COMPARISON OF MOLECULAR TECHNIQUES FOR THE IDENTIFICATION OF DNA MARKERS SPECIFIC TO *FUSARIUM OXYSPORUM* F. SP. *CYCLAMINIS*

by

DARYL ANDY LOUIE

B.Sc., The University of Victoria, 1992

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Plant Science)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1995

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Plant Science

The University of British Columbia
Vancouver, Canada

Date Aug. 30, 1995

DE-6 (2/88)

Abstract

Fusarium wilt of cyclamen is caused by the fungus *Fusarium oxysporum* f. sp. *cyclaminis*. Early detection of this disease in greenhouse plantings has been difficult due to the long latent period which may occur and the ubiquity of morphologically identical nonhost *F. oxysporum* strains present in the greenhouse. A PCR based assay to confirm the identity of *F. o. cyclaminis* would be an improvement over any conventional methods due to its inherent speed, specificity, and sensitivity.

The molecular techniques of random amplified polymorphic DNA (RAPD), combined polymerase chain reaction and restriction fragment length polymorphism (PCR and RFLP), and subtraction hybridization were used to attempt identification of DNA markers specific to *F. o. cyclaminis*. No universal DNA markers were found which would identify the 16 *F. o. cyclaminis* isolates from the 25 nonhost *Fusarium* isolates used in this study. The complicating factor appeared to be the apparent loss of pathogenicity with some of the *F. o. cyclaminis* isolates. Of the methods evaluated, RAPD analysis or PCR and RFLP analysis using the intergenic spacer (IGS) region, proved to be the most promising methods due to their ease of use. Cluster analysis of the RAPD data using the unweighted paired group method with arithmetic averaging (UPGMA) revealed that the pathogenic *F. o. cyclaminis* isolates were found to be exclusive to two clusters. Future research towards identification of DNA markers to this pathogen may best be approached by separation of the isolates with highest genetic similarity into groups and identifying DNA markers to these groups rather than identifying a universal marker to all *F. o. cyclaminis* isolates.

Table of Contents

List of Tables

List of Figures

Acknowledgments

This project was funded by the United Flower Growers Co-op Association and by the Applied Research Partnership Program of the British Columbia Ministry of Agriculture, Fisheries and Food to Dr. R.J. Copeman.

I would like to thank my supervisors, Dr. R.J. Copeman and Dr. J.W. Kronstad, for their guidance and support throughout my studies. Dr. J.E. Carlson for his advice and technical assistance. Dr. N.L. Glass, Dr. C.A. Lévesque, and Gary Donaldson for allowing me use of their primer sets and for providing technical assistance. And thanks to the members of Dr. Kronstad's lab for their helpfulness and experience and members of the Plant Science Department for their support.

Special thanks to Arthur Yee for his wisdom and words of encouragement.

Introduction

Fusarium Link:Fr. is a fungal genus that is common world wide and involved in many diseases of animals, including man, as well as plants (Booth, 1971). Prior to 1935 fusaria were not identified in a consistent manner, resulting in many similar isolates receiving multiple designations. This prompted Wollenweber and Reinking (1935) to propose a classification system based on spore morphology thus reducing approximately 1000 named species to 65 and dividing them into 16 sections. Shortly afterwards, Snyder and Hansen proposed that the section Elegans be collapsed into a single species called *Fusarium oxysporum*, thus reducing the 65 species to nine (Snyder and Hansen, 1940). This nine species system is the classification generally accepted by mycologists and plant pathologists today.

Fusarium oxysporum Schlechtend.:Fr. is a diploid, uninucleate fungus with no known sexual stage; asexual reproduction occurs by spore formation, typically conidia (Puhalla, 1981). This fungus belongs to a group in the Division Eumycota known as the imperfect fungi ie., those which lack a perfect or sexual stage (Agrios, 1988). The species *F. oxysporum* is a common soilborne fungus and many of its strains are economically important plant pathogens. Although this species has a wide host range, individual strains are restricted to a single or limited number of host species (Armstrong and Armstrong, 1981; Booth, 1971). Because of this host specificity, a *forma specialis* concept was proposed by Snyder and Hansen (1940) to further classify pathogenic strains according to the Latin name of their hosts. For example, the form which attacks tomato (*Lycopersicon esculentum*) is designated as *F. oxysporum* f. sp. *lycopersici.*

Fusarium wilt of cyclamen (*Cyclamen persicum* L.) is caused by the fungus *F. oxysporum* f. sp. *cyclaminis* Gerlach. First observed in Germany around 1930 (Gerlach, 1954),

the disease has since been reported in North America (Tompkins and Snyder, 1972). This disease, as with all vascular wilts, results from a root penetration followed by a systemic spread of the fungus and finally a blockage of the xylem vessels with a combination of both fungal and host plant materials to a point where the plant begins to lose turgor and eventually dies (Agrios, 1988). Additional symptoms observed with this disease include chlorosis of the leaf blade, external discolouration of the roots, and internal discolouration of the vascular tissue in the root and corm (Tompkins and Snyder, 1972). Plants of all ages are susceptible throughout their production cycle (Tayama, 1987; Daughtrey and Hoitink, 1988) and may show symptoms at any stage of development due to a lengthy latent period (Rattink, 1986). Studies with a number of isolates of *F. o. cyclaminis* on multiple cyclamen cultivars do not suggest the existence of a race structure (Rattink, 1986).

Detection of diseased planting stocks in the greenhouse is difficult due to the long latent period which may occur. This also makes conventional identification of *F. o. cyclaminis* difficult since inoculation (pathogenicity) tests used to confirm the identity of the pathogen may take weeks or even months to complete. Microscopic identification of cultures is not possible due to the ubiquity of morphologically identical nonhost *F. oxysporum* strains, present on the plant or in the soil, which are nonpathogenic on cyclamen. Losses in the greenhouses of British Columbia have now reached a point where some growers have abandoned cyclamen production in favour of more economically viable crops.

A molecular approach to identification of fungal isolates can overcome the difficulties involved with the differentiation of morphologically similar fungi. The polymerase chain reaction (PCR) was introduced by Mullis and Faloona (1987) and has since become recognized

as a powerful research tool because it has the ability to amplify specific gene sequences from minute starting quantities of DNA. PCR assays for detection and identification have been developed for numerous fungal plant pathogens including *Gaeumannomyces graminis* (Henson *et al.*, 1993), *Phoma tracheiphila* (Rollo *et al.*, 1990), *Phytophthora fragariae* (Stammler and Seemüller, 1993), and *Leptosphaeria maculans* (Taylor, 1993). A PCR based assay to confirm the identity of *F. o. cyclaminis* would be a great improvement over any conventional approach available due to its inherent speed, specificity, and sensitivity.

With the wide array of molecular techniques available to a plant pathologist, it is often difficult to know which technique to use or which one will work the best. Some of the more recent techniques include random amplified polymorphic DNA (RAPD), PCR and restriction fragment length polymorphisms (RFLP), and subtraction hybridization.

Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (RAPD) is a variation of the PCR technique which uses a single short arbitrary primer to amplify small fragments of genomic DNA. Developed independently by two groups working in the United States (Welsh and McClelland, 1990; Williams *et al.*, 1990), RAPD analysis offers both a quick and easy method to detect genetic differences between individuals. The ease and speed with which analyses may be done makes it superior to restriction fragment length polymorphism (RFLP) analysis, another common technique in fungal studies for screening for genetic differences. RAPD analysis can be completed in a single day compared with 3 days for RFLP analysis. Furthermore, RAPD analysis requires only small quantities of purified DNA compared with microgram quantities for

RFLP analysis, and the RAPD technique does not require the use of cloned probes or the use of radioisotopes for detection. RAPD analysis is clearly desirable over RFLP analysis because it allows for a massive throughput of samples in a very short period of time.

The benefits of RAPD analysis over RFLP analysis have been capitalized on by plant pathologists for fungal plant pathogen identification when many isolates needed to be screened. With RAPD analysis, the strains of *F. graminearum* (Ouellet and Seifert, 1993), the two pathotypes of *Leptosphaeria maculans* (Goodwin and Annis, 1991; Schäfer and Wöstemeyer, 1992), and the two pathotypes *of F. oxysporum* f. sp. *ciceris* (Kelly *et al.*, 1994) have been differentiated from one another. Multiple races have been differentiated from one another within *Gremmeniella abietina* (Hamelin *et al.*, 1993), *Bipolaris maydis* (Nicholson *et al.*, 1993), *Colletotrichum orbiculare* (Correll *et al.*, 1993), and *F. solani* f. sp. *cucurbitae* (Crowhurst *et al.*, 1991). In addition, single races can be differentiated from other races in *Cochliobolus carbonum* (Jones and Dunkle, 1993), and in *F. oxysporum* f. sp. *pisi* (Grajal-Martin *et al.*, 1993). Recently, race 2 of *F. oxysporum* f. sp. *dianthi* could be differentiated from nonpathogenic isolates of *F. oxysporum* found on carnation using RAPD markers (Manulis *et al.*, 1994). Although RAPD markers tend to be parts of repetitive sequences within the genome, these markers can be successfully converted into sequence characterized amplified regions (SCARs) by extending the original RAPD primer with knowledge of the regions flanking that marker (Paran and Michelmore, 1993; Maisonneuve *et al.*, 1994; Adam-Blondon *et al.*, 1994).

Often it is necessary to assess the amount of genetic variability and relatedness of fungal isolates within and between *formae speciales* to determine the relationship between molecular markers and pathogenicity or virulence. RAPD data have been combined with cluster analysis to

address this question in *Magnaporthe poae* (Huff *et al.*, 1994), *Puccinia striiformis* (Chen *et al.*, 1993), and *F. o. pisi* (Grajal-Martin *et al.*, 1993). A distinct relationship has now been established with cluster analysis between two pathotypes of *F. o. ciceris* (Kelly *et al.*, 1994), and three races of *F. oxysporum* f. sp. *vasinfectum* (Assigbetse *et al.*, 1994).

Polymerase Chain Reaction And Restriction Fragment Length Polymorphism

Another recent approach to detect genetic differences between individuals has been to digest a PCR product with a restriction enzyme and look for fragment length polymorphisms. This approach is analogous to doing a RFLP analysis, but instead of digesting microgram quantities of genomic DNA with a restriction enzyme, a PCR amplification product is digested. Furthermore, instead of a cloned DNA probe, the PCR primers will amplify a specific region of the genome giving the desired specificity. Also, with PCR and RFLP analysis, no radioisotopes are required for detection. In effect, a RFLP analysis is accomplished with all the benefits of PCR.

With the introduction of the PCR, the ribosomal DNA (rDNA) region has gained much attention with regard to fungal identification. The rDNA is convenient for PCR because it exists in multicopy and the entire internal transcribed spacer (ITS) region can easily be amplified. The rDNA ITS and intergenic spacer (IGS) regions have shown enough variation to make identification of morphologically similar fungi possible. Direct sequencing of the ITS region has lead to the development of specific DNA hybridization probes for *Pythium ultimum* (Lévesque *et al.*, 1994) and a single isolate of *Laccaria bicolor* (Gardes *et al.*, 1991). Specific PCR primers have also been synthesized directly from the sequenced ITS region to detect 14 species of

Basidiomycetes (Gardes and Bruns, 1993), *Ophiosphaerella korrae* or *O. herpotricha* (Tisserat *et al.*, 1994), *Verticillium tricorpus* (Moukhamedov *et al.*, 1994), *V. dahliae* or *V. albo-atrum* (Nazar *et al.*, 1991), and the weakly virulent or highly virulent pathotypes of *Leptosphaeria maculans* (Xue *et al.*, 1992).

Although sequencing provides the best information for the comparison of base pair sequences, it can be time consuming when numerous isolates must be compared. By amplifying part or all of the ITS region, it is possible to examine short base pair sequences using the recognition sequences of various restriction enzymes. Differences in restriction sites may arise due to single base pair changes, or larger insertions or deletions. These differences are then revealed as polymorphic bands when the cut DNA fragments are separated on a gel. This combined PCR and RFLP analysis approach has been successfully used to differentiate *F. oxysporum* f. sp. *lycopersici* from *F. oxysporum* f. sp. *radicis-lycopersici* using the IGS region (Wang, 1993), five different species of *Tuber* using the ITS and IGS regions (Henrion *et al.*, 1994), and 6 species of *Fusarium* using the ITS and other amplified regions (Donaldson *et al.*, 1995). These differences or changes in restriction sites could then be used to synthesize specific PCR primers for use in a PCR assay.

Subtraction Hybridization

A recent strategy for isolating sequences present in one DNA population and absent in another DNA population involves the differential reannealling of DNA between two DNA populations. This technique has been described with names such as genomic subtraction (Straus and Ausubel, 1990), subtractive hybridization (Wieland *et al.*, 1990), or subtraction hybridization

(Bjourson and Cooper, 1988). All of these techniques achieve the same goal and will be referred to collectively as subtraction hybridization. The majority of the research done using this strategy is with prokaryotic organisms. There are probes now available for three strains of *Rhizobium loti* (Bjourson and Cooper, 1988), *R. leguminosarum* bv. *trifolii* (Bjourson et al., 1992), *Pseudomonas solanacearum* (Seal *et al.*, 1992), *P. solanacearum* race 3 (Cook and Sequeira, 1991), and *Erwinia carotovora* subsp. *atroseptica* (Darrasse *et al.*, 1994). However, few attempts have been made using eukaryotic organisms and the only published attempt is with a fungus (Goodwin *et al.*, 1990). Hybridization tests with the two subtraction clones from *Phytophthora citrophthora* revealed no hybridization to 10 other species of *Phytophthora*, but disappointingly revealed hybridization to only a portion of the *P. citrophthora* isolates used in the study (Goodwin *et al.*, 1990). The potential to produce a PCR probe still exists though and was successfully demonstrated with the subtraction probe for *P. solanacearum* (Seal *et al.*, 1992).

Research Objective

The objective of this study was to identify DNA markers specific to *F. o. cyclaminis* for use in a routine PCR-based assay to positively identify this fungus in greenhouse soil and plant material.

Materials And Methods

Fungal Isolates And Media

Forty one isolates of *Fusarium*, mostly *F. oxysporum*, were collected for use in this study (Table 1). These strains were obtained from various geographic locations, and from various hosts or substrates. All cultures were grown as single spore isolates on Potato Dextrose Agar (PDA, Difco) and were stored as chlamydospores in sterile soil (Tousson and Nelson, 1976). All isolates identified as *F. o. cyclaminis* were presumed pathogens of cyclamen. The remaining isolates were presumed pathogens of plants other than cyclamen and thus referred to collectively as the nonhost *Fusarium* of cyclamen, or nonhost *Fusarium*.

Genomic DNA Extractions

DNA was extracted using a miniprep protocol modified from Kim *et al.* (1992). Single spore cultures were grown for 3 days in 250 mL of Potato Dextrose Broth (PDB, Difco) on a rotary shaker at room temperature and harvested by vacuum filtration. The mycelial mat was ground into a fine powder in liquid nitrogen with a mortar and pestle. The mycelial powder was extracted once with CTAB extraction buffer (1 M NaCl, 5 mM Tris pH 8.0, 10 mM EDTA, 1 % ß-mercaptoethanol, 1 % hexadecyltrimethyl-ammonium bromide [CTAB]) at 65° C for 1 h, once with chloroform/isoamyl alcohol (24:1), again with CTAB extraction buffer, isopropanol precipitated at -20° C for 1 h, and resuspended in TE (10 mM Tris, 0.1 mM EDTA pH 8.0).

The DNA was then treated with RNase A (Pharmacia) at 37° C for 1 h followed by Proteinase K (Boehringer Mannheim) at 37° C for 1 h, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) until no debris was visible at the interphase. The final aqueous phase was

Table 1. Geographical origin, source, and host/substrates of *Fusarium* isolates used in this study

Isolate	Identification	Source	Origin	Host/Substrate
DAOM 115700	*F. oxysporum*	a	Quebec	*Linum usitatissimum*
DAOM 149428	*F. oxysporum*	a	Nova Scotia	*Allium cepa*
DAOM 158438	*F. oxysporum* f. sp. *medicaginis*	a	Prince Edward Island	*Medicago sativum*
DAOM 170980	*F. oxysporum*	a	Quebec	*Cucurbita* sp.
DAOM 172550	*F. oxysporum*	a	Ontario	*Dianthus caryophyllus*
DAOM 172551	*F. oxysporum*	a	Ontario	*Dianthus caryophyllus*
DAOM 175160	*F. oxysporum*	a	Manitoba	*Chrysanthemum* sp.
DAOM 193411	*F. oxysporum*	a	Alberta	*Pisum sativum*
DAOM 193413	*F. oxysporum*	a	Alberta	*Pisum sativum*
DAOM 193414	*F. oxysporum*	a	Alberta	*Pisum sativum*
DAOM 193415	*F. oxysporum*	a	Alberta	*Pisum sativum*
DAOM 193416	*F. oxysporum*	a	Alberta	*Pisum sativum*
DAOM 213391	*F. oxysporum*	a	Kenya	*Dianthus* sp.
HRS-SB 82	*F. oxysporum* f. sp. *radicis-lycopersici*	b	Unknown	*Lycopersicon esculentum*
HRS-SB 273	*Fusarium* sp.	b	Unknown	*Rosa* sp.
HRS-SB 274	*Fusarium* sp.	b	Unknown	*Cucumis sativum*
HRS-SB 275	*F. oxysporum*	b	Unknown	*Lilium* sp.
HRS-SB 276	*F. oxysporum*	b	Unknown	*Opuntia* sp.
HRS-SB 277	*F. oxysporum*	b	Unknown	*Cereus* sp.
ATCC 16061	*F. oxysporum* f. sp. *cyclaminis*	c	Germany	*Cyclamen persicum*
ATCC 34371	*F. oxysporum* f. sp. *cyclaminis*	c	France	*Cyclamen persicum*
ATCC 52422	*F. oxysporum* f. sp. *chrysanthemi*	c	Unknown	*Chrysanthemum* sp.
ATCC 11939	*F. oxysporum* f. sp. *dianthi*	c	Unknown	*Dianthus* sp.
ATCC 15642	*F. oxysporum* f. sp. *lilli*	c	Canada	*Lilium auretum*
ATCC 52429	*F. oxysporum* f. sp. *radicis-lycopersici*	c	Canada	*Lycopersicon esculentum*
ATCC 16416	*F. oxysporum* f. sp. *cucumerinum*	c	Florida	*Cucumis sativum*
ATCC 34298	*F. oxysporum* f. sp. *lycopersici*	c	California	*Lycopersicon esculentum*
Brookside	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
Darvonda	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
Westcan	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
Milner	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
Ravenek	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
SV	*F. oxysporum* f. sp. *cyclaminis*	d	British Columbia	*Cyclamen persicum*
Vollebrect Normal	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Sahin	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Evers	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Lazer	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Sakata Scarlet	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Sakata White	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Gloeckner	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed
Mann	*F. oxysporum* f. sp. *cyclaminis*	d	Unknown	Cyclamen Seed

a. Isolates obtained from Carolyn Babcock, Agriculture Canada, Centre for Land and Biological Resources Research, Ottawa, Ontario.
b. Isolates obtained from Susan Barrie, Agriculture Canada Research Station, Harrow, Ontario.
c. Isolates obtained from American Type Culture Collection, Rockville, Maryland.
d. Isolates obtained from Robert Copeman, University of British Columbia, Vancouver, B.C.

ethanol precipitated, and resuspended in TE (10 mM Tris, 1 mM EDTA pH 8.0). All centrifugation was done in an Eppendorf centrifuge model 5415. The DNA was quantified using a Gene Quant RNA/DNA Calculator (Pharmacia) and by gel quantification.

RAPD Analysis

Arbitrary random amplification of genomic DNA was performed with a set of 800 primers (10-mers), GC contents ranging from 50 to 90 %, and a set of 90 simple sequence repeat (SSR) primers (15- to 18-mers), GC contents ranging from 0 to 100 %, obtained from the University of British Columbia Nucleic Acid - Protein Service Unit.

Each 25 μL reaction contained 10 mM Tris pH 8.3, 50 mM KCl, 0.001 % gelatin, 1.5 mM $MgCl_2$, 100 μM of each dNTP (Pharmacia), 0.5 U of *Taq* polymerase (Perkin-Elmer Cetus), 25 ng of genomic DNA, and 0.2 μM primer.

Amplifications were performed using the Perkin-Elmer Cetus Gene Amp PCR System 9600. DNA was amplified for 40 cycles consisting of a denaturation at 94° C for 12 s, annealing at 36° C or 42° C (SSR primers) for 60 s, a 60 s rise to 72° C, and an extension at 72° C for 65 s. This was preceded by an initial denaturation at 94° C for 30 s or 5 min (SSR primers) and followed with a final extension at 72° C for 5 min.

The reaction products were resolved in a 1.4 % agarose gel (high strength analytical grade [Bio Rad]) in a Gibco BRL H4 gel box running at 4 V cm^{-1} for 3 h with TBE running buffer (45 mM Tris-borate, 1 mM EDTA pH 8.0). The products were visualized by UV-fluorescence staining with ethidium bromide and photographed using Polaroid type 667 or 57 film.

The initial screening with *F. o. cyclaminis* isolates compared to nonhost *F. oxysporum*

isolates was performed individually with the first 134 RAPD primers and the remainder by bulk analysis, similar to that of Michelmore *et al.* (1991). The two bulks, or pools, consisted of either 8, 10, or 13 isolates which were used to screen the isolates for possible DNA markers present in the *F. o. cyclaminis* isolates but absent in the *F. oxysporum* isolates. The first set of bulks each consisted of DNA from 8 randomly selected isolates (Table 2). Some of the *F. o. cyclaminis* isolates shared a common banding pattern and the bulks were reorganized to 13 isolates. This was done to include as many isolates as possible in the bulk, remove some *F. o. cyclaminis* isolates with common banding patterns, and minimize the overall screening required (Table 2). The bulks were later changed to 10 isolates. Again, some of the *F. o. cyclaminis* isolates shared similar banding patterns and the nonhost *Fusarium* bulk was changed to be more representative of isolates which may be found in the greenhouse (Table 2). Any primer which revealed a DNA marker specific to the *F. o. cyclaminis* isolates, or bulk, was tested against all 41 isolates.

PCR And RFLP Analysis

Amplification of genomic DNA was performed with Bt-1, ITS, H3-1, and Nts primer sets (Table 3) using the Perkin-Elmer Cetus Gene Amp PCR System 9600. Conditions and thermal cycling parameters for each 100 µL reaction are summarized in Table 4. Following amplification the products were isopropanol or ethanol precipitated, resuspended in sterile distilled water, and gel quantified. Approximately 50 ng (Bt-1, ITS, and H3-1) or 500 ng (Nts) of DNA was digested with restriction enzymes according to manufacturers' recommendations (Pharmacia, Boehringer Mannheim, and Gibco BRL) for at least 1 h. The products were resolved in a 2.5 % agarose gel (high strength analytical grade [Bio Rad]) in a Gibco BRL H4 gel

Table 2. Composition of the bulks used for RAPD analysis

Bulk trial	Nonhost *F. oxysporum* bulk	*F. o. cyclaminis* bulk
1	ATCC 52422	ATCC 16061
	ATCC 11939	Sakata Scarlet
	ATCC 15642	Mann
	ATCC 52429	Brookside
	ATCC 16416	Westcan
	ATCC 34298	Milner
	HRS-SB 275	Ravenek
	DAOM 172550	SV
2	ATCC 52422	Brookside
	ATCC 11939	Darvonda
	ATCC 15642	Westcan
	ATCC 52429	Ravenek
	ATCC 16416	SV
	ATCC 34298	Vollebrect Normale
	HRS-SB 275	Sahin
	DAOM 149428	Evers
	DAOM 170980	Lazer
	DAOM 172550	Sakata Scarlet
	DAOM 172551	Sakata White
	DAOM 175160	Gloeckner
	DAOM 213391	Mann
3	ATCC 52422	ATCC 16061
	ATCC 11939	ATCC 34371
	ATCC 15642	Brookside
	ATCC 52429	Westcan
	ATCC 16416	SV
	ATCC 34298	Vollebrect Normale
	DAOM 172550	Evers
	DAOM 172551	Lazer
	DAOM 175160	Sakata White
	DAOM 213391	Mann

Table 3. Sequence, source, and genes amplified by the primers used in this study

Primer	Sequence (5′ to 3′)	Source	Region amplified
Bt-1a	TTCCCCCGTCTCCACTTCTTCATG	a	ß-tubulin
Bt-1b	GACGAGATCGTTCATGTTGAACTC	a	ß-tubulin
ITS 1	TCCGTAGGTGAACCTGCGG	a	rDNA, internal transcribed spacer
ITS 4	TCCTCCGCTTATTGATATGC	a	rDNA, internal transcribed spacer
H3-1a	ACTAAGCAGACCGCCCGCAGG	a	histone
H3-1b	GCGGGCGAGCTGGATGTCCTT	a	histone
Nts1	TTTTGATCCTTCGATGTCGG	b	rDNA, intergenic spacer
Nts2	AATGAGCCATTCGCAGTTTC	b	rDNA, intergenic spacer

a. Primers obtained from N. Louise Glass, University of British Columbia, Vancouver, B.C.
b. Primers obtained from C. André Lévesque, Pacific Agriculture Research Centre, Vancouver, B.C.

Table 4. PCR conditions using the Bt-1, ITS, H3-1, and Nts primer sets

PCR	Bt-1, ITS, and H3-1 primers	Nts primers
Reagents:		
Tris pH 8.3	10 mM	10 mM
KCl	50 mM	50 mM
gelatin	0.001 %	0.001 %
$MgCl_2$	2 mM	1.5 mM
each dNTP (Pharmacia)	200 μM	200 μM
Taq polymerase (Perkin-Elmer Cetus)	2.5 U	2.5 U
DNA	250 ng	100 ng
each primer	0.1 μM	0.2 μM
Thermal Cycler:		
initial denaturation	94° C, 5 min	94° C, 5 min
denaturation	94° C, 1 min	94° C, 30 s
annealing	60° C, 1 min	65° C, 1 min
ramp to extension	1 min	1 min
extension	72° C, 1 min	72° C, 2 min
final extension	72° C, 6 min	72° C, 6 min
total cycles	30	30

box running at 5 V cm^{-1} for 2 h with TBE running buffer (45 mM Tris-borate, 1 mM EDTA pH 8.0). The products were visualized by UV-fluorescence staining with ethidium bromide and photographed with Polaroid type 57 film. All centrifugation was done in an Eppendorf centrifuge model 5415.

The initial screening was done with three presumed pathogenic isolates compared to three nonpathogenic isolates. The *F. o. cyclaminis* isolates selected for amplification were SV (field isolate), ATCC 34371 (typed isolate), and Sakata Scarlet (seed isolate). The nonhost *F. oxysporum* isolates selected were ATCC 15642 (pathogen of lily), ATCC 11939 (pathogen of carnation), and ATCC 52422 (pathogen of chrysanthemum).

Subtraction Hybridization

The *F. o. cyclaminis* isolate SV was chosen as the probe isolate and 5 µg genomic DNA was partially digested with *Sau*3AI for 1 h according to manufacturer's recommendations (Gibco BRL) to generate fragments less than 1000 bp. The DNA was ethanol precipitated and resuspended in 200 µL TE (10 mM Tris, 1 mM EDTA pH 8.0).

The isolates chosen as subtracter isolates were ATCC 52422, 11939, 15642, 52429, 16416, and 34298. A solution containing 120 µg of genomic DNA in a volume of 240 µL of TE (10 mM Tris, 1 mM EDTA pH 8.0), was obtained by pooling 20 µg of DNA from each isolate. This subtracter pool was sonicated with a Sonic 300 ultrasonicator set at 30 % to give an average size range of 500 to 3000 bp.

Subtraction hybridization was performed at an approximate ratio of 30:1 (subtracter:probe). Approximately 190 µL of the subtracter pool was combined with 200 µL of

the digested probe. The mixture was denatured by boiling at 100° C for 5 min, allowed to reanneal at 65° C for 18 h, and then cooled to room temperature. The reannealled DNA was ethanol precipitated and resuspended in 58 μL sterile distilled water.

A total of 2.5 μg of pUC18 vector DNA was digested with *Bam*HI (Gibco BRL) according to manufacturer's recommendations, dephosphorylated with calf intestinal phosphatase (New England Biolabs) in Boehringer Mannheim dephosphorylation buffer, gel purified in 1 % low melting point agarose (LMP [Gibco BRL]) with TAE running buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0), and recovered in a 13 μL volume of agarose.

Four 20 μL ligations were performed using 14.5 μL aliquots of the subtracted DNA, 1 μL dephosphorylated pUC18, 0.5 U T4 DNA ligase (Gibco BRL), 1 mM dATP pH 7.0, in Promega ligation buffer at 14° C overnight. The ligations were pooled, ethanol precipitated, resuspended in 40 μL sterile distilled water, and dialyzed overnight in sterile distilled water.

The dialyzed DNA was used to transform *Escherichia coli* DH10B by electroporation using the Bio Rad Gene Pulser Electroporation System. Each electroporation event was performed according to manufacturer's recommendations with 2 μL of DNA and pulsed once using a 0.2 cm gap between electrodes. The cells were regenerated for at least 1 h at 37° C in SOC medium (2 % bacto-tryptone [Difco], 0.5 % bacto-yeast extract [BBL (Becton Dickinson)], 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and grown on LB plates (1 % bacto-tryptone, 0.5 % bacto-yeast extract, 1 % NaCl, 1.5 % agar [BBL Granulated Agar (Becton Dickinson)]) with ampicillin selection (100 μg mL^{-1}). Colonies with recombinant plasmids were identified by screening for ß-galactosidase activity on media containing X-gal (Sambrook *et al.*, 1989).

Putative transformants were grown overnight in LB with ampicillin selection (100 μg mL^{-1}) at 37° C. Plasmid DNA was recovered by the miniprep protocol of Zhou *et al.* (1990). The plasmid DNA was digested simultaneously with *Eco*RI (Gibco BRL) and *Hind*III (Gibco BRL) at 37° C for at least 2 h. RNaseA (Pharmacia) was included to digest any contaminating RNA. The inserts were resolved in 1.2 or 2.5 % agarose gels (high strength analytical grade [Bio Rad]). All centrifugation was done in an Eppendorf centrifuge model 5415.

Labeling and detection for all dot blots were performed according to manufacturer's recommendations using the ECL random prime labeling and detection system (Amersham). Membranes used for all dot blots were Biodyne B nylon membranes (Pall). Dot blots with plasmid miniprep DNA were probed with 1 μg of genomic DNA at 68° C overnight and washed with 0.1 X SSC/0.1 % SDS in a Haake SWB 20 shaking waterbath. Dot blots with 2 μg of genomic DNA were probed with DNA, prepared by the Wizard DNA Purification System (Promega), as previously described. Autoradiographs were exposed on Kodak X-OMAT AR or Island Scientific autoradiography film.

Cluster Analysis

The banding patterns produced by specific RAPD primers were assessed by assigning a position to reproducible bands less than 2000 bp. Each band was given a value of 1 or 0 whenever it was present or absent, respectively. The relative band intensity was ignored and bands common to all isolates were not scored as similarities. Lower relative intensity bands which migrated closely with higher relative intensity bands were not scored when the result proved ambiguous.

The data collected were used to construct a similarity matrix using Jaccard's similarity coefficient (Sneath and Sokal, 1973). The matrix was subjected to cluster analysis using the unweighted paired group method with arithmetic averaging (UPGMA) (Sneath and Sokal, 1973) to construct a dendrogram. The computer program SYSTAT for Windows version 5.03 (SYSTAT, Inc.) was used for all computations.

Pathogenicity Tests

Pathogenicity tests were performed on young cyclamen seedlings using fungal spore suspensions. Conidia were harvested from cultures grown on PDA (Difco) plates using sterile distilled water and diluted to a concentration of 1×10^6 spores mL^{-1}. The seedlings were removed from styroblocks, rinsed of media, and had 5 mm of their root tips removed using a sterile scalpel. The roots of the seedlings were dipped in the spore suspension for approximately 10 s and transplanted into 3 inch pots containing a soil mix of two parts sterile soil and one part peat. The seedlings were further inoculated with 1 mL of spore suspension directly above the crown. Each treatment was replicated using three plants. The plants were grown in a greenhouse from January to July 1995 with no supplemental lighting, a setting of 10° C and 24° C, and a daily temperature fluctuation range of between 15° C to 35° C. Fungal isolates were scored for their ability to kill the plant and whether or not typical symptoms were observed. The presence of *F. oxysporum* within the corms of living and dead plants was determined by plating onto modified Komada agar (MKA)(Komada, 1975). Corms were harvested, rinsed of media, and cut open latitudinally. Pieces of the vascular tissue, 2 to 3 mm^2, were removed aseptically and incubated on MKA.

Results

Pathogenicity Tests

The isolates on hand were not tested prior to their use by this researcher. The seed and greenhouse *F. o. cyclaminis* isolates were presumed to be pathogenic based on random pathogenicity tests of eight seed and corm isolates (R.J. Copeman, personal communication). Further investigation revealed that seven of the 16 putative *F. o. cyclaminis* isolates used in this study had been previously tested for pathogenicity (Table 5). Of the 16 putative *F. o. cyclaminis* isolates tested in the current study, only five caused typical wilt symptoms (Fig. 1) and killed the plants within 4 to 8 weeks after their inoculation. The remaining *F. o. cyclaminis* inoculated plants did not develop wilt symptoms up to 17 weeks after inoculation. All 16 *F. o. cyclaminis* isolates were tested again with the same results. The five isolates determined to be pathogenic by this study were ATCC 16061, ATCC 34371, Brookside, Darvonda, and Ravenek (Table 5). The SV, Sahin, Evers, and Lazer isolates had apparently lost pathogenicity. The remaining putative *F. o. cyclaminis* isolates were not tested prior to this study and it is possible that some of these were not pathogenic. The control and nonhost *Fusarium* inoculated plants did not exhibit external wilt symptoms after 9 or 17 weeks, nor were any of these plants killed by the 25 nonhost *Fusarium* isolates tested.

Upon examination of the corms from the dead plants, they all were found to have some degree of vascular discolouration (Fig. 2). *F. oxysporum* was recovered from these corms regardless of the presence, absence, or degree of vascular discolouration. The corms of all plants which did not die were also examined for vascular discolouration. The living control plants, nonhost *Fusarium* inoculated plants, and living *F. o. cyclaminis* inoculated plants all exhibited

Table 5. Pathogenicity test results for the putative *F. oxysporum* f. sp. *cyclaminis* isolates used in this study

Isolate	From a previous study	From this study
ATCC 16061	+	+
ATCC 34371	+	+
Brookside	+	+
Darvonda	n.t.	+
Westcan	n.t.	-
Milner	n.t.	-
Ravenek	n.t.	+
SV	+	-
Vollebrect Normale	n.t.	-
Sahin	+	-
Evers	+	-
Lazer	+	-
Sakata Scarlet	n.t.	-
Sakata White	n.t.	-
Gloeckner	n.t.	-
Mann	n.t.	-

+ = pathogenic
- = nonpathogenic
n.t. = not tested

A

B

Figure 1. External disease symptoms of *F. oxysporum* f. *sp. cyclaminis* on cyclamen. Comparison of a (A) healthy, and (B) diseased host plant.

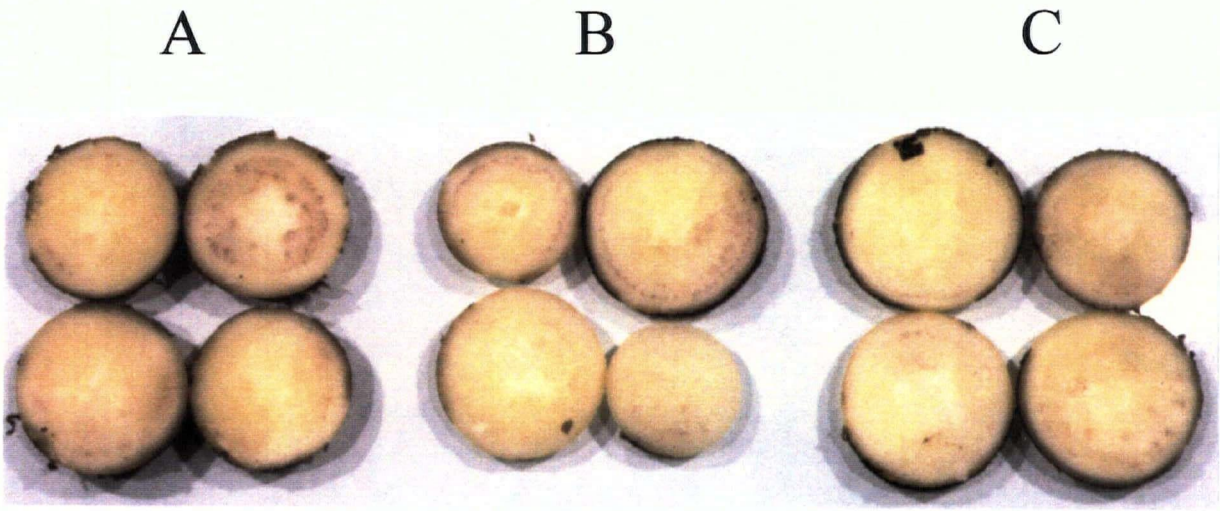

Figure 2. Corms of cyclamen exhibiting varying degrees of vascular discolouration. Comparison of corms from (A) living control plants, (B) living nonhost *Fusarium* inoculated plants, and (C) living or dead *F. oxysporum* f. sp. *cyclaminis* inoculated plants.

some degree of vascular discolouration similar to the dead plants and could not be distinguished from the corms of the dead plants killed by *F. o. cyclaminis* (Fig. 2). A random sampling of the corms from living plants revealed no *F. oxysporum* present regardless of the presence, absence, or degree of vascular discolouration. This suggested that there may be other factor(s) influencing vascular discolouration.

RAPD Analysis

The first technique used to identify DNA markers specific to *F. o. cyclaminis* was RAPD analysis. A total of 890 primers was tested; most primer sets worked well with the *Fusarium* DNA but approximately one third of the primers failed to give any amplification when examined by sets and collectively (Table 6). The exception was primer set 100/1. This set failed to give any amplification with approximately half of its primers. There did not appear to be anything which distinguished primer set 100/1 from sets 100/2 through 100/8. For future reference, primer set 100/1 may not be a good set to begin screening due to the poor efficiency at which it amplifies *Fusarium* DNA.

All potential diagnostic markers for the *F. o. cyclaminis* isolates were found either to be absent in some of these isolates and/or present in the 25 nonhost *Fusarium* isolates. For example, a DNA marker of approximately 1500 bp was identified by bulk analysis as present in the *F. o. cyclaminis* bulk but absent in the nonhost *F. oxysporum* bulk. When examined as individual isolates, the DNA marker was found to be present in seven nonpathogenic isolates of *F. o. cyclaminis* and also present in one nonhost isolate, 193416 (Fig. 3). Because of the number of shared DNA markers between the *F. o. cyclaminis* and nonhost *Fusarium* isolates, no marker was found to be unique to the collection of *F. o. cyclaminis* isolates on hand. Eleven of the *F. o. cyclaminis* isolates were observed to have lost pathogenicity at the end of this study but some had not been tested prior to this study (Table 5). This fact may explain the difficulty in obtaining DNA markers specific for the *F. o. cyclaminis* isolates because between five to ten of these apparently nonpathogenic isolates were included in the strains used for bulk analysis.

Table 6. Summary statistics of amplification for the RAPD primer sets used in this study

Primer set	Primer numbers	Percentage of primers which did not amplify
100/1	1-100	53
100/2	101-200	28
100/3	201-300	32
100/4	301-400	30
100/5	401-500	34
100/6	501-600	17
100/7	601-700	34
100/8	701-800	14
100/9	801-890	23
100/1-100/9	1-890	30

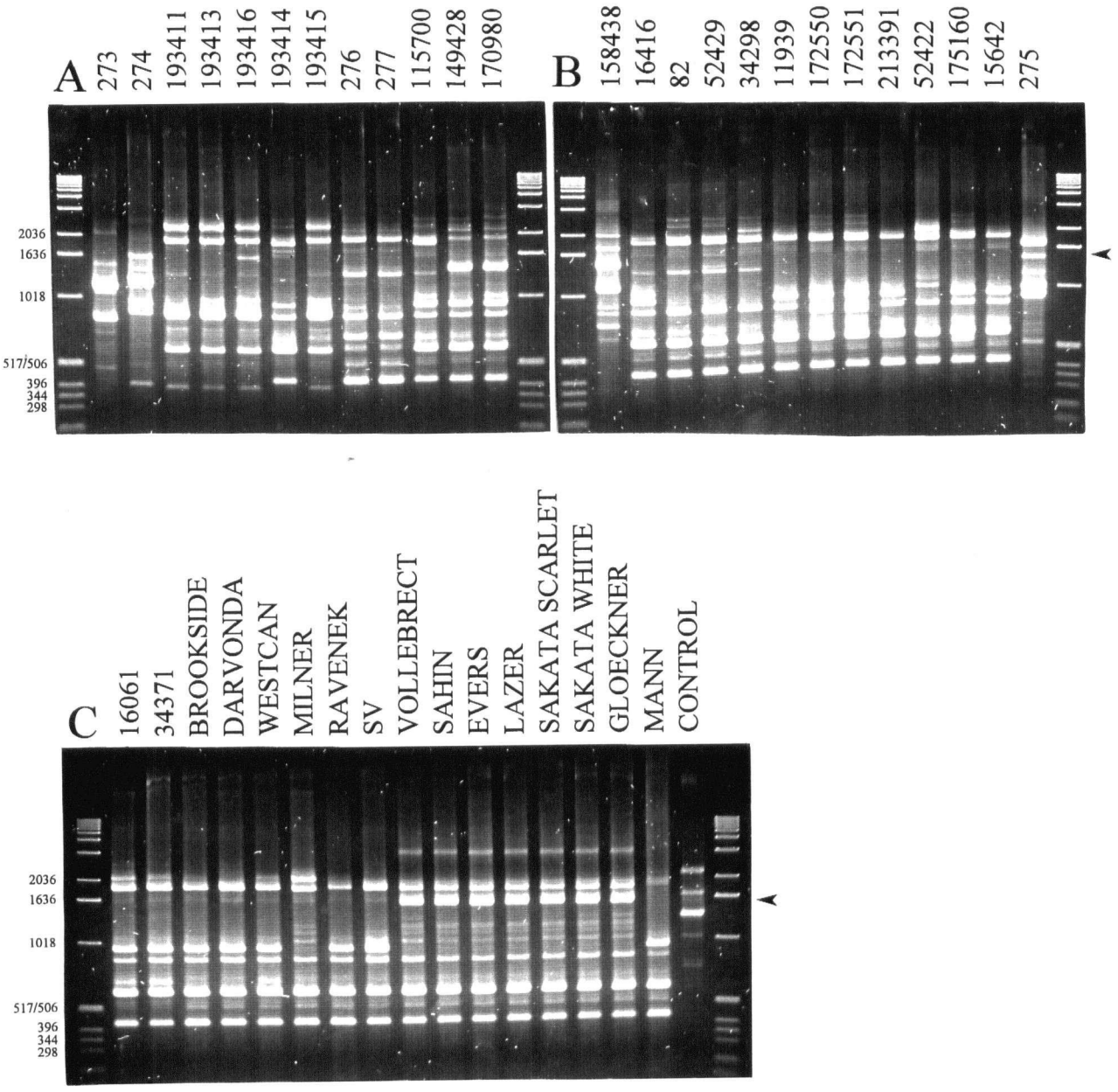

Figure 3. RAPD analysis of *Fusarium* isolates with primer 890. The amplified DNAs from (A) 12 isolates of nonhost *Fusarium*, (B) 13 isolates of nonhost *F. oxysporum*, and (C) 16 isolates of *F. oxysporum* f. sp. *cyclaminis* electrophoresed in a 1.4 % agarose gel and stained with ethidium bromide. Isolates are identified above each lane. Size markers are indicated in base pairs on the left. The 1500 bp marker identified by bulk analysis is indicated by an arrow on the right.

PCR And RFLP Analysis

Four PCR primer sets were used in RFLP analysis to identify DNA markers specific to *F. o. cyclaminis*. Six isolates of *F. oxysporum* were amplified with the Bt-1, ITS, and H3-1 primer sets. The Bt-1 primers amplified a fragment of approximately 570 bp for all six isolates, the ITS primers amplified a fragment of approximately 550 bp, and the H3-1 primers amplified a fragment of approximately 515 bp. Within each primer set, the PCR products appeared identical in apparent molecular weight. Each region was digested with *Acc*I, *Alu*I, *Ava*I, *Ban*II, *Bst*UI, *Bst*YI, *Dde*I, *Fok*I, *Hae*III, *Hinc*II, *Hinf*I, *Hph*I, *Mbo*I, and *Taq*I. The H3-1 region was not cut with *Ava*I, *Bst*YI, and *Dde*I, while the ITS region was not cut with *Acc*I, *Ava*I, *Ban*II, *Fok*I, and *Hph*I. Otherwise, all six isolates appeared identical with most DNA and enzyme combinations, and fragment length polymorphisms were limited to the Bt-1 region digested either with *Dde*I or *Fok*I (Fig. 4). Because of the lack of variability among the six isolates, no marker was found which could differentiate the three putative *F. o. cyclaminis* isolates from the three nonhost *F. oxysporum* isolates used and further screening was not performed. It was concluded that these three primer sets would not likely be useful for intraspecific identification of *F. oxysporum*.

The same isolates were amplified with the Nts primer set with an observed amplification product of approximately 2900 bp for all six isolates (Fig. 5A). This IGS region was digested with *Acc*I, *Alu*I, *Ava*I, *Ban*II, *Bst*UI, *Bst*YI, *Cfo*I, *Hae*III, *Hinf*I, *Hph*I, *Mbo*I, *Mva*I, *Rsa*I, and *Taq*I. All enzymes, which were tested, cut within this region and the IGS was found to be more variable than the previous three regions, using the same isolates. Only in digests with *Ban*II or *Cfo*I were no fragment length polymorphisms observed between the isolates. Digestion with *Rsa*I revealed a possible diagnostic doublet of approximately 570 and 635 bp (Fig. 5B) for the

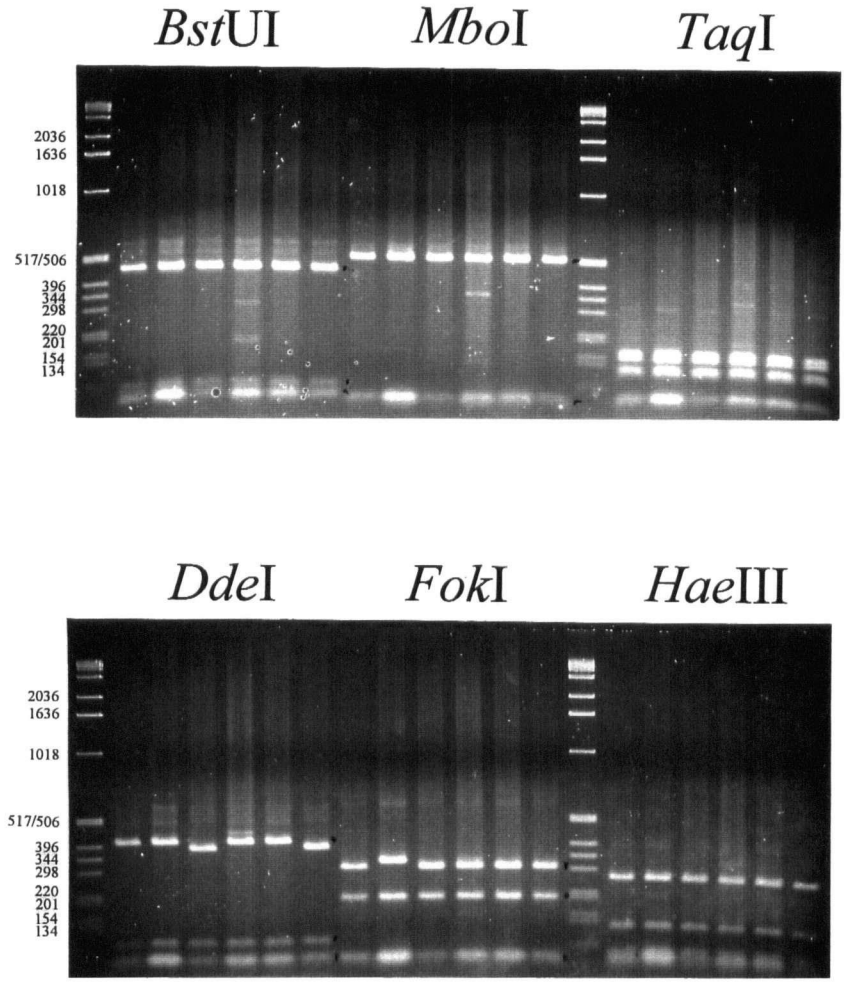

Figure 4. PCR and RFLP analysis of the ß-tubulin (Bt-1) region. Restriction fragments were electrophoresed in a 2.5 % agarose gel and stained with ethidium bromide. Restriction enzymes used are indicated above each photograph. The isolates used with each of the six restriction enzymes were: (left to right) the *F. oxysporum* f. sp. *cyclaminis* isolates SV, 34371, Sakata Scarlet, and the nonhost *F. oxysporum* isolates 15642, 11939, 52422. Size markers are indicated in base pairs on the left.

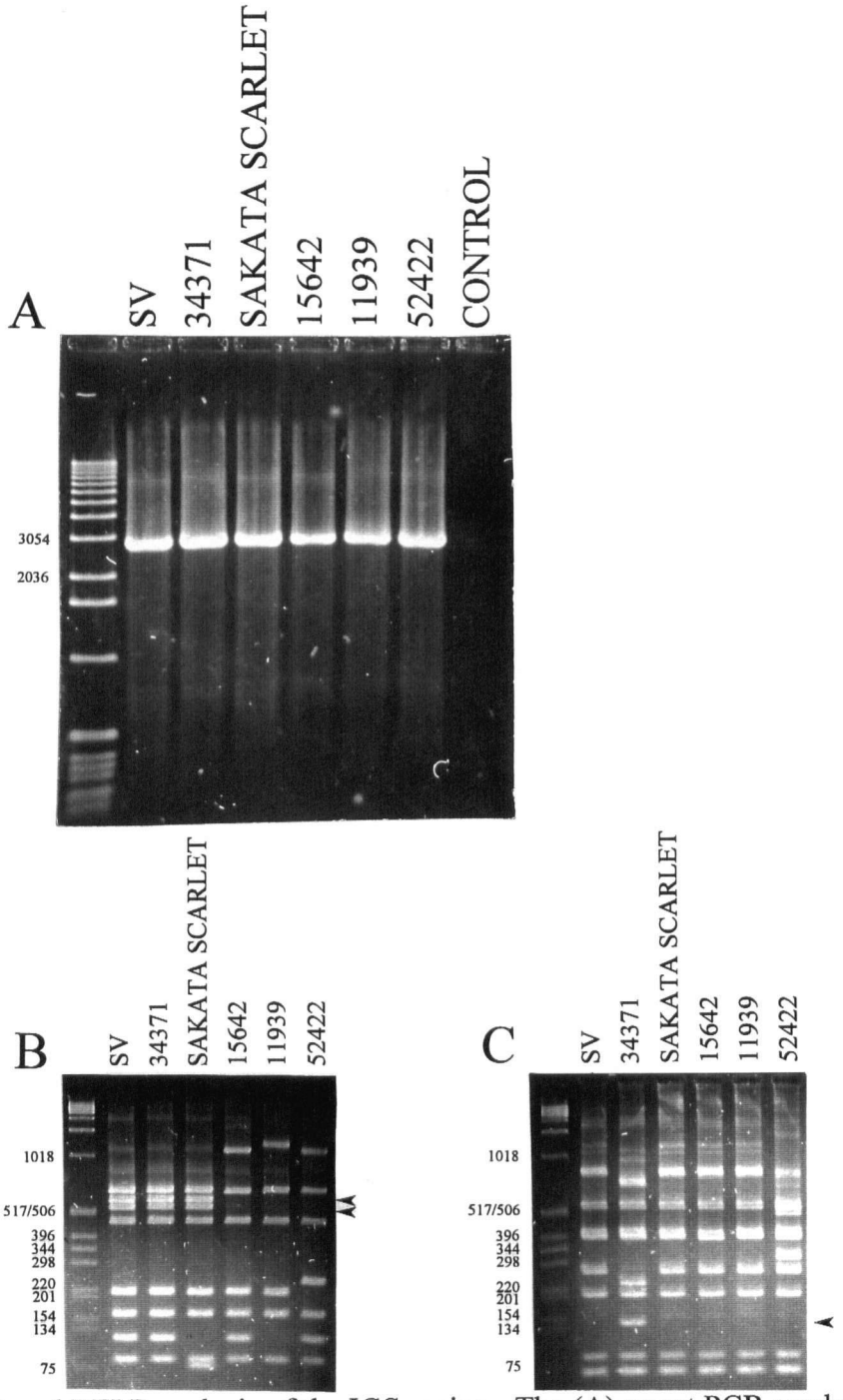

Figure 5. PCR and RFLP analysis of the IGS region. The (A) uncut PCR products, (B) PCR products digested with *Rsa*I, and (C) PCR products digested with *Alu*I are shown following electrophoresesis in 1.4 % and 2.5 % agarose gels respectively and stained with ethidium bromide. The *F. oxysporum* f. sp. *cyclaminis* isolates SV, 34371, Sakata Scarlet, and the nonhost *F. oxysporum* isolates 15642, 11939, 52422 are identified above each lane. Size markers are indicated in base pairs on the left. The diagnostic doublet of 570/635 bp is indicated by arrows on the right of the *Rsa*I digests (B), and a potential DNA marker to *F. oxysporum* f. sp. *cyclaminis* is indicated by an arrow on the right of the *Alu*I digests (C).

three putative *F. o. cyclaminis* isolates. Upon amplification of the remaining 13 putative *F. o. cyclaminis* and 21 nonhost *Fusarium* isolates, size polymorphisms were observed only in the nonpathogenic group of isolates, these uncut products ranged in size from 2600 to 3300 bp. Digestion of all 16 putative *F. o. cyclaminis* and 24 nonhost *Fusarium* isolates with *Rsa*I however revealed that neither fragment of the diagnostic doublet was associated with all *F. o. cyclaminis* isolates nor were they specific to the *F. o. cyclaminis* isolates as a group (Fig. 6).

It was later observed that, of the three putative *F. o. cyclaminis* isolates used in the initial screenings, only the ATCC 34371 isolate was pathogenic. Reinterpretation of the data did not appear to change the results of the analysis using the ITS or H3-1 primer sets, but may have affected the analyses using the Bt-1 and Nts primer sets. There were differences in restriction patterns between the single pathogenic and the remaining five nonpathogenic isolates of the Bt-1 region digested with *Fok*I restriction enzyme (Fig. 4) and of the IGS region digested with *Alu*I (Fig. 5C), *Hinf*I, *Mbo*I, *Mva*I, and *Taq*I restriction enzymes. These differences may have proven useful for identification purposes upon further examination.

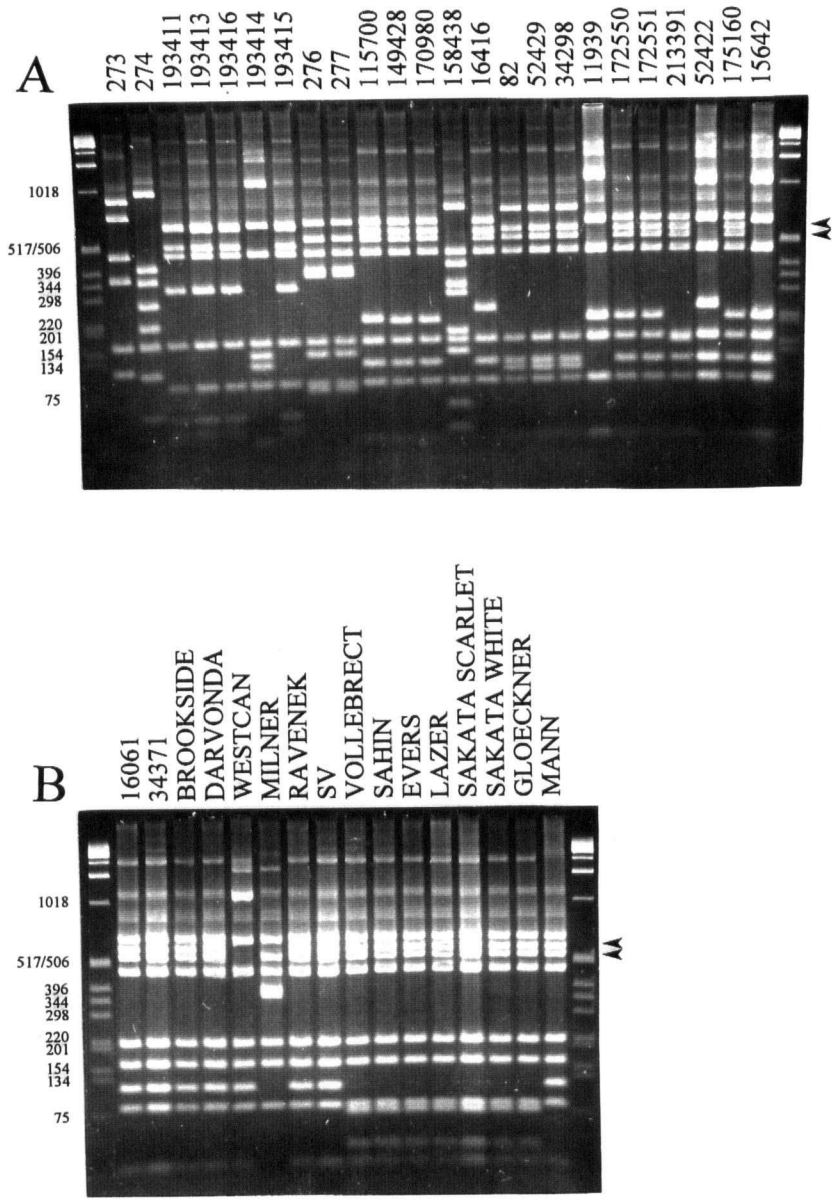

Figure 6. PCR and RFLP analysis of the IGS region digested with *Rsa*I. The amplified DNAs from (A) 24 isolates of nonhost *Fusarium*, and (B) 16 isolates of *F. oxysporum* f. sp. *cyclaminis* digested with *Rsa*I, were electrophoresed in a 2.5 % agarose gel, and stained with ethidium bromide. Isolates are identified above each lane. Size markers are indicated in base pairs to the left. The diagnostic doublet of 570 and 635 bp are indicated by arrows on the right.

Subtraction Hybridization

The subtraction hybridization technique was also used to identify DNA markers specific to *F. o. cyclaminis*. The SV isolate was chosen for generation of a probe because of its pathogenicity in a previous study. A total of 34 clones were recovered from subtraction hybridization with inserts ranging in size from less than 75 bp up to 4550 bp. The plasmid DNA from these clones were dotted identically on two membranes, and then probed either with SV or subtracter pool genomic DNA to determine whether any of the clones contained insert DNA specific to the SV isolate. One clone was found which hybridized with the SV genomic DNA and not the subtracter pool genomic DNA. The 125 bp insert from this clone was then used to probe a dot blot containing genomic DNA from all 41 *Fusarium* isolates used in this study (Fig. 7). There appeared to be some vector carryover with the probe, noticeable at high stringency with the pUC18 and pBluescript II KS vector controls. The 125 bp insert was not found to be useful because only three of the putative *F. o. cyclaminis* isolates were detected; SV, Ravenek, and Mann. Additionally, the nonhost *F. oxysporum* isolates 149428, 172550, and 172551 were detected. The SV isolate was later observed to have lost pathogenicity, raising concern as to the suitability of identifying DNA markers to pathogenic *F. o. cyclaminis* isolates from an apparently nonpathogenic isolate.

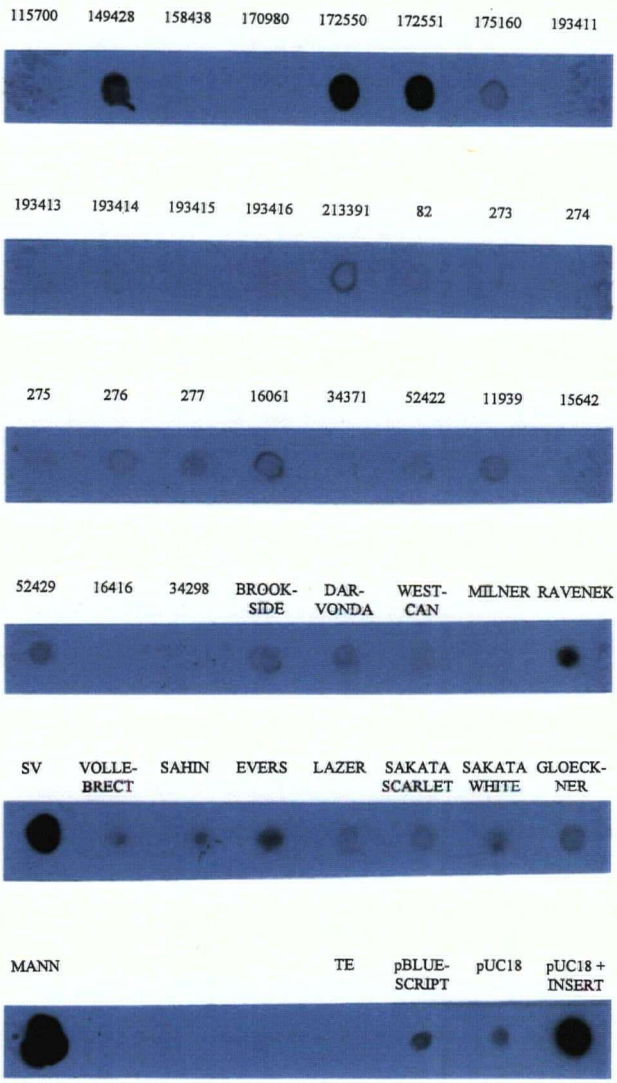

Figure 7. Specificity of the subtraction hybridization probe. Genomic dot blots of 41 isolates of *Fusarium* probed with the 125 bp insert recovered from the isolate SV by subtraction hybridization. Approximately 2 µg of genomic DNA was used for each dot blot. The blot was washed under high stringency conditions (68° C, 0.1 X SSC, 0.1 % SDS). Isolates are identified above each dot blot.

Cluster Analysis

An additional benefit of performing RAPD analysis is that the data are suitable for population studies. RAPD analysis using primers which preferentially anneal to repetitive DNA sequences is similar to DNA fingerprinting using Southern blotting and a short repetitive DNA probe, such as variable numbers of tandem repeats (VNTRs). Because of the potential of repetitive elements to reveal variation in populations, the RAPD data using the SSR primer set was selected for use in cluster anlaysis. Cluster analysis was performed using the data from six RAPD primers individually; each of these primers amplified 10 to 23 scorable markers. The scorable markers from all six primers, when analyzed individually, revealed a dendrogram with mainly unresolved clusters of individuals with 100 % similarity to each other (Fig. 8). By comparison, when all 103 RAPD markers were combined into a single data set a better resolution of the isolates within the previously unresolved clusters was observed (Fig. 9). Examination of all 16 putative *F. o. cyclaminis* isolates revealed that there was a group of seven seed isolates which appeared identical to each other and which exhibited 45 % similarity to the remaining nine *F. o. cyclaminis* isolates. The remaining nine isolates, consisting of mostly greenhouse isolates, also shared more similarity (53 %) to over half of the nonhost *Fusarium* isolates than to the seven putative *F. o. cyclaminis* seed isolates. Two flower pathogens, *F. o. lilii* and *F. o. dianthi*, also exhibited 67 % similarity to a cluster of seven pathogenic isolates which themselves shared 81 % similarity.

When the pathogenicity of all isolates was tested near the completion of this study, only five of the *F. o. cyclaminis* isolates were pathogenic, indicated as (+), and the remainder had apparently lost pathogenicity or were not previously tested and were now determined to be

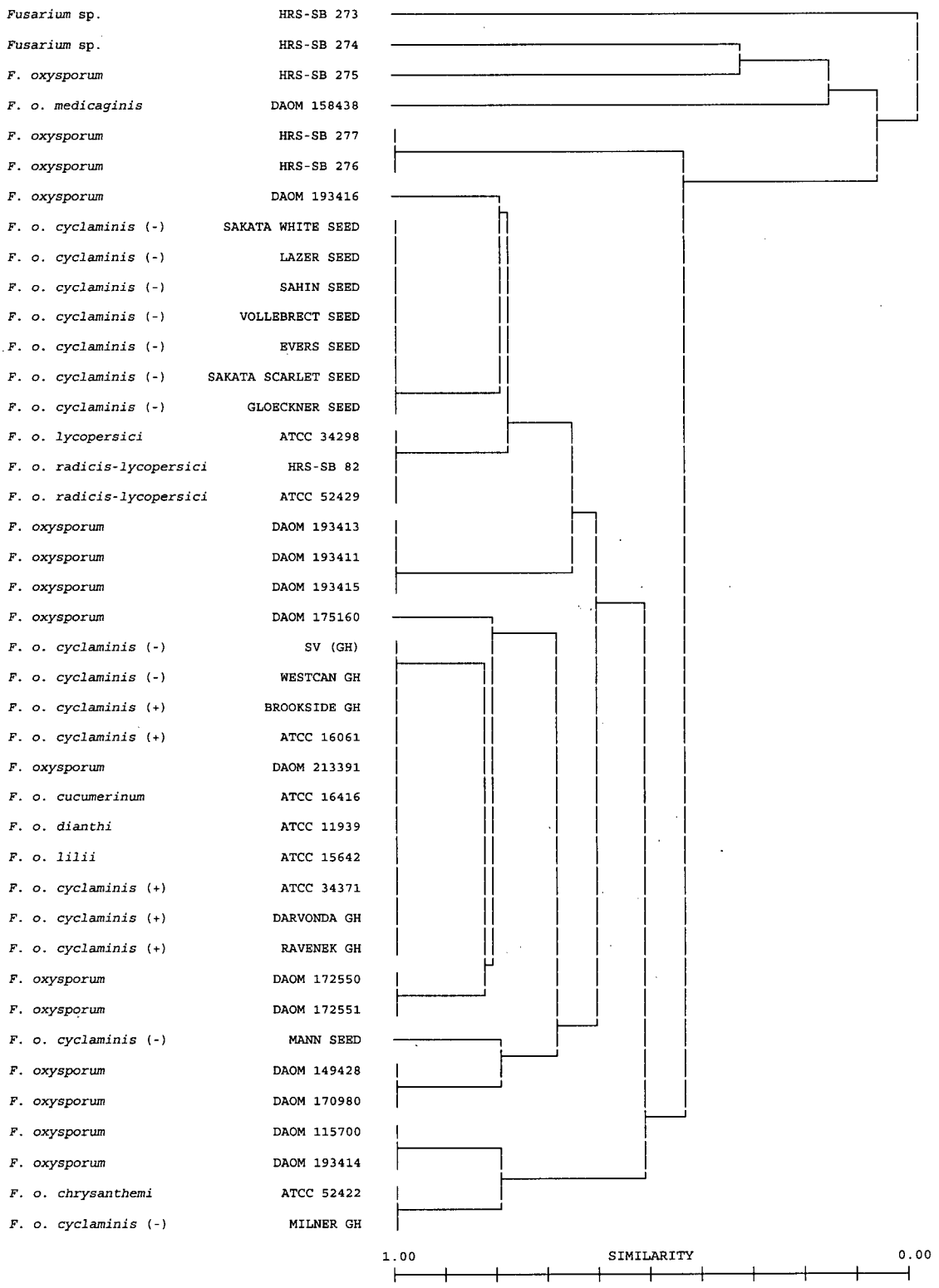

Figure 8. UPGMA cluster analysis of the RAPD data from primer 890. Dendrogram generated by the UPGMA cluster analysis of Jaccard coefficients of similarity from 15 markers generated by RAPD primer 890 using 41 isolates of *Fusarium*. *F. oxysporum* f. sp. *cyclaminis* isolates are indicated as pathogenic (+), or as nonpathogenic (-), as determined by pathogenicity testing.

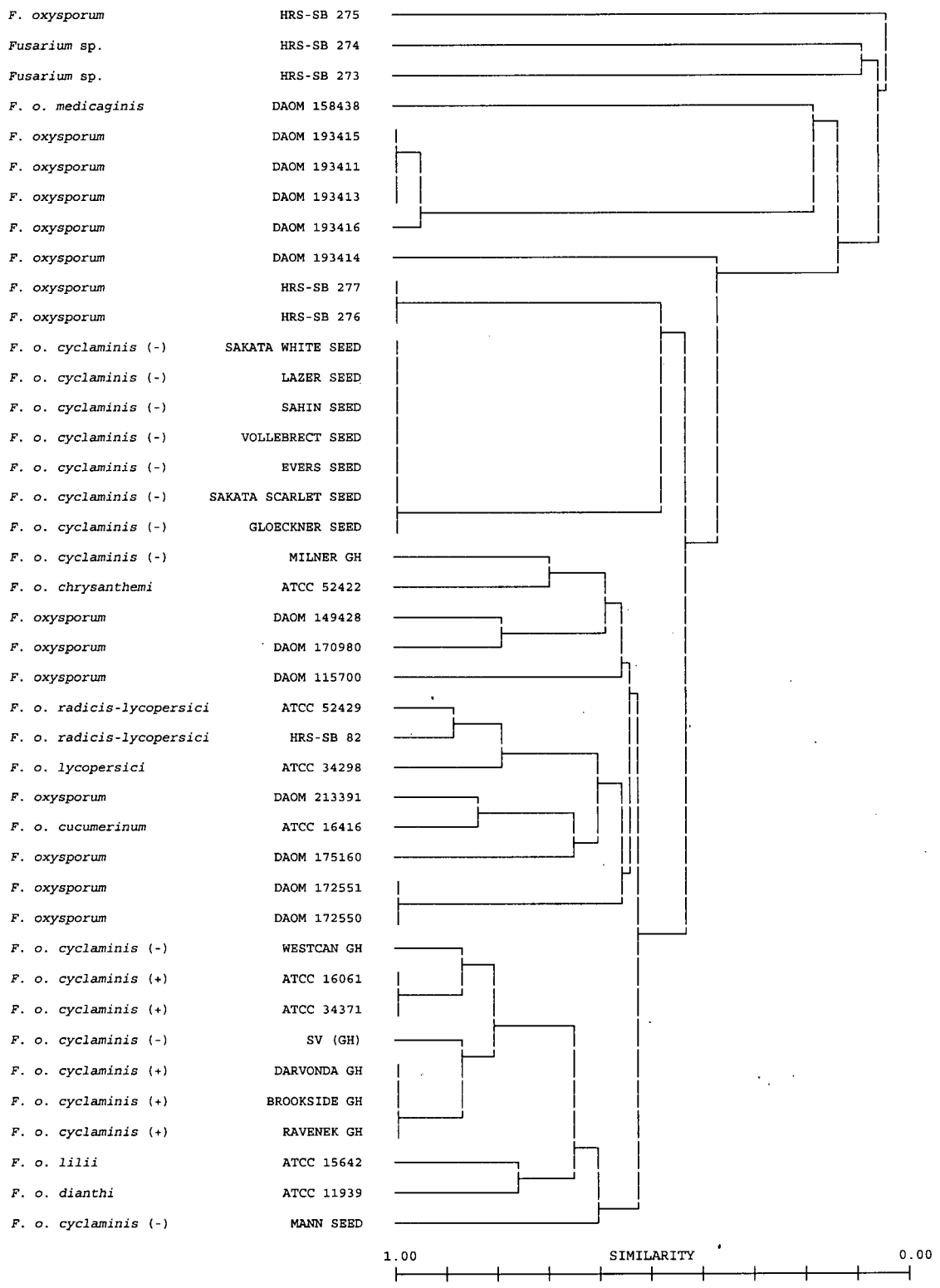

Figure 9. UPGMA cluster analysis of the RAPD data from six primers. Dendrogram generated by the UPGMA cluster analysis of Jaccard coefficients of similarity from 103 markers generated by six RAPD primers using 41 isolates of *Fusarium*. *F. oxysporum* f. sp. *cyclaminis* isolates are indicated as pathogenic (+), or as nonpathogenic (-), as determined by pathogenicity testing.

nonpathogenic, indicated as (-). If only the five pathogenic isolates are examined, then they fall into two clusters (Fig. 9). The first cluster, which consisted of the ATCC 16061 and 34371 isolates, shared 81 % similarity to the second cluster, which consisted of the Darvonda, Brookside, and Ravenek isolates. The first cluster also shared 88 % similarity to the nonpathogenic Westcan isolate, while the second cluster also shared 87 % similarity to the nonpathogenic SV isolate. These seven *F. o. cyclaminis* isolates also shared 67 % similarity to the remaining 34 *Fusarium* isolates.

Cluster analysis using the 35 markers from the *Rsa*I digested IGS region of 40 *Fusarium* isolates is shown for comparison to the RAPD data and revealed several large clusters of isolates with 100 % similarity (Fig. 10). This result was similar to the results using data from individual RAPD primers (Fig. 8). Examination of all putative *F. o. cyclaminis* isolates revealed that the seven seed isolates identified using the RAPD data again clustered together with 100 % similarity. Also, seven of the remaining nine *F. o. cyclaminis* isolates which clustered identically using the IGS data (Fig. 10) were represented in a single cluster with the RAPD data (Fig. 8) which consisted of eight of the nine greenhouse isolates. When only the five *F. o. cyclaminis* pathogenic isolates, indicated as (+), are examined, still no clear picture was obtained. The markers from a single restriction digest did not provide sufficient data to resolve this cluster.

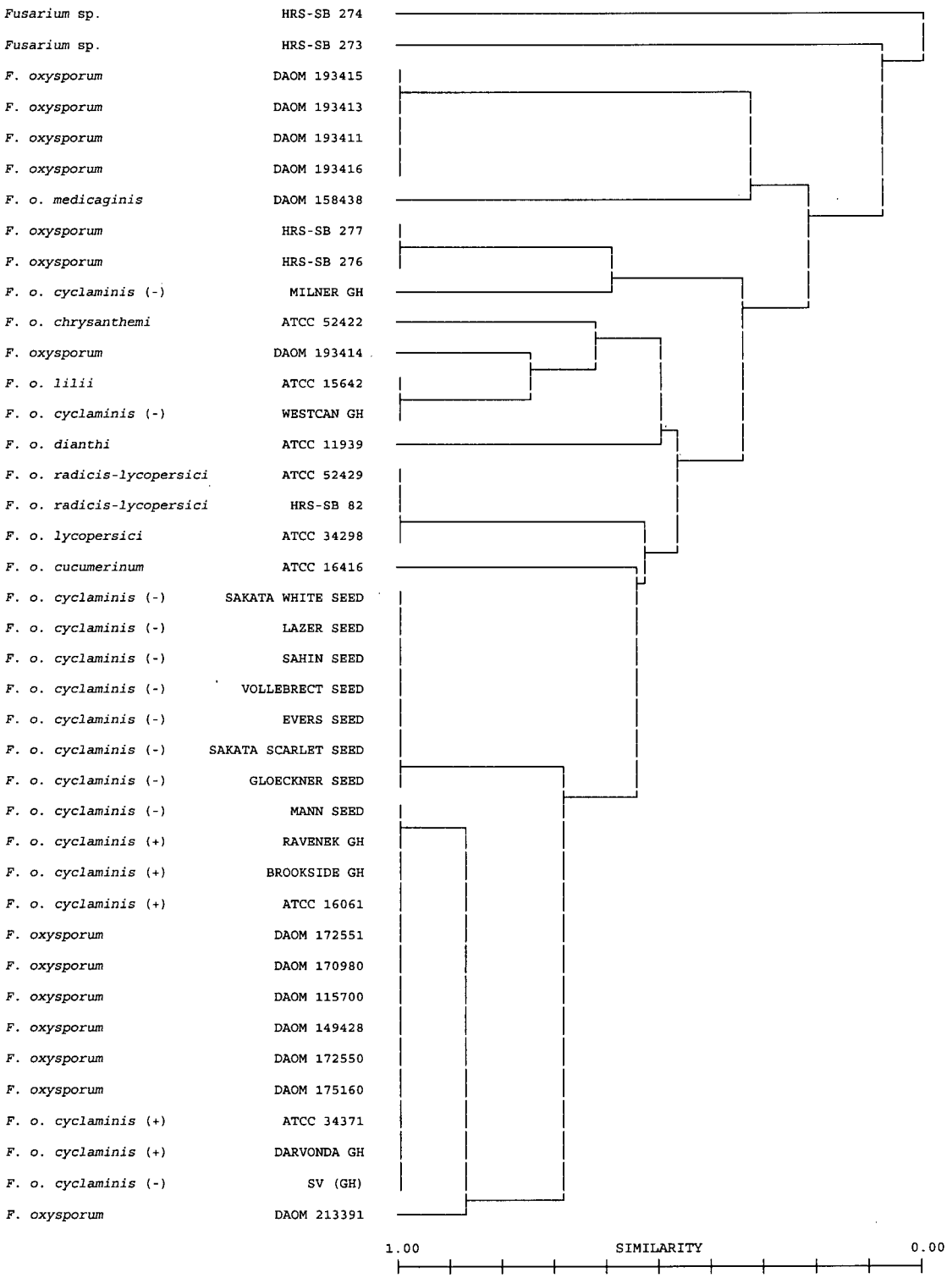

Figure 10. UPGMA cluster analysis of the PCR and RFLP data. Dendrogram generated by the UPGMA cluster analysis of Jaccard coefficients of similarity from 35 markers generated from the restriction digestion of the IGS region with *Rsa*I using 40 isolates of *Fusarium*. *F. oxysporum* f. sp. *cyclaminis* isolates are indicated as pathogenic (+), or as nonpathogenic (-), as determined by pathogenicity testing.

Discussion

The objective of this study was to identify DNA markers to the fungal pathogen *F. o. cyclaminis*. No universal DNA markers were found which would identify all the *F. o. cyclaminis* isolates from the nonhost *Fusarium* isolates. However, cluster analysis suggested that it may be possible to differentiate smaller groups of *F. o. cyclaminis* isolates from the nonhost *Fusarium* isolates. The complicating factor appeared to be the apparent loss of pathogenicity with some of the *F. o. cyclaminis* isolates.

Changes in Pathogenicity

Rather alarming was the discovery that some of the *F. o. cyclaminis* isolates lost pathogenicity on cyclamen when tested. The two ATCC cultures behaved as expected, but only three of the 14 field and seed isolates exhibited pathogenicity. The Brookside, Sahin, Evers, and Lazer isolates killed small cyclamen within 3 to 10 weeks after inoculation in a previous study. In this study the ATCC 16061, ATCC 34371, Brookside, Darvonda, and Ravenek isolates killed small cyclamen within 4 to 8 weeks after inoculation, and these results are consistent with the results from the previous pathogenicity tests. The remaining *F. o. cyclaminis* isolates were nonpathogenic on cyclamen. Because the single spore SV isolate lost pathogenicity during this study, the original polyspore SV culture stored in soil was examined to confirm its pathogenicity. Three plants were inoculated with 1×10^6 chlamydospores using the original polyspore SV soil culture directly. Two of the three plants died within 4 to 5 weeks after inoculation. Three plants were also inoculated with 1×10^6 conidia using an actively growing culture derived from the original polyspore SV soil culture. All three plants died within 3 to 4 weeks after inoculation.

The loss of pathogenicity observed with the single spore SV isolate suggested that this phenomena could have occurred with at least some of the other single spore *F. o. cyclaminis* isolates which were not pathogenic on cyclamen at the end of this study.

This loss of pathogenicity could be due to mycoviruses, double stranded (ds) RNAs, or transposable elements. It is believed that these mycoviruses or dsRNAs may somehow influence virulence or pathogenicity in some fungi (Nuss and Koltin, 1990). A dsRNA was recently found in an isolate of *F. oxysporum* but its presence or relationship with pathogenicity was not explored (Wang, 1993). More recently, transposable elements have also been identified in *F. oxysporum*; these elements may affect gene structure and function through insertion, imprecise excision, or chromosomal rearrangement (Daboussi and Langin, 1994). No evidence exists for the presence of these genetic elements in the isolates which lost pathogenicity but their presence could explain the change in pathogenicity and some of the variability observed between all the *F. oxysporum* isolates used in this study. Also, *Fusarium* is well known for its variability in growth on artificial media, as exhibited by sectoring, but this is believed to be in response to the rich artificial media used to maintain fungi (Booth, 1971). This variable random sectoring could be partly influenced by these genetic elements.

Some researchers have observed that single spore derived cultures can grow as either normal mycelial or degenerate pionnotal cultures. Single spore mycelial cultures derived from four isolates of *F. oxysporum* f. sp. *apii* have been observed to be more virulent than single spore pionnotal cultures derived from the same four isolates (Awuah and Lorbeer, 1988). If this phenomena also exists in *F. o. cyclaminis* then it is possible that during generation of the single

spore cultures, some spores may have been inadvertently selected which gave rise to colonies with reduced virulence or loss of pathogenicity.

A loss in pathogenicity could also complicate the interpretation of the results. If a change occurred, would this necessarily make the nonpathogenic *F. o. cyclaminis* isolates distinguishable from the pathogenic *F. o. cyclaminis* isolates? For instance, would a mutation to a putative pathogenicity gene that inhibited expression of this gene or changed the product expressed, have any effect on the identification of DNA markers if one is targeting repetitive elements linked to this gene? Another concern is whether some *F. o. cyclaminis* isolates lost pathogenicity before or after DNA extraction and therefore, it would be difficult to know for certain whether the extracted DNA was from a pathogenic or nonpathogenic isolate at the time of the DNA extraction.

To avoid confusion in the future, it would be best to test pathogenicity at the time of DNA extraction. If a loss of pathogenicity is associated with sustained growth of the fungus in culture, then it may be best to minimize the culture period of the fungus. This may involve the use of polyspore rather than single spore isolates. Or one could even consider the use stocks of soil with a known spore titre for plant inoculations rather than generating a new culture for use each time.

DNA Markers to *F. oxysporum* f. sp. *cyclaminis*

RAPD analysis was initially chosen because DNA markers could be identified without prior knowledge of any DNA sequences of the organism, the relative ease with which it could be accomplished, and the almost unlimited screening potential associated with this technique.

However, the results of the RAPD analysis must be interpreted with caution because some *F. o. cyclaminis* isolates had apparently lost pathogenicity at the end of this study when tested by this researcher. The inclusion of nonpathogenic *F. o. cyclaminis* isolates into the *F. o. cyclaminis* bulks may have resulted in markers from the *F. o. cyclaminis* isolates being inadvertently overlooked. The bulks were examined side by side for moderate or intense bands which were present in the *F. o. cyclaminis* bulk but absent in the nonhost *Fusarium* bulk. The inclusion of DNA from nonpathogenic *F. o. cyclaminis* isolates would have diluted the total amount of pathogenic *F. o. cyclaminis* DNA present in the reaction and resulted in weaker bands. These bands may have been unique to the pathogenic *F. o. cyclaminis* bulk but excluded from further screening because of their intensity. However, the available RAPD data suggested that many markers would be shared among the isolates used in this study and it now appears unlikely that a single DNA marker would be sufficient for all the *F. o. cyclaminis* isolates.

Looking at the results of the cluster analysis, the five pathogenic *F. o. cyclaminis* isolates were present exclusively in two clusters but had high similarity to a number of nonpathogenic *F. o. cyclaminis* isolates. Because of their high similarity, these two clusters may represent individuals from two clonal populations or two vegetative compatibility groups (VCGs) of *F. o. cyclaminis*. Within the species *F. oxysporum* several VCGs are known to exist and strains within a VCG have been argued to be more genetically similar than those between VCGs because isolates will only form heterokaryons with isolates from the same VCG (Correll, 1991). And two VCGs have been found to be exclusive to *F. o. cyclaminis* (Woudt *et al.*, 1993).

In order to fairly assess RAPD analysis, isolates from either of the two distinct pathogenic clusters should be used in their own separate bulks and compared against

nonpathogenic bulks consisting of the isolates with the highest similarity to those pathogenic isolates. This would maximize the likelihood of finding markers to a specific group of pathogenic isolates rather than a universal DNA marker for all the pathogenic isolates. This strategy of using more similar isolates would more closely approximate work done using near-isogenic lines, but using fungal pathogenicity rather than plant resistance (Barua *et al.*, 1993; Michelmore *et al.*, 1991; Paran *et al.*, 1991). Recent work with repetitive elements (Namiki *et al.*, 1994; DeScenzo and Harrington, 1994) and RAPD analysis (Assigbetse *et al.*, 1994; Kelly *et al.*, 1994; Manulis *et al.*, 1994) suggest that it should be possible to differentiate the pathogenic from the nonpathogenic isolates of *F. oxysporum*.

The other reason for choosing RAPD analysis was the usefulness of the data for the assessment of genetic variability between the isolates. Without cluster analysis, it would have been difficult to identify the pathogenic isolates with the highest genetic similarity to each other. This data may have proven more useful if VCG data for the of *F. o. cyclaminis* isolates used were examined to determine if a relationship existed between VCGs and clustering since correlations between VCGs and *formae speciales* have been observed (Correll, 1991).

The technique of combined PCR and RFLP analysis was chosen because of the availability of primer sets, restriction enzymes, and the ease with which analyses may be performed. In choosing primer sets for intraspecific identification purposes, it would appear that the ITS, ß-tubulin, and histone regions were too highly conserved and not suitable for use in a PCR and RFLP analysis to differentiate *F. oxysporum* at the intraspecific level. The loss of pathogenicity with some isolates did not appear to affect the results of the ITS and H3-1 regions but may have affected the results of the Bt-1 region. The overall lack of variability observed

between isolates suggested that these amplified regions were highly conserved within the species *F. oxysporum*. This work supported that of Donaldson *et al.* (1995); these investigators reported that with the isolates of *F. oxysporum* they tested, there was always a single or predominant restriction pattern for every combination of restriction enzyme and amplified region examined. In all cases where similar enzyme and amplified region combinations were tested, the results were identical to the reported predominant restriction pattern. As the data suggested, these three primer sets appeared to be better suited for interspecific identification of the fusaria rather than for identification of *F. oxysporum* at the intraspecific level.

The IGS region appeared less conserved and would appear to be a better choice for intraspecific identification. This is likely due to the fact that although this amplified region is a conserved nontranscribed region, it will tolerate some intraspecific variation, and also because this amplified region is approximately six times larger than the other three regions tested and the probability of detecting differences becomes greater with size. Unfortunately, only the ATCC 34371 isolate was pathogenic and so the initial screening was really with five nonpathogenic isolates compared against one pathogenic isolate. Had the initial screening been balanced between isolates from each group as intended and more representative of isolates likely to be found in the greenhouse, more useful data may have been obtained. However, the usefulness of this region for intraspecific identification should be explored further since it was successfully used to differentiate *F. o. lycopersici* from *F. o. radicis-lycopersici* (Wang, 1993), and to characterize strains of *F. oxysporum* (Edel, *et al.*, 1995).

The subtraction hybridization technique was chosen simply as an alternative to the PCR strategies and because of its past success in distinguishing between closely related bacterial

strains. The choice of the SV isolate for subtraction was unfortunate. Because a loss of pathogenicity was observed, it may not have been possible to identify any DNA associated with pathogenicity since the mechanism of change was not examined nor is the genetic basis of pathogenicity known. Although no suitable probe was recovered, the technique did work and could be useful for probe isolation with more time and effort. From the 34 clones recovered, one did not contain DNA from the six subtracter strains used. When this clone was tested against all isolates in a dot blot, only two additional *F. o. cyclaminis* isolates were detected, and unfortunately three out of a possible 25 nonhost *Fusarium* isolates were also detected. If one considers that it may be easier to find probes for specific groupings of *F. o. cyclaminis* isolates, rather than for all *F. o. cyclaminis* isolates, then the numbers seem favourable. Goodwin *et al.* (1990) were unable to isolate a universal *Phytophthora citrophthora* probe possibly because they did not subtract against a pool of isolates, and used a low subtraction ratio. The use of a subtraction pool should minimize the chances of isolating DNA not associated with pathogenicity which may have arisen from isolate to isolate variation. Also, the use of a high subtraction ratio should remove more of the nonspecific DNA from the probe strain and minimize the number of clones which contain nonspecific DNA and maximize the number of clones which can be screened.

Internal Disease Symptoms

Vascular discolouration was previously described as a symptom associated with *F. o. cyclaminis* infection but it was also noted that symptoms may not develop until flowering or a mature stage of growth (Tayama, 1987; Tompkins and Snyder, 1972). Vascular discolouration

may be an unreliable symptom associated with *F. o. cyclaminis*, especially at the early stages of growth, because it may not be visible until the plants are mature and may be influenced by other unknown factors. Vascular discolouration was noted in most of the plants used in the pathogenicity tests regardless of whether they were inoculated with the pathogen. It should also be noted that at harvest the oldest plants were approximately six months old and had not yet begun to flower but *F. oxysporum* was successfully recovered only from plants killed by the pathogenic isolates. If this is the case then vascular discolouration must be influenced by other factors, in addition to the presence of the pathogen.

Conclusions

Of the molecular techniques evaluated, only PCR and RFLP analysis using either the ITS, ß-tubulin, or histone regions exhibited insufficient intraspecific variation to warrant further research. In choosing a technique for continuation of this research, either PCR and RFLP analysis using the IGS region or RAPD analysis would be a good choice. PCR and RFLP analysis is recommended because of the ease with which it may be performed; unfortunately this technique is limited by the availability of restriction enzymes. RAPD analysis has the advantage of its almost unlimited screening potential. Subtraction hybridization would not be recommended due to the greater amount of time required to screen colonies and the greater technical skills required for performing blots. Cluster analysis combined with RAPD analysis was useful in the assessment of genetic variability of the *Fusarium* isolates, but may have been more useful with knowledge of VCGs. In future studies involving *F. o. cyclaminis*, the pathogenicity of single spore isolates must be confirmed. Vascular discolouration did not appear to be a reliable disease symptom because it may be influenced by other factors.

Bibliography

Adam-Blondon, A.F., Sévignac, M., Bannerot, H., and Dron, M. 1994. SCAR, RAPD and RFLP markers linked to a dominant gene (*Are*) conferring resistance to anthracnose in common bean. Theor. Appl. Genet. 88:865-870.

Agrios, G.N. 1988. Plant pathology. Academic Press, Inc., San Diego, CA. 803 pp.

Armstrong, G.M., and Armstrong, J.K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt disease. Pages 391-399 in *Fusarium*: Disease, biology, and taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., eds. The Pennsylvania State University Press, University Park, PA.

Assigbetse, K.B., Fernandez, D., Dubois, M.P., and Geiger, J.-P. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 84:622-626.

Awuah, R.T., and Lorbeer, J.W. 1988. Nature of cultural variability in *Fusarium oxysporum* f. sp. *apii* race 2. Phytopathology 78:385-389.

Barua, U.M., Chalmers, K.J., Hackett, C.A., Thomas, W.T.B., Powell, W., and Waugh, R. 1993. Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. Heredity 71:177-184.

Bjourson, A.J., and Cooper, J.E. 1988. Isolation of *Rhizobium loti* strain-specific DNA sequences by subtraction hybridization. Appl. Environ. Microbiol. 54:2852-2855.

Bjourson, A.J., Stone, C.E., and Cooper, J.E. 1992. Combined subtraction hybridization and polymerase chain reaction amplification procedure for isolation of strain-specific *Rhizobium* DNA sequences. Appl. Environ. Microbiol. 58:2296-2301.

Booth, C. 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, England. 237 pp.

Chen, X., Line, R.F., and Leung, H. 1993. Relationships between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology 83:1489-1497.

Cook, D., and Sequeira, L. 1991. The use of subtractive hybridization to obtain a DNA probe specific for *Pseudomonas solanacearum* race 3. Mol. Gen. Genet. 227:401-410.

Correll, J.C. 1991. The relationship between formae speciales, races, and vegetative compatibility groups in *Fusarium oxysporum*. Phytopathology 81:1061-1064.

Correll, J.C., Rhoads, D.D., and Guerber, J.C. 1993. Examination of mitochondrial DNA restriction fragment length polymorphisms, DNA fingerprints, and randomly amplified polymorphic DNA of *Colletotrichum orbiculare*. Phytopathology 83:1199-1204.

Crowhurst, R.N., Hawthorne, B.T., Rikkerink, E.H.A., and Templeton, M.D. 1991. Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. 20:391-396.

Daboussi, M.J., and Langin, T. 1994. Transposable elements in the fungal plant pathogen *Fusarium oxysporum*. Genetica 93:49-59.

Darrasse, A., Kotoujansky, A., and Bertheau, Y. 1994. Isolation by genomic subtraction of DNA probes specific for *Erwinia carotovora* subsp. *atroseptica*. Appl. Environ. Microbiol. 60:298-306.

Daughtrey, M., and Hoitink, H. 1988. Control of Fusarium wilt of cyclamen. Greenhouse Grower 2:34-39.

Donaldson, G.C., Ball, L.A., Axelrood, P.E., and Glass, N.L. 1995. Primer sets developed to amplify conserved genes from filamentous ascomycetes are useful in differentiating *Fusarium* species associated with conifers. Appl. Environ. Microbiol. (In press).

Edel, V., Steinberg, C., Avelange, I., Laguerre, G., and Alabouvette, C. 1995. Comparison of three molecular methods for the characterization of *Fusarium oxysporum* strains. Phytopathology 85:579-585.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes-Application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Gardes, M., White, T.J., Fortin, J.A., Bruns, T.D., and Taylor, J.W. 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can. J. Bot. 69:180-190.

Gerlach, W. 1954. Untersuchungen über die Welkekrankheit des Alpenveilchens (*Fusarium oxysporum* f. sp. *cyclaminis*). Phytopathol. Z. 22:125-176.

Goodwin, P.H., and Annis, S.L. 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. 57:2482-2485.

Goodwin, P.H., Kirkpatrick, B.C., and Duniway, J.M. 1990. Identification of *Phytophthora citrophthora* with cloned DNA probes. Appl. Environ. Microbiol. 56:669-674.

Grajal-Martin, M.J., Simon, C.J., and Muehlbauer, F.J. 1993. Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f. sp. *pisi*. Phytopathology 83:612-614.

Hamelin, R.C., Ouellette, G.B., and Bernier, L. 1993. Identification of *Gremmeniella abietina* races with random amplified polymorphic DNA markers. Appl. Environ. Microbiol. 59:1752-1755.

Henrion, B., Chevalier, G., and Martin, F. 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. Mycol. Res. 98:37-43.

Henson, J.M., Goins, T., Grey, W., Mathre, D.E., and Elliot, M.L. 1993. Use of polymerase chain reaction to detect *Gaeumannomyces graminis* DNA in plants grown in artificially and naturally infested soil. Phytopathology 83:283-287.

Huff, D.R., Bunting, T.E., and Plumley, K.A. 1994. Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Magnaporthe poae*. Phytopathology 84:1312-1316.

Jones, M.J., and Dunkle, L.D. 1993. Analysis of *Cochliobolus carbonum* races by PCR amplification with arbitrary and gene-specific primers. Phytopathology 83:366-370.

Kelly, A., Alcalá-Jiménez, A.R., Bainbridge, B.W., Heale, J.B., Pérez-Artés, E., and Jiménez-Díaz, R.M. 1994. Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. Phytopathology 84:1293-1298.

Kim, D.H., Martyn, R.D., and Magill, C.W. 1992. Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporum* f. sp. *niveum*. Phytopathology 82:346-353.

Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev. Plant Prot. Res. 8:114-125.

Lévesque, C.A., Vrain, T.C., and DeBoer, S.H. 1994. Development of a species-specific probe for *Pythium ultimum* using amplified ribosomal DNA. Phytopathology 84:474-478.

Maisonneuve, B., Bellec, Y., Anderson, P., and Michelmore, R.W. 1994. Rapid mapping of two genes for resistance to downy mildew from *Lactuca serriola* to existing clusters of resistance genes. Theor. Appl. Genet. 89:96-104.

Manulis, S., Kogan, N., Reuven, M., and Ben-Yephet, Y. 1994. Use of the RAPD technique for identification of *Fusarium oxysporum* f. sp. *dianthi* from carnation. Phytopathology 84:98-101.

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. 88:9828-9832.

Moukhamedov, R., Hu, X., Nazar, R.N., and Robb, J. 1994. Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84:256-259.

Mullis, K.B., and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. Methods Enzymol. 155:335-350.

Namiki, F., Shiomi, T., Kayamura, T., and Tsuge, T. 1994. Characterization of the formae speciales of *Fusarium oxysporum* causing wilts of cucurbits by DNA fingerprinting with nuclear repetitive DNA sequences. Appl. Environ. Microbiol. 60:2684-2691.

Nazar, R.N., Hu, X., Schmidt, J., Culham, D., and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. Physiol. Mol. Plant Pathol. 39:1-11.

Nicholson, P., Rezanoor, H.N., and Su, H. 1993. Use of random amplified polymorphic DNA (RAPD) analysis and genetic fingerprinting to differentiate isolates of race O, C, and T of *Bipolaris maydis*. J. Phytopathol. 139:261-267.

Nuss, D.L., and Koltin, Y. 1990. Significance of dsRNA genetic elements in plant pathogenic fungi. Annu. Rev. Phytopathol. 28:37-58.

Ouellet, T., and Seifert, K.A. 1993. Genetic characterization of *Fusarium graminearum* strains using RAPD and PCR amplification. Phytopathology 83:1003-1007.

Paran, I., Kesseli, R., and Michelmore, R.W. 1991. Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines. Genome 34:1021-1027.

Paran, I., and Michelmore, R.W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985-993.

Puhalla, J.E. 1981. Genetic considerations of the genus *Fusarium*. Pages 291-305 in *Fusarium*: Disease, biology, and taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., eds. The Pennsylvania State University Press, University Park, PA.

Rattink, H. 1986. Some aspects of the etiology and epidemiology of Fusarium wilt on cyclamen. Meded. Fac. Landbouwwet. Rijksuniv. Gent. 51:617-623.

Rollo, F., Salvi, R., and Torchia, P. 1990. Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. Appl. Microbiol. Biotechnol. 32:572-576.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: A laboratory manual. Second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 3 vols.

DeScenzo, R.A., and Harrington, T.C. 1994. Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. Phytopathology 84:534-540.

Schäfer, C., and Wöstemeyer, J. 1992. Random primer dependent PCR differentiates aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). J. Phytopathol. 136:124-136.

Seal, S.E., Jackson, L.A., and Daniels, M.J. 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. 58:3751-3758.

Sneath, P.H.A., and Sokal, R.R. 1973. Numerical taxonomy: The principles and practice of numerical classification. W.H. Freeman and Company, San Francisco, CA. 573 pp.

Snyder, W.C., and Hansen, H.N. 1940. The species concept in *Fusarium*. Am. J. Bot. 27:64-67.

Stammler, G., and Seemüller, E. 1993. Specific and sensitive detection of *Phytophthora fragariae* var. *rubi* in raspberry roots by PCR amplification. Z. Pflanzenkr. Pflanzenschutz 100:394-400.

Straus, D., and Ausubel, F.M. 1990. Genomic subtraction for cloning DNA corresponding to deletion mutations. Proc. Natl. Acad. Sci. 87:1889-1893.

Tayama, H.K. 1987. Control of cyclamen Fusarium wilt- A preliminary report. Ohio Florist's Assn. Bull. 693:1-3.

Taylor, J.L. 1993. A simple, sensitive, and rapid method for detecting seed contaminated with highly virulent *Leptosphaeria maculans*. Appl. Environ. Microbiol. 59:3681-3685.

Tisserat, N.A., Hulbert, S.H., and Sauer, K.M. 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

Tompkins, C.M. and Snyder, W.C. 1972. Cyclamen wilt in California and its control. Plant Dis. Rep. 56:493-497.

Toussoun, T.A., and Nelson, P.E. 1976. A pictorial guide to the identification of *Fusarium* species. Second edition. The Pennsylvania State University Press, University Park, PA. 43 pp.

Wang, Q. 1993. Analysis of physiological and genetic variability in *Fusarium* spp. with particular regard to *Fusarium oxysporum* pathotypes of tomato. M.Sc. Thesis. Simon Fraser University, Burnaby, Canada. 62 pp.

Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. 18:7213-7218.

Wieland, I., Bolger, G., Asouline, G., and Wigler, M. 1990. A method for difference cloning: Gene amplification following subtractive hybridization. Proc. Natl. Acad. Sci. 87:2720-2724.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18:6531-6535.

Wollenweber, H.W., and Reinking, O.A. 1935. Die Fusarien, ihre Beschreibung, Schadwirkung und Bekampfung. Paul Parey, Berlin. 355 pp.

Woudt, L.P., Sikkema, A., Leslie, J.F., Neuvel, A., deLange, M., deMilliano, W.A.J., and van Grinsven, M.Q.J.M. 1993. Identification of isolates of *Fusarium oxysporum* f. sp. *cyclaminis* by DNA-fingerprinting, vegetative compatibility and PCR. (Abstr.) 6th International Congress of Plant Pathology.

Xue, B., Goodwin, P.H., and Annis, S.L. 1992. Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. Physiol. Mol. Plant Pathol. 41:179-188.

Zhou, C., Yang, Y., and Jong, A.Y. 1990. Mini-prep in 10 minutes. Biotechniques 8:172-173.