a]	82[a]	106[a]	H41/33[a]	H51/2[a]
	H61/12[a]	H61/14[a]	H646/7[a]	*Fod* P[d]	*Fod* R[d]
	Fod W[d]	DAR 28063[e]	WCS 816(VCG 0021)[f,g]		
D	73[a]				
E	B1D204[b]				
F	H3BC39[a]	H3BC40[a]			
G	H4BC16[a]				
H	H3BC21[a]				
I	H3BC42[a]				
J	H41/38[a]	H41/42[a]			
K	H5T1/6[a]				
L	H5T2/3[a]				
M	H61/35[a]				
N	H61/59[a]				
O	H746/7[a]	H9BC6[a]	H1146/2[a]		
P	H81/4[a]	H81/5[a]			
Q	H8BC14[a]	H12T4/2[a]			
R	B9D204/4[b]	B9D244/2[b]			
S	B9D244/1[b]	B9D244/3[b]			
T	H1046/1[a]	H111/2[a]	H111/7[a]	H111/10[a]	H1146/1[a]
U	H111/4[a]	H111/8[a]	H111/9[a]	H111/11[a]	H1146/3[a]
	H1146/4[a]	H1146/5[a]	H1146/6[a]		
V	WA 2407[c]				
W	WA 2581[c]				
0020[g]	WCS 838[f]				
0022[g]	WCS 827[f]	WCS 840[f]			

Australian isolates from: [a] grower A or [b] B Victoria, [c] D.Emery, Western Australia, [d] carnation growers, Queensland, [e] M.Priest, New South Wales, [f] isolates from R.Baayen, The Netherlands, [g] classified in Aloi and Baayen, 1993.

More recently, a new DNA polymorphism assay has been developed, known as the random amplified polymorphic DNA (RAPD) assay (Williams et al., 1990). This technique has been applied for strain identification, determination of inheritance patterns and genetic mapping in a wide variety of organisms. The RAPD assay has previously been used for race typing of *F. oxysporum* f. sp. *pisi* (Hall) Snyd. & Hans. (Grajal-Martin et al., 1993) and *F. solani* f. sp. *cucurbitae* Snyd. & Hans. (Crowhurst et al., 1991). Recently, RAPDs have been shown to be able to differentiate between pathogenic and non-pathogenic *F. oxysporum* isolated from carnation plants in Israel, and possibly to distinguish *Fod* race 2 from race 4 (Manulis et al., 1994).

The studies reported in this paper deal with a collection of *F. oxysporum* isolates from carnation plants from Australia where there is currently no information available on the variability within *Fod* populations, or of races present.

Table 2. Results of characterisation of 69 isolates of *Fusarium oxysporum* from carnations by pathogenicity testing and RAPD analysis

VCG	Isolate(s)	Pathogenicity[a]	RAPD pattern[b]	500bp band[c]
A	21*; 24; 36; 75; 123; 6/1W; H8BC12*; B9D223/1*; B9D242/1; B9D242/2*; B9D243/1; B9D243/4;	N	1	N
B	68*; 70*; 121*; H9BC16; B6D214/2*; B8D291/1*; B9D202/1*; B9D221/1*; B9D221/2*; B9D221/3*; B9D221/4*; B9D221/5*;	P	2	Y
C	47*; 82*; 106*; *Fod*R*; *Fod*P*; *Fod*W*; H41/33*; H51/2*; H61/12; H61/14*; H646/7*; DAR28063*;	P	3	Y
D	73*	N	1	N
E	B1D204*	N	1	N
F	H3BC39; H3BC40	N		
G	H4BC16*	N	1	N
H	H3BC21	N		
I	H3BC42*	N	1	N
J	H41/38; H41/42	N		
K	H5T1/6	N		
L	H5T2/3	N		
M	H61/35	N		
N	H61/59	N		
O	H746/7; H1146/2*	N	1	N
P	H81/4; H81/5	N		
Q	H8BC14; H12T4/2*	N	1	N
R	B9D204/4; B9D244/2	N		
S	B9D244/1*	N	1	N
T	H1046/1; H111/7; H1146/1*;	N	1	N
U	H111/4; H111/8; H111/9; H111/11; H1146/3; H1146/4; H1146/5; H1146/6;	N		

* RAPD banding pattern determined

[a] N = non-pathogenic; P = pathogenic on carnation cultivar Crowley

[b] 1 = DNA fragments of 1.2 kb, 0.95 kb; 2 = DNA fragments of 1.2 kb, 0.5 kb; 3 = DNA fragments of 1.2 kb, 0.95 kb, 0.5 kb

[c] N = not present; Y = present.

Vegetative compatibility grouping

A total of 82 Australian isolates of *F.oxysporum* were used in this study (Table 1). Mutants unable to utilise nitrate were readily recovered from chlorate-amended media and classified as *nit1* or nitM according to Correll et al. (1987). During VCG testing, all compatibility reactions were easily discernible. There was no evidence of the self-incompatibility reported in other studies (Correll et al., 1986b; Jacobson and Gordon, 1988), nor were there any weak reactions as has been observed in other studies (Elmer and Stephens, 1989; Gordon and Okamoto, 1991; Katan et al., 1991). The isolates sorted into 23 VCGs arbitrarily named A to W (Table 1). Three of these groups (A, B and C) contained 12 or more isolates, while the other groups were smaller, often consisting of only one or two isolates. No isolate was compatible with more than one VCG. All of the isolates were re-tested after re-isolation from the plants used in pathogenicity testing, with all groupings confirmed.

Once the VCGs were identified, representatives of each group were tested for compatibility with

four isolates obtained from Dr. R. Baayen (Research Institute for Plant Protection, Agricultural Research Department, Wageningen, The Netherlands). The only compatible response observed was that between isolate 106 (representative of VCG C) and isolate WCS 816. VCG C should therefore be formally classified as VCG0021 (Aloi and Baayen, 1993).

Pathogenicity testing

A total of 69 of the Australian isolates was screened for pathogenicity by dipping rooted cuttings of carnation cultivar Crowley (six cuttings per isolate) in conidial suspensions ($0.2–1.0 \times 10^7$ spores ml^{-1}) or sterile water as control. Cuttings were potted up in a sterile mixture of coarse sand, peat moss and perlite (40:50:10 v/v/v) and maintained in a glasshouse (19 °C–22 °C, 60% relative humidity, natural light). Plants were assessed after seven weeks, when 24 isolates were shown to be pathogenic and 45 isolates non-pathogenic (Table 2). Pathogenic isolates incited typical wilt symptoms, with plants showing a gradual chlorosis and wilting of the plant. Vascular discolouration was visible in the stems. In the later stages of disease, fungal mycelium and sporodochia were visible on the butts of the plants. Root systems of diseased plants were discoloured and small compared to those of healthy plants, and broke easily away from stems at harvest. In some cases the roots had completely rotted. No diseases symptoms were seen over the seven week period in plants inoculated with isolates assessed to be non-pathogenic. Uninoculated control plants also remained healthy throughout the course of the trials.

All of the isolates in two of the large VCG groups (VCG B and VCG C(VCG0021)) were pathogenic and should, therefore, be considered as *Fod*, while the isolates in the remaining groups were non-pathogenic. A correlation between VCG and pathogenicity has also been demonstrated in a study of *F. oxysporum* from carnations in Israel (Katan et al., 1989). In that study, 132 out of a total of 170 isolates proved to be pathogenic and all of these sorted into a single VCG. None of the non-pathogenic isolates were compatible with the pathogenic group. Correlation between VCG and pathogenicity has also been reported for other formae speciales, for example *F. oxysporum* from cotton (Katan and Katan, 1988) and gladiolus (Roebroeck and Mes, 1992). In other cases, however, the correlation between VCG and pathogenicity is not absolute (Correll et al., 1986b). In the case of the Australian *F. oxysporum* isolates studied here, we have identified two distinct populations of pathogens and a non-pathogenic population highly variable with respect to vegetative compatibility.

Unfortunately, it was not possible to perform race identification using differential cultivars (Garibaldi, 1977; Demmink et al., 1987) as the required cultivars are unavailable in Australia. However, the compatibility of isolate 106 (representative of VCG C) with isolate WCS 816 (representative of VCG 0021 and of race 2 (Baayen et al., 1988; Aloi and Baayen, 1993)) suggests that Group C isolates may be race 2 although recent work suggests that VCG0021 may also contain races 5 and 6 (Aloi and Baayen, 1993). Isolate 68 (representative of the other pathogenic group, VCG B) was not compatible with any of the four tester strains suggesting that this group of isolates does not represent race 1,2,4,5,6,or 8 (Aloi and Baayen, 1993). Whether this group is representative of races 3 or 7 or whether it represents an entirely new race remains to be resolved.

Simple race-VCG relationships have been demonstrated in a number of *F. oxysporum* formae speciales (Katan and Katan, 1988; Katan et al., 1989; Larkin et al., 1990). In the case of *Fod* (Katan et al., 1989) 132 pathogenic isolates from Israel were classified into a single VCG and, on the basis of cultivar trials, were all shown to be race 2. In more recent work, however, the relationships have been found to be more complex, with some *Fod* VCGs apparently containing members of several races (Aloi and Baayen, 1993), or a single race containing members of two VCGs (Manicom et al., 1990). In other *F. oxysporum* formae speciales, a similarly complex situation occurs (Ploetz and Correll, 1988; Brake et al., 1990; Ploetz, 1990; Correll, 1991) and it is clear that the race status of pathogenic isolates cannot be defined purely on the basis of VCG. The identification of which race both VCG B and VCG C (VCG0021) isolates belong to therefore remains to be resolved.

The large number of VCGs identified for the non-pathogenic isolates in this study indicates that there is a great deal of genetic variability amongst them. This has similarly been demonstrated in other studies, for example in non-pathogenic *F. oxysporum* isolates from celery roots (Correll et al., 1986a) and from soils infested with *F. oxysporum* f. sp. *melonis* (Gordon and Okamoto, 1991). In the light of the vital role that these isolates can play in wilt disease suppression (Correll et al., 1986a; Louvet et al., 1981) and in their potential use as biocontrol agents (Mandeel and Baker, 1991; Rattink, 1987; Rattink, 1991; Tramier et al., 1987)

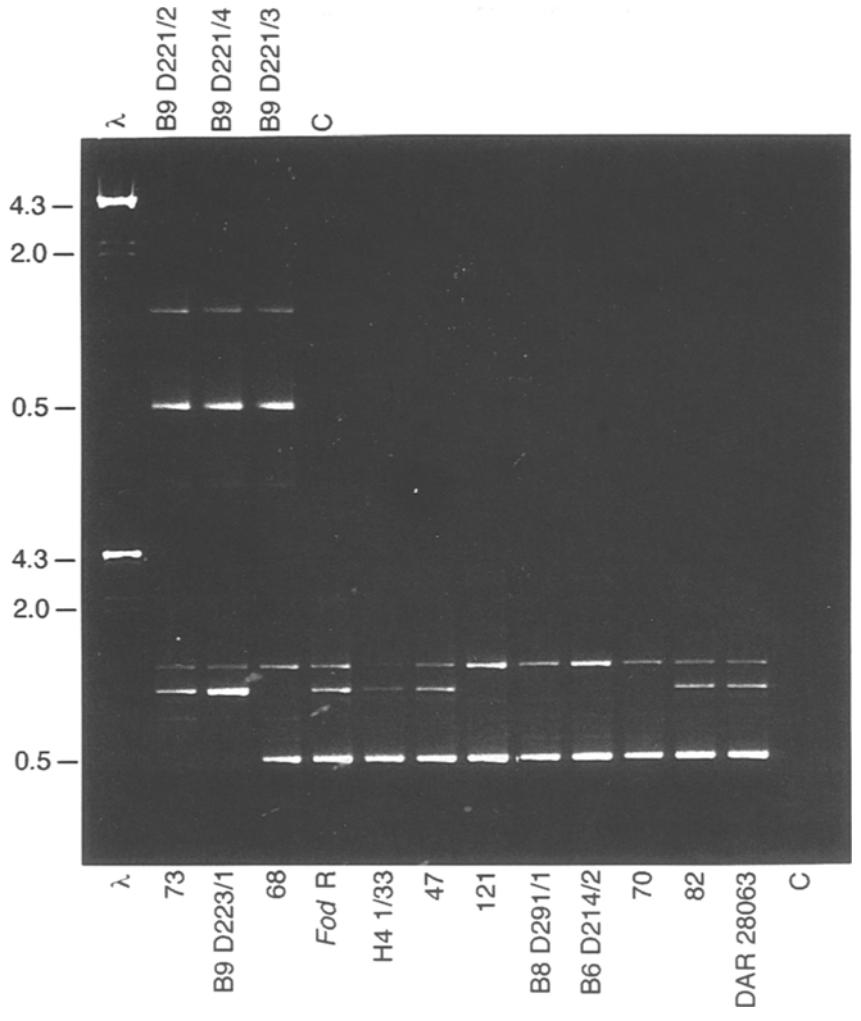

Figure 1. RAPD analysis of *F. oxysporum* amplified with Primer P2. Results obtained for isolates 73, 68 and 47 illustrate the three main banding patterns denoted 1, 2 and 3 respectively in Table 2. The 0.5 kb fragment is present in all pathogenic isolates. C-Control.

further information regarding the genetic composition of these isolates is required.

Random amplified polymorphic DNA (RAPD) analysis

A total of 34 isolates representative of 11 VCGs were characterised by RAPD analysis of genomic DNA preparations with a decamer primer labelled P2 (sequence CTGTTGCTAC). Representative results are shown in Figure 1 where analysis of isolates 73, 68 and 47 illustrate the three main banding patterns, denoted 1, 2 and 3 respectively in Table 2. On the basis of these banding patterns this method enabled classification of all isolates into three groups (Table 2). A 1.2 kb fragment was present in all cases, while a 0.95 kb fragment was only present in patterns 1 and 3, and a 0.5 kb fragment was only present in patterns 2 and 3.

The data in Table 2 demonstrates that the 0.5 kb RAPD band correlates with pathogenicity, even for isolates from widely dispersed geographical sources within Australia. The two pathogenic VCGs could also be differentiated on the basis of the banding patterns observed. Before any firm conclusions can be drawn on the degree of relatedness of these strains, or the connection between the 0.5kb RAPD band and pathogenicity, a more extensive analysis should be performed using, for example, a larger number of RAPD primers, and isolates from a wider range of sources including other formae speciales.

Recently, Manulis et al. (1994) carried out a similar study examining the genetic diversity of *F. oxysporum* isolated from carnation plants in Israel. They demonstrated that RAPD analysis could distinguish between pathogenic and non-pathogenic isolates, and in the majority of cases between isolates of race 2 and one isolate of race 4.

In the present study, a high degree of correlation was demonstrated between the results obtained from pathogenicity testing, VCG testing and the RAPD assay (Table 2). The RAPD assay is easier and quicker than the other tests, and it has several advantages over other DNA polymorphism assays. This technique may in future prove to be very useful for classification of *F. oxysporum* isolates or as a general diagnostic tool. DNA sequencing of a diagnostic RAPD fragment(s) followed by synthesis of specific PCR primers would enable construction of a robust, highly specific test.

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