ANALYSIS OF PHYSIOLOGICAL AND GENETIC VARIABILITY IN FUSARIUM SPP. WITH PARTICULAR REGARD TO FUSARIUM OXYSPORUM PATHOGENS OF TOMATO

by

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Title of Thesis/Project/Extended Essay

Analysis of physiological and genetic variability in Fusarium spp. with particular regard to Fusarium oxysporum pathogens of tomato

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ABSTRACT

Physiological and genetic variation among *Fusarium* spp. and particularly between two formae speciales, *Fusarium oxysporum* (Schlechtend.:Fr.) f.sp. *lycopersici* (Sacc.) Snyder & Hans. (FOL) and *F. oxysporum* (Schlectend.:Fr.) f.sp. *radicis-lycopersici* Jarvis & Shoemaker (FORL) were studied in the laboratory. Both formae speciales are pathogenic on tomato plants. FOL is a widespread pathogen and causes a vascular wilt disease, while FORL is apparently a new biotype of FOL and causes a crown and root rot disease. A commonly held view is that FORL originated as a variant of FOL. In my research, I tested this hypothesis by using two techniques: vegetative compatibility grouping (VCG) test using nitrate nonutilizing (*Nit*) mutants; and restriction fragment length polymorphism (RFLP) analysis of total DNA and polymerase chain reaction (PCR) amplified DNA.

In the vegetative compatibility tests, both FOL and FORL isolates occurred in numerous VC groups. Six isolates of FORL occurred in three VC groups, and each of three FOL isolates tested represented a distinct VC group. Using RFLP analysis, FOL and FORL isolates were differentiated in *HindIII* digests of total DNA but not *EcoRI* digests. Restriction digests with *AluI* of PCR-amplified nuclear rDNA clearly distinguished between FOL and FORL isolates. Several different restriction enzymes revealed RFLPs in

PCR-amplified rDNA which were consistent with the VC groupings seen in FORL isolates. Although the number of isolates studied was relatively small, it is clear that considerable genetic variation occurs both among and between isolates of FOL and FORL. The results do not reveal the mechanism of origin of FORL.

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DEDICATION

To my dear mother and father, for their love

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CHAPTER 1. GENERAL INTRODUCTION

Introduction:

The fungal genus *Fusarium* consists of many species. Wollenweber divided the genus into several sections, *e.g. Elegans*, *Martiella*, *Discolor*, and *Gibbosum* (Wollenweber, 1913). In 1940, Snyder and Hansen proposed that all *Fusarium* species and varieties in the *Elegans* section be combined into one, named *F. oxysporum*. In 1945, Snyder and Hansen reduced all *Fusarium* to nine species, which has become known as the "nine species" system (Toussoun & Nelson, 1968). The current status of the taxonomy of *Fusarium* was presented by Snyder and Toussoun in 1965 (Snyder and Toussoun, 1965). Spore morphology is the major character used in the identification of *Fusarium*. Conidia range from hyaline, oval, single-celled microconidia through to straight or curved, multi-septate macroconidia. Chlamydospores constitute a resting stage in many species (Booth, 1971).

Fusarium oxysporum Schlechtend.:Fr. is a genetically diverse soil-borne fungal species that is widely distributed in cultivated and noncultivated soils. *F. oxysporum* was the first of nine species to be described in the section *Elegans* by Wollenweber in 1913. In 1940, Snyder and Hansen designated 25 forms (formae speciales) of *F. oxysporum* based on their pathogenic patterns and

capabilities. Gordon (1965) and as well as Messiaen and Cassini (1968) listed many new formae speciales, some of which have been found to consist of two or more races (Booth, 1971).

The distribution of *F. oxysporum* is world wide. It occurs chiefly as a soil saprophyte and it is typically one of the most common and prevalent fungi of cultivated soils. Strains of the species are serious wilt pathogens of many crop plants, and most of these strains are morphologically indistinguishable (Booth, 1971; Nelson, 1981). The pathogenic strains cause diseases in many economically important crops, including tomato (Walker, 1971; Jones and Woltz, 1981; Nelson, 1981; MacHardy and Beckman, 1981).

Diversity within the pathogenic strains of *F. oxysporum* has received much attention. Strains of *F. oxysporum* are typically host-specific (Correll *et al.*, 1987), and are designated as formae speciales (and races) based on their ability to cause disease on a particular host or group of hosts. In the latest compilation, there were 122 formae speciales and races of *F. oxysporum* (Armstrong and Armstrong, 1981).

Fusarium oxysporum (Schlechtend.:Fr.) f.sp. *lycopersici* (Sacc.) Snyder & Hans (FOL) and *F. oxysporum* (Schlechtend.:Fr.) f.sp. *radicis-lycopersici* Jarvis & Shoemaker (FORL) are two pathogenic forms virulent on tomato plants. FOL is a widespread pathogen and causes

tomato vascular wilt. FORL is apparently a new biotype of FOL and causes a crown and root rot disease of tomato (Jarvis, 1988).

Fusarium wilt of tomato was initially described by Massee in 1895 (Walker, 1971) and, according to Jones and Woltz (1981), the disease occurs in at least 32 countries, and in most of the tomato-growing regions of the world. Tomato wilt is a warm weather disease and it causes most severe symptoms at 28 C. In North America, the disease is widespread and destructive in the field in southern locations, but is limited by temperature, primarily to the greenhouse crops, in northern areas (Jones and Woltz, 1981). In the field, the symptoms usually occur first on the lower leaves, which become yellow and die; later, symptoms appear in younger leaves. When stem sections are examined, the vascular system shows browning and discoloration (Walker, 1971). In the greenhouse, young plants show clearing of the veinlets and drooping of the petioles (epinasty).

Fusarium crown and root rot (or foot and root rot) was first recognized in Japan in 1969 on tomatoes grown in plastic-covered greenhouses (Jarvis, 1988). Fusarium crown and root rot of tomatoes is caused by *F. oxysporum* f.sp. *radicis-lycopersici* (FORL). Although FORL is a relatively recently recognized pathogen, it has already spread to many tomato-growing areas in the world, and it is now reported

in nine countries (Katan *et al.*, 1991). In Canada, the disease has been reported from Ontario (Jarvis *et al.*, 1975), Quebec (Thibodeau and Simard, 1978) and British Columbia (Jarvis, 1988). In the greenhouse, crown and root rot is characterized by a wilt, but is also distinguished by a brown cortical rot at soil level, and a reddish-brown vascular discoloration which extends upward from the crown for 5-10 cm or more (Jarvis, 1988).

There has been much speculation about the origin and long-range dispersal of FORL. All investigators have agreed that the causal fungus is *F. oxysporum* Schlecht., and it is generally assumed that FORL originated as a variant of FOL (Jarvis, 1988). FORL differs from FOL in symptomology, epidemiology, and cultivar susceptibility (Benhamou *et al.*, 1988; Jarvis, 1988; Jarvis and Shoemaker, 1978; Menzies *et al.*, 1990; Rowe and Farley, 1981). FOL rapidly invades and moves through the host vascular tissue, while FORL lacks movement through the vascular system. The optimum temperature for FORL is around 18 C while FOL is favored by temperatures around 28 C. These considerations led Jarvis and Shoemaker (1978) to propose that the causal agent of fusarium crown and root rot was a new forma specialis of *F. oxysporum*, and they designated the pathogen as *F. oxysporum* f.sp. *radicis-lycopersici*.

In addition to pathogenic differences, a number of physiological, biochemical and molecular techniques have

been used in recent years to assess variation within and among formae speciales of *F. oxysporum*. These techniques include vegetative compatibility tests (VCG) (Puhalla, 1985), isozyme analysis (Biles and Martyn, 1988; Bosland and Williams, 1987), RNA sequence comparison (Guadet *et al.*, 1989), karyotyping (Mills and McCluskey, 1990), DNA-DNA reassociation kinetics (Kuninaga and Yokosawa, 1991) and restriction fragment length polymorphisms (RFLPs) (Coddington *et al.*, 1987; Kim *et al.*, 1990; Kim *et al.*, 1992; Manicom *et al.*, 1990). Among these procedures, RFLPs have the advantage of potentially detecting numerous polymorphisms at the DNA level (Michelmore and Hulbert, 1987; Kim *et al.*, 1992)

The objectives of my research project were (i) to learn the techniques of vegetative compatibility and restriction fragment analysis; (ii) to use these techniques to assess compatibility relationships between and among isolates of *Fusarium* spp., particularly FOL and FORL; and (iii) if possible, to differentiate FORL from FOL using molecular analysis. The isolates of *Fusarium* used in my research, and information regarding their sources and geographic origins, are presented in Table 1.

Table 1. Geographical origin and sources of isolates of *Fusarium* species used in this study.

Isolate	Species	Origin	Source
1	*Fusarium oxysporum* (FO)	Guelph, Ont.	a
2	*Fusarium solani* (FS)	Guelph, Ont.	a
3	(FO)	Guelph, Ont.	a
4	*Fusarium oxysporum* f.sp. *lyccopersici* (FOL)	Unknown	a
5	*Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL)	British Columbia	a
6	(FORL)	Harrow, Ont.	a
7	(FORL)	Harrow, Ont.	b
8	(FORL)	Ont.	b
9	(FORL)	Ont.	b
10	(FORL)	Ont.	b
11	(FOL)	Welling, Alb.	c
12	(FOL)	Princess, Alb.	c
13	(FOL)	Welling, Alb.	c
14	(FOL)	Louisiana U.S.A.	d
15	(FORL)	Zaadunie, Holland	e
16	(FORL)	Zaadunie, Holland	e

a. Isolates obtained from Dr. R.J. Copeman, Department of Plant Science, University of British Columbia, Vancouver, B.C.
b. Isolates obtained from Dr. J.G. Menzies, Agriculture Canada Research Station, Agassiz, B.C.
c. Isolates obtained from Dr. R. Howard, Alberta Special Crops and Horticultural Research Center, Brooks, Alberta.
d. Isolates obtained from Dr. K.S. Elias, United states Department of Agriculture, New Orleans, Louisiana, USA.
e. Isolates obtained from Dr. B. Woudt, Zaadunie, Holland.

CHAPTER 2. VEGETATIVE COMPATIBILITY TEST

2-1. Introduction:

No sexual stage has been described for *F. oxysporum*. Consequently, other mechanisms of genetic exchange between individuals determines genetic diversity in different populations of this fungus (Ploetz and Correll, 1988; Katan *et al.*, 1991).

Puhalla (1985) introduced a technique by which he selected spontaneous nitrate metabolism (*Nit*) mutants of *F. oxysporum* without using a mutagen. He obtained two complementary, but uncharacterized, *Nit* mutants and used these to assess heterokaryon formation and vegetative compatibility. Growth of heterokaryons was observed macroscopically. Using this technique, Puhalla proposed an evolutionary model for the origin of formae speciales and races, based on the results from 21 isolates representing 12 formae speciales of *F. oxysporum*.

Correll *et al.* (1987) refined Puhalla's system by prescribing physiological tests for selecting *Nit* mutants of known phenotype that are deficient in specific regulatory and structural genes. This system assured that potentially complementary strains were being paired so that false negatives could be minimized (Elias and Schneider, 1991). In such tests, auxotrophic mutants have been used almost exclusively as forcing markers to determine whether

or not strains are capable of forming heterokaryons with one another. Puhalla and Correll argued that strains that were vegetatively compatible were much more likely to be genetically similar than strains that were vegetatively incompatible (Correll *et al.*, 1987).

Several investigators have since used *Nit* mutants for vegetative compatibility group (VCG) analysis in several formae speciales of *F. oxysporum* and other *Fusarium* species (Table 2). Vegetative compatibility tests are proving to be a particularly powerful tool for studying genetic diversity in *F. oxysporum*. There have been numerous VCG studies in several species of fungi including several formae speciales of *F. oxysporum*. The advantages of using *Nit* mutants over other types of mutants for vegetative compatibility tests are that *Nit* mutants can be readily recovered, are stable, and can grow on unsupplemented media (Correll *et al.*, 1988). Using VCGs to define distinct populations may provide valuable insight into relationships between new and established infections and the pattern of disease spread (Jacobson and Gordon, 1988).

The objective of this study was to assess compatibility relationships between and among crown root rot and vascular wilt tomato Fusaria using *Nit* mutant complementation testing.

Table 2. List of *Fusarium* and other fungal species for which vegetative compatibility studies have been reported.

Species	Reference
Fusarium oxysporum f.sp. *apii*	Correll *et al.*, 1986*a*
F. o. f.sp. *cubense*	Ploetz and Correll, 1988
F. o. f.sp. *melonis*	Jacobson and Gordon, 1988
F. o. f.sp. *vasinfectum*	Katan and Katan, 1988
F. o. f.sp. *asparagi*	Elmer and Stephens, 1989
F. o. f.sp. *radicis-lycopersici*	Katan *et al.*, 1991
F. o. f.sp. *lycopersici*	Elias and Schneider, 1991
F. o. f.sp. *tuberosi*	Venter *et al.*, 1992
Fusarium oxysporum	Puhalla, 1985
F. oxysporum	Correll *et al.*, 1986*b*
F. oxysporum	Correll *et al.*, 1987
F. oxysporum	Bosland and Williams, 1987
F. oxysporum	Hadar *et al.*, 1989
F. oxysporum	Gordon and Okamoto, 1991
Gibberella fujikuroi (*F. moniliforme*)	Puhalla and Spieth, 1985
F. proliferatum	Elmer, 1991
Verticillium dahliae	Puhalla and Hummel, 1983
V. albo-atrum	Correll *et al.*, 1988
V. dahliae	Joaquim and Rowe, 1991
Colletotrichum spp.	Brooker *et al.*, 1991

2-2. Materials and methods:

2-2.1. Fungi

The 13 isolates used in this study are described in Table 1. Conidia from each isolate were transferred to Nash and Snyder (NS) medium (Nash and Snyder, 1962). Cultures derived from a single spore transfer were made for each isolate onto cornmeal agar. For long-term storage, each isolate was subcultured to potato dextrose agar (PDA) onto which a sterilized Whatman No.3 filter paper disk (7cm diameter) was placed. After 8 days, the colonized filter paper disk was removed and allowed to air-dry in a sterile petri plate. The disk was then cut into 2 to $3mm^2$ pieces, and placed in sterilized No.2 dram screw-cap vials, and stored at 4 C (Correll *et al.*, 1986*a*). All cultures were kept at room temperature (22-24 C).

2-2.2. Media

A basal medium was prepared following the methods of Puhalla (1985) and Correll *et al.* (1987), and contained per liter of distilled water: sucrose, 30 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4.7H_2O$, 10 mg; agar, 20 g; and trace element solution 0.2 ml.

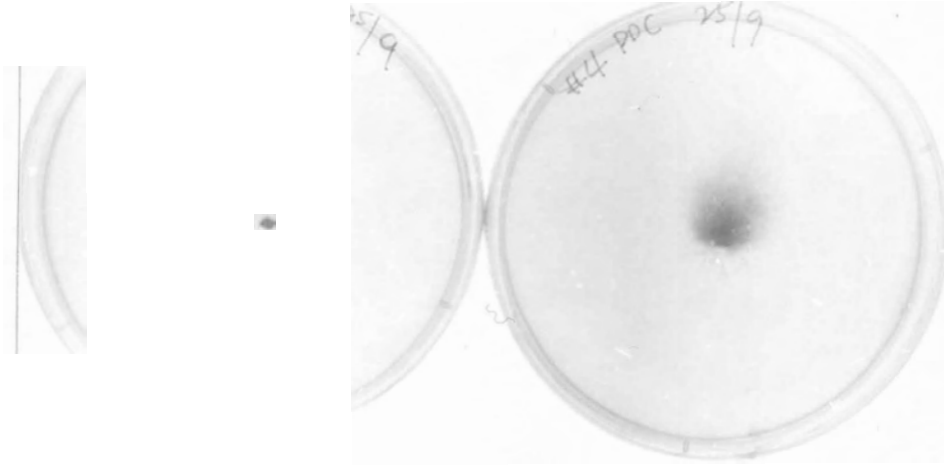
Minimal medium (MM) was prepared by adding 2 g of $NaNO_3$ to 1 L of basal medium. Complete medium (CM) was made

by adding the following to 1 L of basal medium: $NaNO_3$, 2 g; N-Z amine (Sheffield), 2.5 g; yeast extract (Difco), 1 g; and 10 ml of a vitamin solution (Correll *et al.*, 1987).

Following the method of Puhalla (1985) and Correll *et al.* (1987), two chlorate-containing media were used to generate nitrate nonutilizing (*Nit*) mutants: minimal medium amended with chlorate (MMC) and potato-dextrose agar amended with chlorate (PDC). MMC consisted of basal medium (MM) amended with 1.6 g/L L-asparagine; 2 g/L $NaNO_3$; and 15 g/L $KClO_3$. PDC contained the following: 1 L of distilled water; 24 g dehydrated potato-dextrose broth (Difco); 20 g agar; and 15 g $KClO_3$.

2-2.3. Recovery of nitrate nonutilizing mutants

Nit mutants were recovered from each isolate of *Fusarium* spp. following the technique described by Puhalla (1985) and Correll *et al.* (1987). Plates containing either MMC or PDC were inoculated in the centre with a single conidium or a mycelial plug (1 mm^3 block) of each isolate, and incubated at 22-24 C. Transfers of mycelium from chlorate-resistant sectors occurring in wild type colonies (Fig. 1) were made after 7-10 days onto MM. Sector transfers that grew on MM as thin expansive colonies with no aerial mycelium were considered to be *Nit* mutants. All *Nit* mutants showed wild type growth on CM.



a b

Fig. 1. Growth of wild parental isolate #4 of *Fusarium oxysporum* f.sp. *lycopersici* on potato dextrose agar containing chlorate. a. Restricted colony. b. A restricted colony with fast growing sector from which a putative *Nit* mutant was obtained.

2-2.4. *Nit* mutant phenotypes

Phenotypes of the *Nit* mutants obtained from the 13 isolates were determined by observing their colony morphologies on media containing one of five different nitrogen sources, following the methods described by Correll *et al.* (1987). The five *Nit* mutant screening media were: 1) nitrate medium = MM, 2) nitrite medium = basal medium plus 0.5 g /L $NaNO_2$; 3) hypoxanthine medium = basal medium plus 0.2 g /L hypoxanthine; 4) ammonium medium = basal medium plus 1 g /L NH_4 tartrate, and 5) uric acid medium = basal medium plus 0.2 g /L uric acid. The inoculated plates were incubated at room temperature, and colony morphology was scored relative to the wild type parents after 4 days
(Fig. 2).

2-2.5. Selection of *Nit* mutant testers

The *Nit* mutants of each isolate were first paired among themselves on MM. Different *Nit* mutants from the same wild type parent that were able to complement one another were designated "A" and "B". The 26 mutants (A1-A13, and B1-B13) were then paired on MM in all possible combinations to determine which isolates were vegetatively compatible with one another. Complementation tests were done by placing mycelium transferred from two different *Nit* mutant

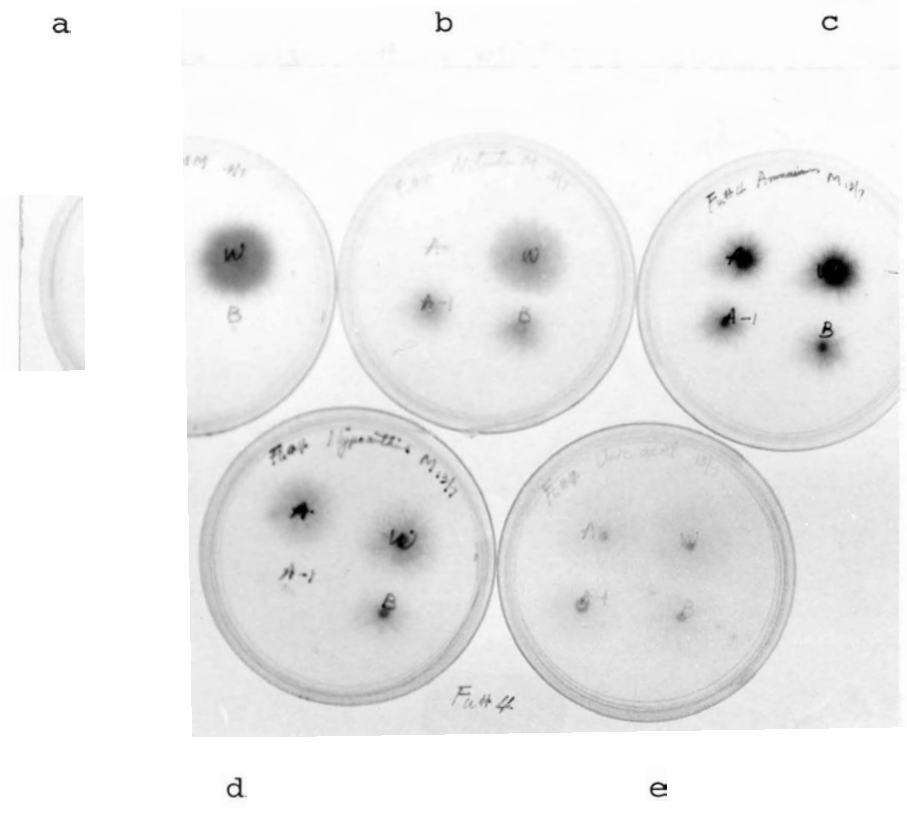


Fig. 2. Types of growth of three *Nit* mutant phenotypes (A, A-1, B) and wild-type (W) isolate #4 of *Fusarium oxysporum* f.sp. *lycopersici* on media with one of five different nitrogen sources: a. Nitrate medium, b. Nitrite medium, c. Ammonium medium, d. Hypoxanthine medium, e. Uric acid medium. Mutants A, A-1, and B are designated *Nit*3, *Nit*M and *Nit*1 respectively, on the basis of the colony morphology occurring on these media.

testers (designated "A" and "B") 1.5 cm apart on MM (Fig. 3). The plates were kept at room temperature for 7-14 days and then scored for complementation, *i.e.* wild-type growth where two sparsely growing *Nit* mutant colonies came in contact. When mutants of two different isolates formed a heterokaryon (as indicated by wild-type growth), their parent isolates were assigned to the same VCG.

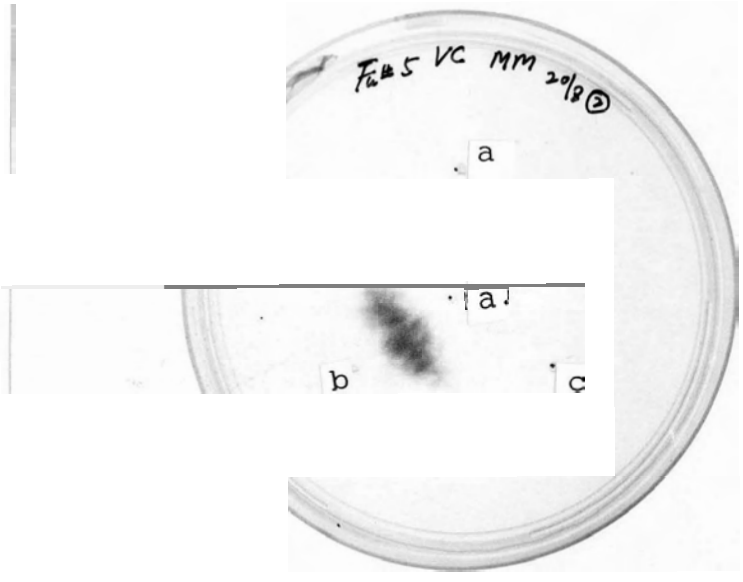


Fig. 3. Complementation test among three *Nit* mutants of a single isolate (#5) of *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Complementation between mutants a and b is manifested by wild-type growth in the region where the mutant colonies meet, whereas mutants a and c are noncomplementary with each other.

2-3. Results:

2-3.1. *Nit* mutant isolation

Using the standard MMC and PDC media, *Nit* mutants were generated from 11 of the 13 isolates (exceptions were isolates #2 and #3). Chlorate-resistant mutants were obtained from these latter isolates when the concentration of $KClO_3$ in MMC and PDC was increased to 3-5%. The frequency of chlorate-resistant sectors ranged from 0.2 and 2.0 (mean=0.88) per colony and 0.2 to 1.0 (mean=0.31) per colony on MMC and PDC, respectively (Table 3). Most (82%) of the isolates obtained from chlorate-resistant sectors were unable to utilize nitrate as a sole nitrogen source and showed thin expansive growth on MM; these isolates were considered to be *Nit* mutants (Table 3). At least three *Nit* mutants were recovered from each of the parental isolates used in this study (Table 3).

2-3.2. *Nit* mutant phenotype identification and selection of testers

All three phenotypes of *Nit* mutants were obtained (*Nit*1, *Nit*3 and *Nit*M). *Nit*1 and *Nit*3 mutants occurred on both PDC and MMC (Table 3), but the frequency of both *Nit*1 and *Nit*3 mutants recovered was considerably higher from PDC. Consequently, the relative frequency of *Nit*M mutant recovered was higher on MMC than PDC. *Nit* mutants of

Table 3. Frequency and phenotype of nitrate nonutilizing (*Nit*) mutants recovered on two media.

Isolate	Medium	Sector observed /colony(n)	Number of putative mutants examined	Nit mutant classes *Nit*1	*Nit*3	*Nit*M
1 (FO)	PDC	1.7(10)	15	1	6	1
	MMC	0 (10)				
2 (FS)	PDC	0.4(20)	7	1	3	1
	MMC	0 (20)				
3 (FO)	PDC	2.0(20)	28	2	1	0
	MMC	0 (20)				
4 (FOL)	PDC	0.2(20)	7	2	2	2
	MMC	0.2(20)				
5 (FORL)	PDC	1.7(10)	18	3	13	1
	MMC	0.7(10)				
6 (FORL)	PDC	1.3(10)	13	6	5	0
	MMC	0.2(10)				
7 (FORL)	PDC	0.7(10)	13	3	1	1
	MMC	0.6(10)				
8 (FORL)	PDC	0.5(10)	10	5	3	1
	MMC	0.5(10)				
9 (FORL)	PDC	0.7(10)	7	2	2	2
	MMC	1.0(10)				
10 (FORL)	PDC	0.6(10)	12	4	0	1
	MMC	0.6(10)				
11 (FOL)	PDC	1.0(10)	13	4	4	0
	MMC	0.3(10)				
12 (FOL)	PDC	0.4(10)	5	3	1	1
	MMC	0.1(10)				
13 (FOL)	PDC	0.2(10)	8	3	2	0
	MMC	0.6(10)				

FO= *Fusarium oxysporum*
FS= *Fusarium solani*
FOL= *Fusarium oxysporum* f.sp. *lycopersici*
FORL= *Fusarium oxysporum* f.sp. *radicis-lycopersici*
PDC= Potato dextrose agar amended with chlorate
MMC= Minimal medium amended with chlorate

at least two different phenotypes were identified within each isolate. One complementary pair of mutants was obtained from each of the 13 isolates of *Fusarium* except #3 and #4. In later tests, the #3 isolate gave a delayed compatible reaction which was either missed or did not occur in the initial screening of mutants. The members of a compatible pair of mutants within each isolate were designated "A" and "B" (Table 4).

2-3.3. Complementation tests

The 26 tester mutants that were selected from the 13 isolates (Table 4) were paired in all combinations on MM (Fig. 4). Based on the results of these tests, the 13 isolates were placed into 10 vegetative compatibility groups (Table 5). The six isolates of FORL were in three VCGs, while the other seven isolates, including four isolates of FOL, each represented a unique VCG. None of the isolates of FORL was vegetatively compatible with any of the isolates of FOL. No correlation between vegetative compatibility group and geographical origin was observed.

Table 4. Phenotypes of complementary pairs of *Nit* mutants (arbitrarily designated A & B) obtained from isolates of *Fusarium*.

Isolate	Classes of complementary Nit mutants		
	*Nit*1	*Nit*3	*Nit*M
1 (FO)	A1	-	B1
2 (FS)	A2	-	B2
3 (FO)	A3	B3	-
4* (FOL)	a4	b4	-
5 (FORL)	A5	-	B5
6 (FORL)	A6	B6	-
7 (FORL)	A7	-	B7
8 (FORL)	A8	-	B8
9 (FORL)	-	A9	B9
10 (FORL)	A10	-	B10
11 (FOL)	A11	B11	-
12 (FOL)	-	A12	B12
13 (FOL)	A13	B13	-

* self-incompatible
FO= *Fusarium oxysporum*
FS= *Fusarium solani*
FOL= *Fusarium oxysporum* f.sp. *lycopersici*
FORL= *Fusarium oxysporum* f.sp. *radicis-lycopersici*

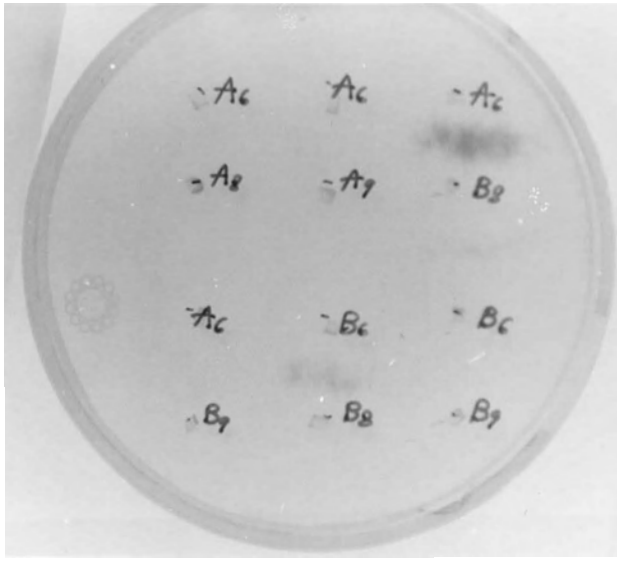


Fig. 4. Pairings of *Nit* mutants from each of three different isolates of *Fusarium*. Two mutant testers (A6B6, A8B8, and A9B9) from isolate #6, #8, #9 of *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) respectively (note wild-type growth between mutant tester A6 and B8, and B6 and B8 from isolates #6 and #8.

Table 5. Isolates of *Fusarium*, geographical origin and vegetative compatibility group.

Isolate	Species	Origin	VCG
1	*Fusarium oxysporum* (FO)	Guelph, Ont.	01
2	*F. solani* (FS)	Guelph, Ont.	02
3	(FO)	Guelph, Ont.	03
4	*Fusarium oxysporum* f.sp. *lycopersici* (FOL)	Unknown	04
5	*Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL)	British Columbia	05
6	(FORL)	Harrow, Ont.	06
7	(FORL)	Ont.	05
8	(FORL)	Ont.	06
9	(FORL)	Ont.	07
10	(FORL)	Ont.	07
11	(FOL)	Welling, Alb.	08
12	(FOL)	Princess,Alb.	09
13	(FOL)	Welling, Alb.	10

CHAPTER 3. RESTRICTION FRAGMENT LENGTH POLYMORPHISMS OF TOTAL DNA AND POLYMERASE CHAIN REACTION AMPLIFIED DNA

3-1. Introduction:

Since minor nucleotide variations may cause morphological, physiological and biochemical differences, investigations of DNA sequences can provide information about the genetic background of organisms and, thereby, permit conclusions about relatedness. Digestion of DNA and subsequent analysis of restriction fragment length polymorphism (RFLP), has been shown to be a useful tool in the taxonomy of fungi (Bruns *et al.*, 1991).

Recent reports of RFLP analysis in formae speciales of *F. oxysporum* have included studies of mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) from *F. oxysporum* (Kistler *et al.*, 1987); mtDNA from *F. oxysporum* f.sp. *niveum* (Kim *et al.*, 1992); total DNA from *F. oxysporum* f.sp. *pisi* (Coddington *et al.*, 1987) and *F. oxysporum* f.sp. *dianthi* (Manicom *et al.*, 1990); total DNA, mtDNA, and plasmid DNA from *F. oxysporum* f.sp. *melonis* (Jacobson and Gordon, 1990).

In the short time since its introduction in 1985, the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987) has become a standard tool for DNA analysis. PCR involves *in vitro* enzyme catalyzed synthesis of a specific DNA segment that can be selected by

experimentally chosen primers (White *et al.*, 1990). Only minute amounts of template DNA are needed. The PCR test provides a simple and fast way of typing fungal isolates (Rollo *et al.*, 1990). As a result, genetic information from rare or obligately parasitic fungi can now be accessed (Bruns *et al.*, 1991). PCR has recently been used to differentiate *Verticillium* spp. (Nazar *et al.*, 1991) and *Rhizoctonia* spp. (Cubeta *et al.*, 1991).

PCR amplified rDNA from mitochondrial and nuclear DNA are particularly suitable for RFLP analysis, following digestion by restriction endonucleases (Kistler *et al.*, 1987). This technique may provide a more sensitive way to investigate divergence in formae speciales than other methods, and as a means for rapid identification of pathogenic isolates.

In chapter 2, ten VCG were identified among 13 isolates of *F. oxysporum* collected from British Columbia, Ontario and Alberta. In the research described here variation within total DNA, mitochondrial and nuclear rDNA of *F. oxysporum* was examined to further study the variation within and among FOL and FORL, and the relationships between VCG and RFLP.

3-2. Materials and Methods:

3-2.1. Fungal isolates

The isolates of *F. oxysporum* that were used in this study are described in Table 1. Single-spore cultures were made from each isolate onto NS medium, and subsequently maintained on PDA. Mycelium for DNA extraction was grown in 50 ml potato dextrose broth (PDB) (Difco) in 250 ml Erlenmeyer flasks which was inoculated with 5mm diameter plugs from PDA cultures. After growth in the dark at 22-24 C for 5-7 days, mycelial mats of individual isolates were harvested, washed by vacuum filtration on Whatman No. 1 filter paper with cold 0.1 M NaCl and weighed.

3-2.2. DNA extraction

DNA was extracted using a modified version of the procedure described by Curran *et al.* (1986). Washed mycelium was mixed with extraction buffer (0.1 M Tris-base, 0.05 M Na_2EDTA, 0.2 M NaCl, 1.0% SDS, pH 8.0) at 4 ml per g of mycelium, and frozen in liquid nitrogen. The frozen mycelium was ground to a fine powder with a mortar and pestle and after lysis at room temperature, transferred to a centrifuge tube. A half volume of phenol (Bethesda Research Laboratories, Inc.) was added to the lysed mycelium and mixed by gentle inversion, followed by centrifugation at 3500 rpm for 5 min, and this was repeated

three times. The upper aqueous phase was removed and treated twice with chloroform-isoamyl alcohol (24:1, V:V). DNA was precipitated from the aqueous phase by addition of two volumes of 95% ethanol, suspended after centrifugation in 70% ethanol, collected by centrifugation and resuspended in 0.5 ml of TE buffer (10 mM Tris-HCL, 1 mM Na_2EDTA, pH 8.0). The suspension of DNA in TE buffer was treated with 5 μg RNAse (Sigma) and incubated at 37 C for 30 min. Further purification was done by adding 4 M NaCl to bring the concentration up to 0.2 M and precipitation with ethanol, as just described. The pellet obtained after addition of 70% ethanol was dried under vacuum and then dissolved in 50-100 μl of TE and stored at 4 C.

The extracted total DNA from each sample was examined by electrophoresis in a 0.7% agarose gel in TBE buffer (89 mM Tris-HCl, 2.5 mM Na_2EDTA, 89 mM boric acid, pH 8.3). Agarose gels contained ethidium bromide and were photographed over a UV transilluminator (300 nm).

3-2.3. Digestion with restriction enzymes

Various restriction enzymes were used to digest total DNA. Approximately 1 μg total DNA from each isolate was incubated with *EcoRI* or *HindIII* at 37 C for 2 h. The digests were then mixed with loading buffer (10 X = 15% Ficoll 400, TBE 8 X, 0.25% bromophenol blue, 0.25% xylene cynole) and loaded onto gels. Electrophoresis was done

overnight at 0.5 V cm^{-1} in 0.7% agarose gel with ethidium bromide in a 1 X TBE buffer. A 1 kb DNA ladder (BRL) was used as a size marker. The gels were photographed over a 300 nm UV light source to record banding patterns.

3-2.4. Primer development and synthesis

Oligodeoxyribonucleotide primers ITS1-ITS4, ML1-ML4, ML7-ML8 (sequence published in White *et al.*, 1990), and NTS1 and NTS2 (pers. comm. C.A. Lévesque) were synthesized at Simon Fraser University, and used to amplify portions of mitochondrial rDNA (ML) and nuclear rDNA (ITS & NTS). Primers NTS1 (TTTTGATCCTTCGATGTCGG) and NTS2 (AATGAGCCATTCGCAGTTTC) are located at the 5' end of the 18S gene and the 3' end of the 28S gene, respectively, and amplify the rDNA intergenic spacer (IGS). The primer sequence on the 18S gene has 100% homology in five fungal and three plant genera. The primer sequence on the 28S gene has 100% homology in one species of slime mold, two plant and two animal genera (pers. communication, C.A. Lévesque).

3-2.5. DNA amplification with polymerase chain reaction (PCR)

Reaction mixtures containing 1.0 μM of each primer, 1.25 unit of Taq polymerase (Promega Biotec), 200 μM of each of 4 μl deoxyribonucleotide triphosphates (dNTP), and 1.5 μl of 1.5 mM $MgCl_2$, and 1 μl of template DNA in a

total volume of 1 X TAQ buffer (Promega) brought up to 25 μl were run through 40 cycles on an Ericomp thermocycler (Ericomp Inc., San Diego, California). The following parameters were used for PCR amplification of related regions of DNA: 1 min denaturation step at 94 C (NTS, ML, and ITS), annealing at 65 C for 2 min (NTS) or 55 C for 2 min (ML) or 54 C for 2 min (ITS) and primer extension at 72 C for 3 min (NTS and ML) or for 2 min (ITS). After amplification, a 3 μl aliquot from each sample was subjected to electrophoresis in 1.5% agarose gel with ethidium bromide in 1 X TBE buffer at 3.3 V cm^{-1} for 1 h.

3-2.6. Digestion with restriction enzymes

For restriction analysis of PCR products, 0.5-1.0 μg aliquots of DNA were digested with *AluI*, *CfoI*, *DdeI*, *HaeIII*, *HinfI*, *HpaII*, *RsaI*, or *TaqI* following the manufacturers' directions (BRL or Promega). After digestion, samples were subjected to electrophoresis in 2% agarose gel with ethidium bromide for 1 h at 3.3 V cm^{-1}, and photographed over a UV transilluminator (300 nm).

Dr. C.A. Lévesque, Agriculture Canada Research Station, 6660 N.W. Marine Drive, Vancouver, B.C. V6T 1X2.

3-3. Results:

3-3.1. Preparations of total DNA

After electrophoretic analysis in 0.7% agarose gel, each isolate showed a single band of DNA. Isolate #3 of *F. oxysporum* contained additional bands in the 2-3 kb region (Fig. 5). The following treatments of the total DNA preparation of isolate #3 were used to test the hypothesis that these bands could be double-stranded RNA (dsRNA): digestion using DNAse at 37 C for 2 h; digestion using RNAse at 37 C for 2 h; and digestion using 0.1 N NaoH solution at 37 C for 2 h. The results of these tests support the hypothesis that the extra bands are double-stranded RNA (Fig. 6).

3-3.2. Restriction analysis of total DNA

After digestion of total DNA with *EcoRI*, isolates #1-#10 of *F. oxysporum* could be differentiated into one group of eight isolates containing a similar pattern of repeat sequence DNA, and isolates #2 and #3 which produced unique banding patterns (Fig. 7). The eight isolates which had a common pattern of repeat sequence bands included both FOL and FORL isolates. The single isolate of *F. solani*, and one isolate of *F. oxysporum* produced unique patterns of repeat sequence DNA after digestion with *EcoRI*.

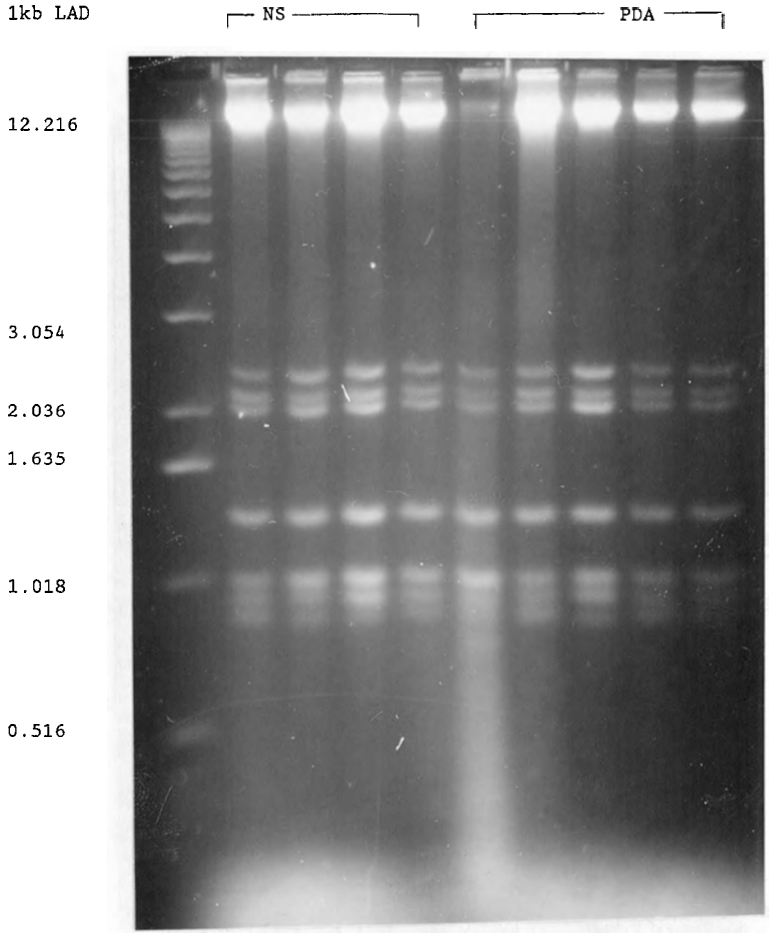


Fig. 5. Electrophoretic analysis of uncut DNA of nine preparations of total DNA from isolate #3 of *Fusarium oxysporum* showing identical low molecular bands. Inoculum used to produce mycelium for DNA extraction was obtained from single spore cultures growing on Nash and Snyder (NS) or potato dextrose agar (PDA) media, as indicated. (0.7% agarose gel, with ethidium bromide, electrophoresed at 0.5 V cm^{-1} for 18 hr).

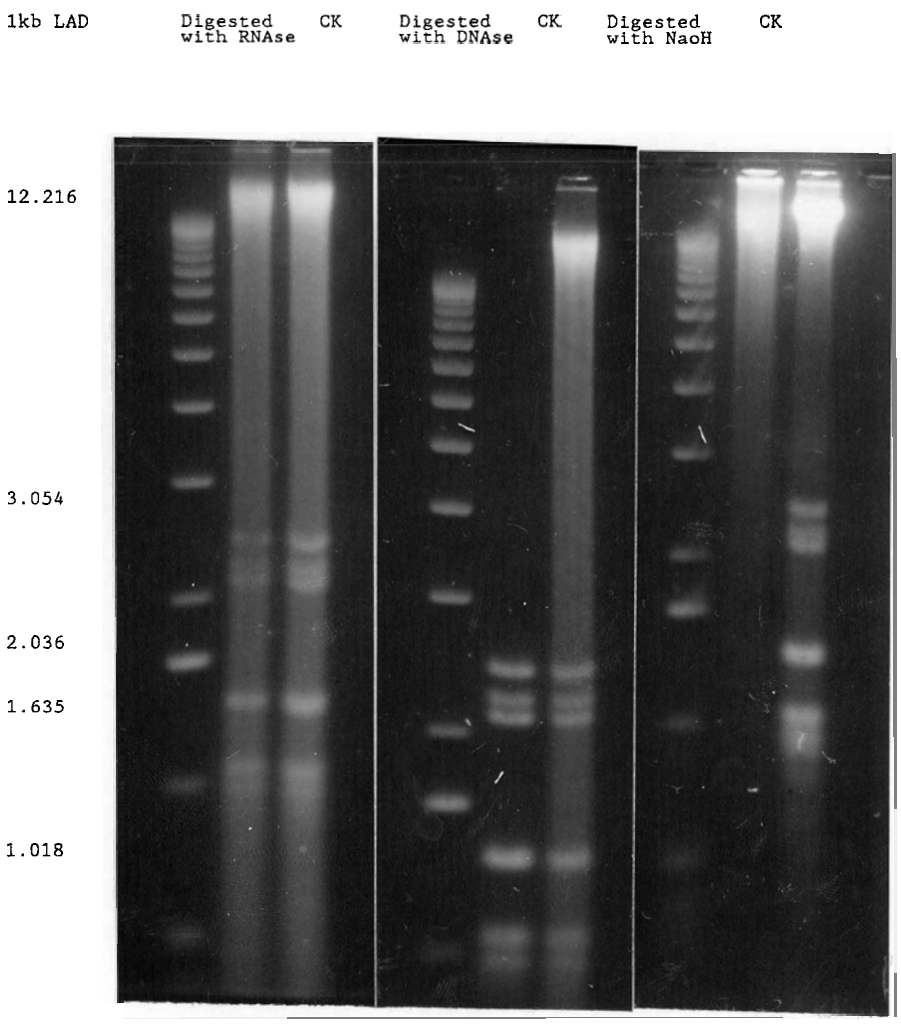


Fig. 6. Total DNA of isolate #3 of *Fusarium oxysporum* after digestion with RNAse, DNAse, and NaoH compared with control treatment (CK). (0.7% agarose gel with ethidium bromide, electrophoresed at 0.5 V cm^{-1} for 18 hr).

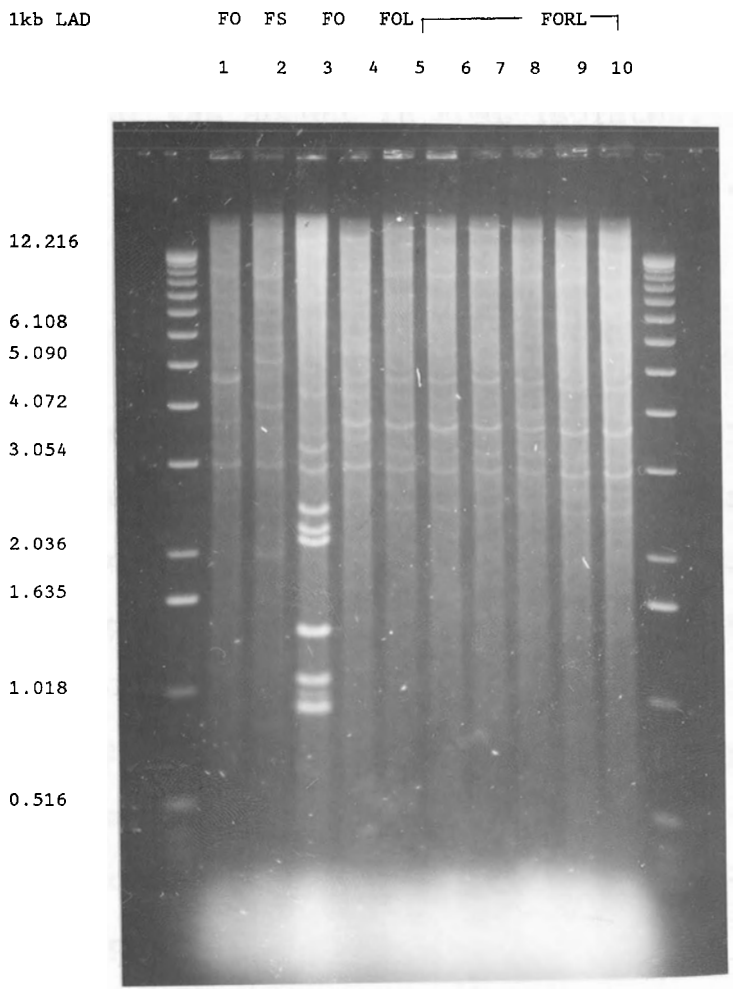


Fig. 7. Electrophoretic analysis of repeat sequence fragments obtained after digestion of total DNA from 10 isolates of *Fusarium* spp. with *EcoRI* (0.7% agarose gel electrophoresed at 0.5 V cm^{-1} for 14 hr and stained with ethidium bromide).

Restriction enzyme digestion of total DNA with *HindIII* gave a complex pattern of bands (data not shown). Of the 10 isolates examined, there was a slight difference between the FOL and FORL isolates. The FOL isolate had a band about 9 kb in size, that was absent in FORL isolates. The remaining four isolates (including two isolates of FO, and one FS) gave unique banding patterns.

3-3.3. PCR-generated DNA fragments

In a preliminary study, the primers ML1-ML4, ML7-ML8, ITS1-ITS4, and NTS1-NTS2 were used to amplify mitochondrial (ML) and nuclear rDNA (ITS, NTS) of five isolates of *Fusarium* spp. (one FO, two FOL, and two FORL) respectively. With the ML7-ML8 primers, isolate #10 produced a single band of 0.3 kb, but isolates #4, #11, #5 and #1 yielded unexpected multiple bands of 1.6 kb to 0.2 kb which probably were artifacts or errors in amplification (Fig. 8). One fragment, approximately 2.8 kb in size, was obtained from all isolates examined in the preliminary and subsequent studies using primers NTS1 and NTS2 (Fig. 9). A 0.5 kb fragment was the product generated from primers ITS1 and ITS4. A 1.0 kb fragment was the product generated from primers ML1 and ML4 (Fig. 9).

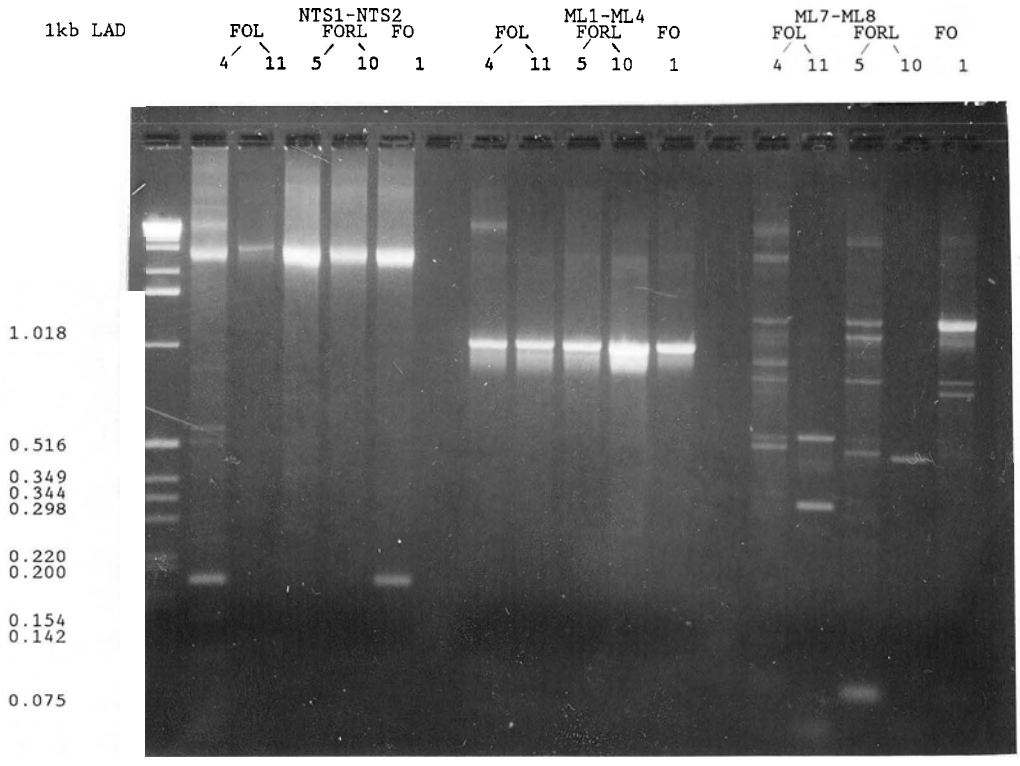


Fig. 8. PCR products generated after amplification of total DNA from five isolates of *Fusarium* spp. using primers NTS1-NTS2, ML1-ML4, and ML7-ML8 (1.5% agarose gel electrophoresed at 3.3 V cm^{-1} for 1 hr).

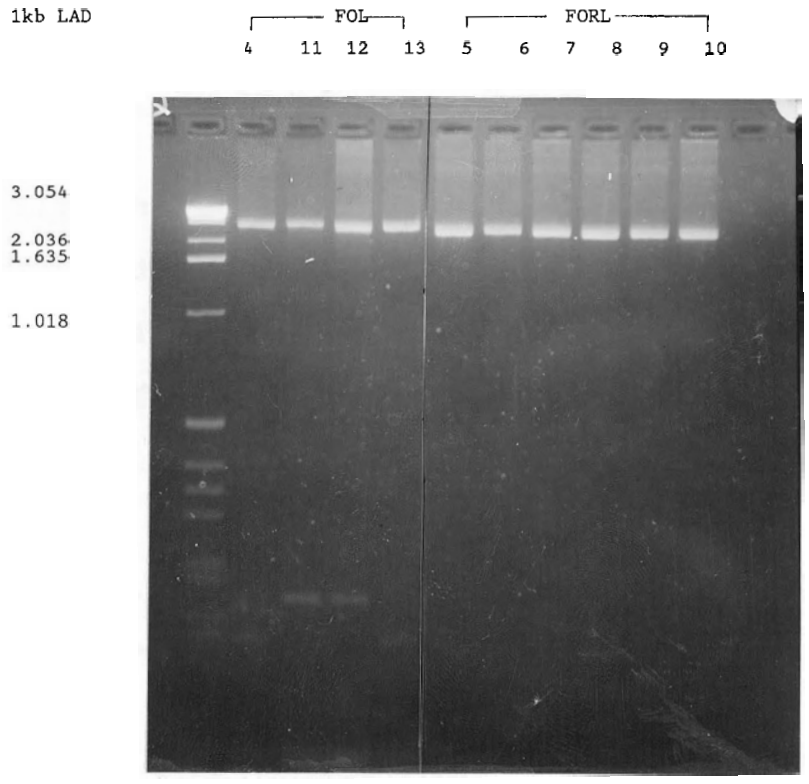


Fig. 9. PCR products generated after amplification of total DNA from 10 isolates of *Fusarium oxysporum* using primers NTS1 and NTS2 (1.5% agarose gel elestrophoresed at 3.3 V cm^{-1} for 1 hr).

3-3.4. Restriction analysis of amplified DNA

When the ML1-ML4 and ITS1-ITS4 PCR products obtained in the preliminary study of five isolates were digested with three restriction enzymes (*AluI*, *DdeI*, and *TaqI*) similar banding patterns were produced and none of the digests clearly differentiated these isolates from each other. Generally similar patterns of restriction fragments were also produced after digestion of amplified mitochondrial rDNA (ML1-ML4) from each of the various *Fusarium* isolates with *AluI*, *CfoI*, *DdeI*, *HinfI*, *HpaII*, *RsaI*, and *TaqI*, except for DNA from isolate #12 of FOL which always yielded fragments in addition to those fragments common to all of the isolates. Photographs of representative gels are shown in Fig. 10 and 11. Isolate #13 sometimes produced weak bands that matched some of the additional bands produced by isolate #12.

Each of eight restriction endonucleases yielded restriction fragments after digestion of amplified intergenic rDNA from isolates of *F. oxysporum*, and the patterns of restriction fragments produced by each of these endonucleases were different. In the preliminary study of a selected subset of isolates that included one FO, two FOL and two FORL isolates, PCR-amplifed mitochondrial rDNA (ML1-ML4) and nuclear rDNA (ITS1-ITS4) were digested with *AluI*, *DdeI*, and *TaqI* restriction endonuleases. All isolates produced similar banding patterns, and no differences were

seen among them. PCR-amplified nuclear rDNAs (NTS1-NTS2) of the same five isolates (one FO, two FOL and two FORL) were digested using *HpaII*, *HinfI*, and *HaeIII* restriction endonucleases. Although many of the bands in these digests were common to all five isolates of *Fusarium*, differences between the FO, FOL and FORL isolates were also evident (Fig. 12). When restriction digests of PCR-amplified nuclear rDNA (NTS1-NTS2) from four isolates of FOL and six isolates of FORL were compared, only the *AluI* endonuclease generated products that clearly distinguished all of the FORL isolates from all of the FOL isolates. Digests of all FOL isolates showed a band of 0.18 kb that was absent in digests of all FORL isolates, and digests of all FORL isolates showed a band of 0.39 kb that was absent in digests of all FOL isolates (Fig. 13A).

Each of the eight endonucleases produced digests of PCR-amplified nuclear rDNA (NTS1-NTS2) with some unique character that differentiated isolate #12 from the other isolates being compared. The patterns of restriction fragments produced by endonucleases *HpaII* and *DdeI* were similar among all of the isolates being compared except for isolate #12. The remaining six endonucleases and *AluI* produced digests which differentiated at least some of the isolates. Notably, patterns of restriction fragments were produced by these latter endonucleases within the group of

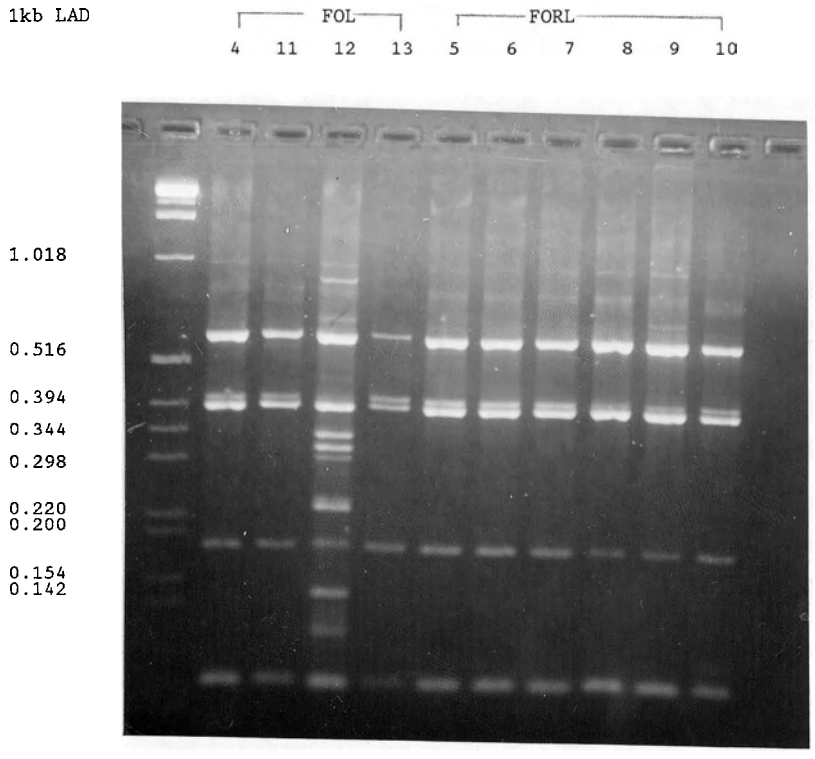


Fig. 10. Amplified mitochondrial rDNA (ML1-Ml4) of 10 isolates of *Fusarium oxysporum* digested with *HinfI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

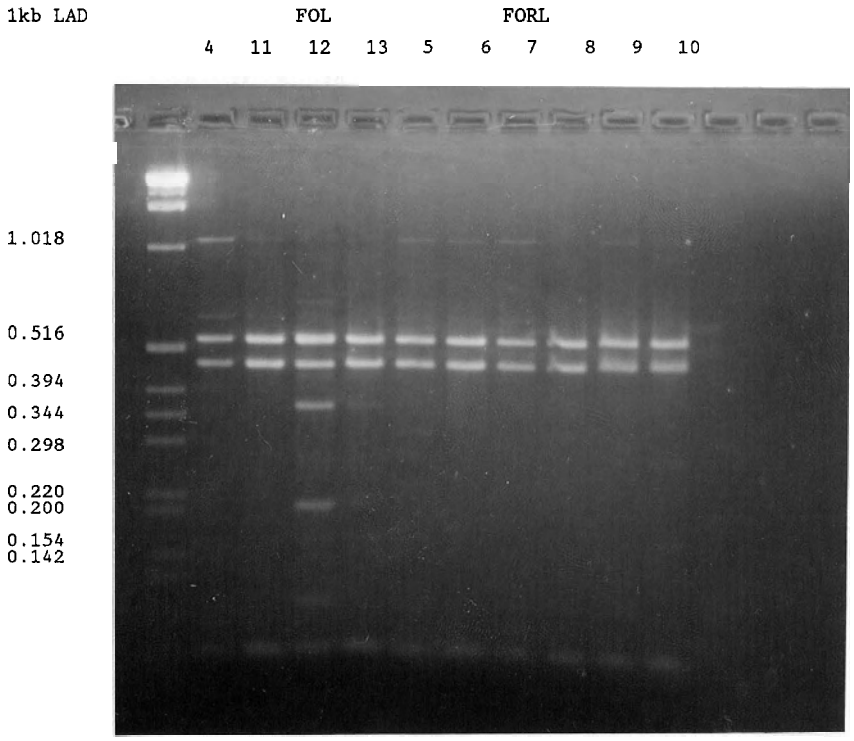


Fig. 11. Amplified mitochondrial rDNA (ML1-ML4) of 10 isolates of *Fusarium oxysporum* digested with *TaqI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

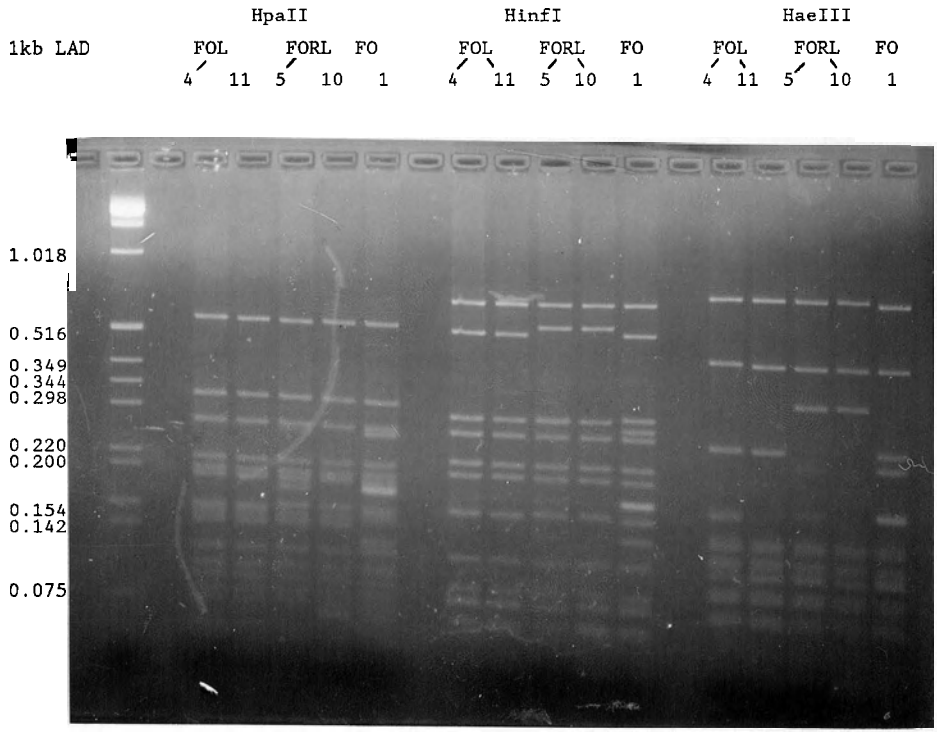


Fig. 12. Amplified nuclear rDNA (NTS1-NTS2) of five isolates of *Fusarium* spp. digested with *HpaII*, *HinfI* and *HaeIII* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

six FORL isolates that were consistent with the pattern of vegetative compatibility described previously (Chapter 2). Isolates #6 and #8 (VCG 06) were differentiated from isolates #5, #7, #9 and #10 (VCG 05, and 07) and the two groups of isolates always had the same banding pattern among themselves (Fig. 13-17). None of the endonucleases produced a banding pattern that was inconsistent with the pattern of vegetative compatibility observed among these six isolates.

The potential value of the 0.18 kb and 0.39 kb bands produced in *AluI* digests of PCR-amplified nuclear rDNA (NTS1-NTS2) for distinguishing FOL from FORL isolates was tested using two new isolates of FORL from Holland and one isolate of FOL from Louisiana, USA that were obtained near the completion of my study. *AluI* digests of PCR-amplified DNA (NTS1-NTS2) from the new FORL isolates also contained a 0.39 kb band that was absent in the new FOL isolate from Louisiana, but the 0.18 kb fragment observed in all previously studied FOL isolates was absent in *AluI* digests of NTS1-NTS2 amplified DNA from the Louisiana FOL isolate (Fig. 13B).

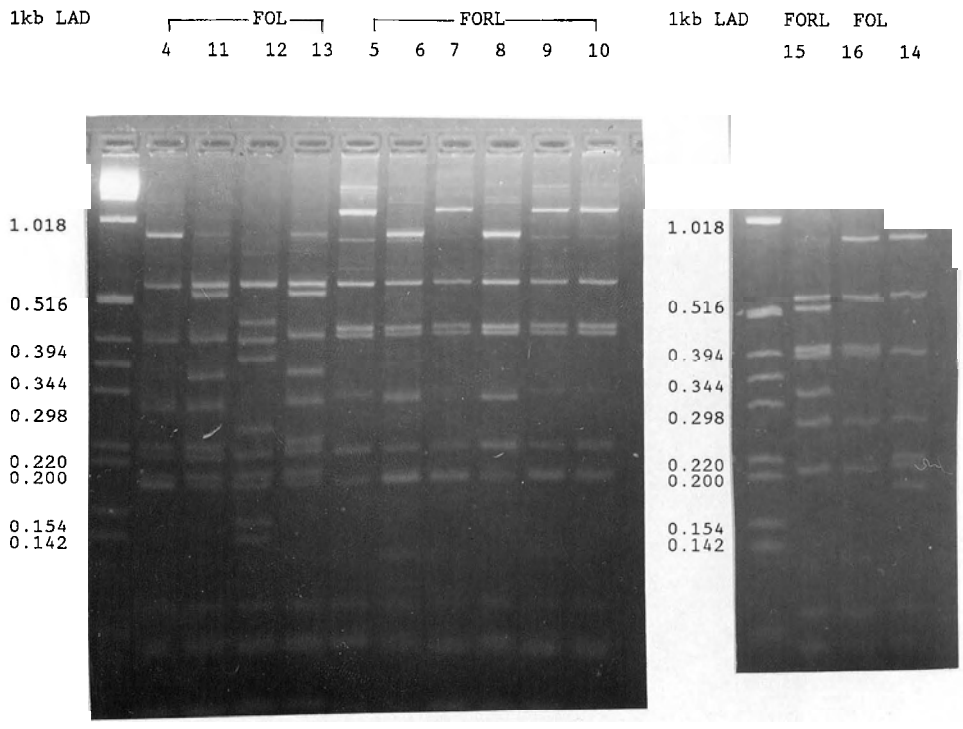


Fig. 13. A. Amplified nuclear rDNA (NTS1-NTS2) of 13 isolates of *Fusarium oxysporum* digested with *AluI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left. B. Amplified nuclear rDNA (NTS1-NTS2) three additional isolates of *Fusarium oxysporum* digested with *AluI* and electrophoresed as described for Fig. 13A.

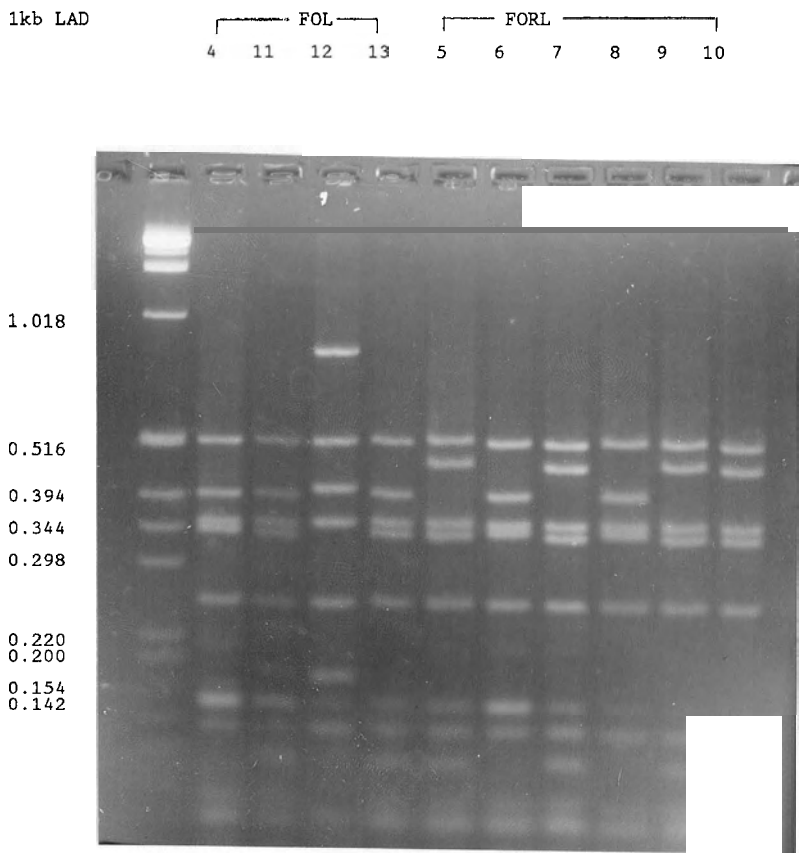


Fig. 14. Amplified nuclear rDNA (NTS1-NTS2) of 10 isolates of *Fusarium oxysporum* digested with *TaqI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

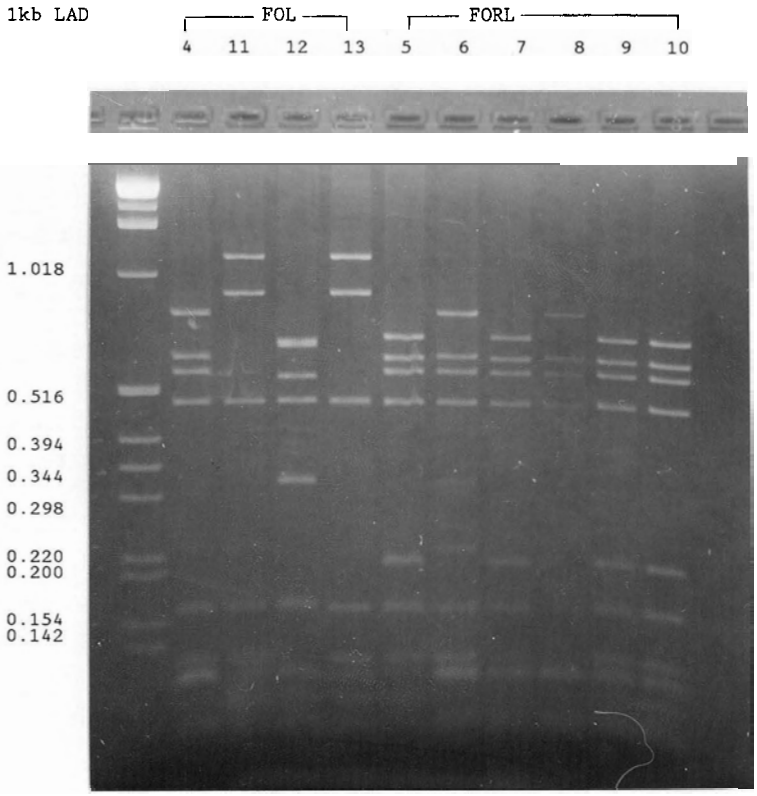


Fig. 15. Amplified nuclear rDNA (NTS1-NTS2) of 10 isolates of *Fusarium oxysporum* digested with *RsaI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

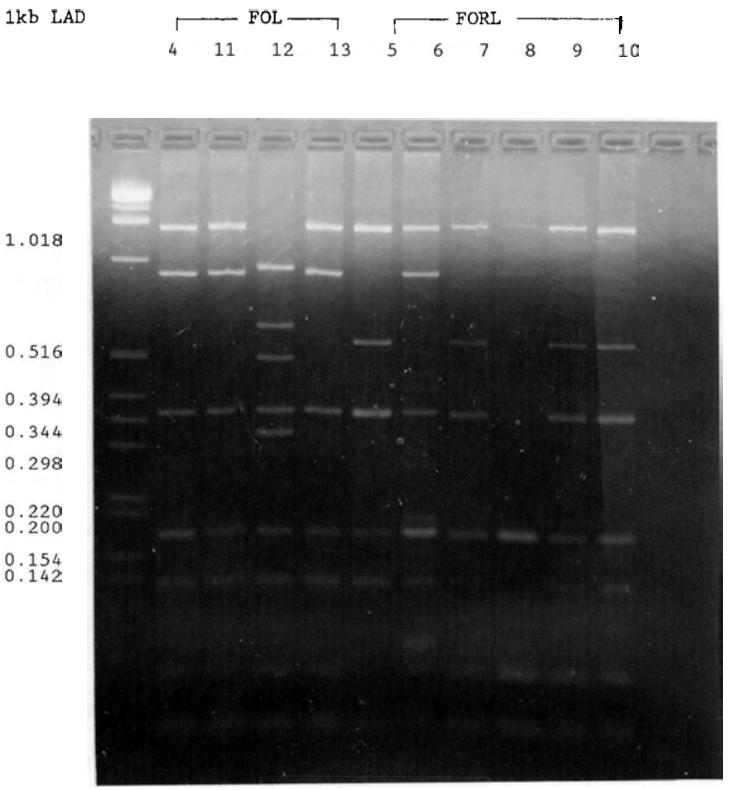


Fig. 16. Amplified nuclear rDNA (NTS1-NTS2) of 10 isolates of *Fusarium oxysporum* digested with *CfoI* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

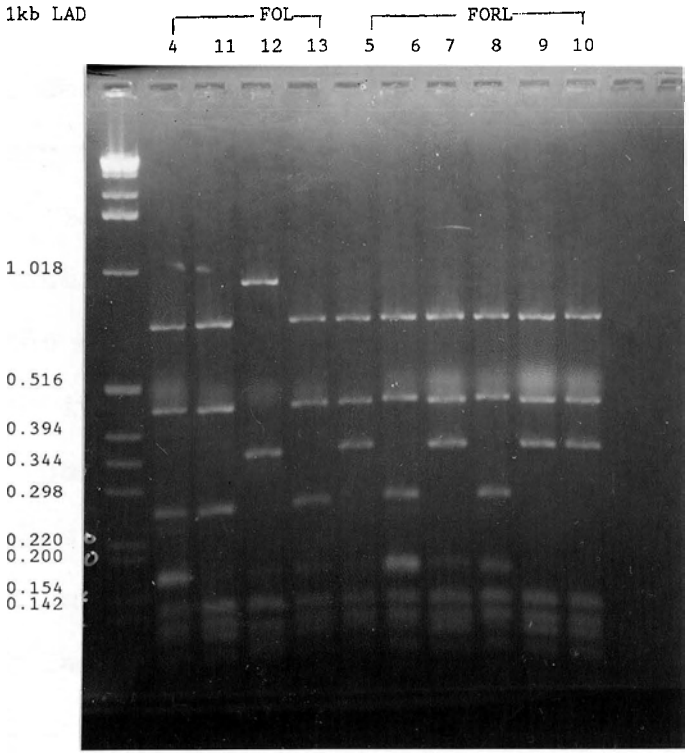


Fig. 17. Amplified nuclear rDNA (NTS1-NTS2) of 10 isolates of *Fusarium oxysporum* digested with *HaeIII* after electrophoresis in 2.0% agarose gel at 3.3 V cm^{-1} for 1 hr and stained with ethidium bromide. Size markers (kb) are indicated on the left.

CHAPTER 4. DISCUSSION

Discussion:

Fusarium crown and root rot (or foot and root rot) was first described from Japan, and the pathogen has spread rapidly through many countries, specially within greenhouse crops (Jarvis, 1988; Farley *et al.*, 1975). The losses from the disease have been considerable (Farley *et al.*, 1975) and the disease has become a severe threat to the respective industries.

There are different opinions on the host range of isolates of FORL, ranging from pathogenicity on different tomato cultivars to pathogenicity on various hosts in five plant families (Yamamoto *et al.* 1974; Rowe, 1978; Menzies *et al.*, 1990). The differences between these studies may be attributable to differences in cultivars or inoculation techniques. However, these results of a wide host range may explain the spread of this pathogen over short and long distances on infected plant materials.

Previously, identification of formae speciales and races of *F. oxysporum* was based commonly on greenhouse virulence tests (Booth, 1971). Such tests provide limited information and sometimes contradictions with regard to the precise relationships among various pathotypes found on tomato. Variation in environmental conditions, methods of inoculation, and host age can give inconclusive and

sometimes misleading results (Bosland and Williams, 1987). Availability of other techniques that are less time-consuming and more precise than pathogenicity tests would be useful in differentiating *F. oxysporum* isolates. My research supports the conclusions of others in several recent publications already cited that VCG tests and PCR amplification of nuclear-encoded rDNA and digestion with restriction enzymes are useful techniques for the analysis of variation in *F. oxysporum* and for the more precise separation of isolates into distinct groups.

Ten distinct vegetative compatibility groups were revealed among 13 isolates of *Fusarium* spp. tested. The six isolates of FORL fell into three VCGs, while the other seven isolates, including four isolates of FOL, each represented a unique VCG. There was no apparent relationship between geographical origin and VCG. None of the isolates of FORL was vegetatively compatible with any of the isolates of FOL. The three VCGs found among the six isolates of FORL were consistently characterized by two patterns of RFLP in digests of PCR-amplified rDNA that differentiated the members of VCG 06 from VCGs 05 and 07 (Figs. 13-17). The digests showed homology between individual members of VCG 06, and among the members of VCGs 05 and 07. This putative correlation of RFLPs with VCG indicate that at least some VCGs of *F. oxysporum* isolates pathogenic on tomato may be distinguished by observed

genetic variation occurring within their DNA. Overall, the results from my study show that a high degree of genetic diversity exists within isolates of *F. oxysporum* pathogenic on tomato. Even though only four isolates of FOL were studied, each isolate represented a distinct VCG, and six isolates of FORL represented three VCGs.

PCR amplified rDNA digested with *AluI* endonuclease generated products that distinguished all of the eight FORL isolates from the five FOL isolates examined. Thus, two separate methods, VCG testing and RFLP analysis, have independently produced evidence for genetic differences between FOL and FORL within *F. oxysporum*.

Although it was possible to distinguish all FORL isolates from all FOL isolates studied on the basis of a 0.39 kb restriction fragment produced by *AluI* digestion of PCR-amplified rDNA, many other RFLP's were observed among the isolates. It is not likely that the produced RFLP which differentiated FORL and FOL would have received particular attention if the identity of isolates as FORL and FOL had been unknown. In addition, the total number of FOL and FORL isolates distinguished by this RFLP is small. The overall conclusion is that evidence for substantial genetic variation was observed among 13 isolates of *F. oxysporum* pathogenic on tomato, including a single RFLP which differentiated isolates of FORL from isolates of FOL. Genetic variation within the eight isolates of FORL

examined was less than among the five isolates of FOL, but the total number of isolates examined is too few to draw conclusions of this type with regard to the natural populations of FORL and FOL.

It is often argued that vegetatively compatible strains of fungi are more similar genetically than are incompatible strains. Based on studies of sexually reproducing fungi such as *Gibberella fujikruoi* (Puhalla and Spieth, 1985) and *Aspergillus nidulans* (Croft and Jinks, 1977), it has been argued that a high degree of genetic homology is needed for vegetative compatibility. Correll *et al.* (1986*b*) placed 50 out of 110 strains of *F. oxysporum* into 14 VCGs, but also found many VCGs are represented by single strains. A similar finding was made in my study: 13 isolates of *Fusarium* spp. were assigned to 10 VCGs, including seven single-member VCG and three VCGs each with two members. Elias and Schneider (1991) found that 50 of 115 isolates of FOL occurred as single-member VCGs. Elmer and Stephens (1989) found that 34 of 97 isolates of *F. oxysporum* f.sp *asparagi* occurred in unique VCGs. Elias and Schneider (1991) suggested that certain members of major VCGs may have undergone simple mutations, which could no longer form heterokaryons with the other members. It seems that biochemical or molecular measures of genetic diversity could provide explanations for this phenomenon of many single-member VCGs.

It is expected that additional VCGs will be found if more isolates are examined. Studies with other formae speciales of *F. oxysporum* revealed that some of these pathogens consisted of single VCGs (Katan *et al.*, 1989; Katan and Katan, 1988) while others were more diverse and consisted of several VCGs (Correll *et al.*, 1986*b*; Elias and Schneider, 1988; Jacobson and Gordon, 1988; Ploetz and Correll, 1988). Studies with populations of *F. oxysporum* f.sp. *radicis-lycopersici* revealed seven VC groups among 218 isolates. Diversity was evident among isolates of FORL from France and Canada, where two or three VCGs were found among a small number of isolates tested from each country (Katan *et al.*, 1991). Puhalla found 16 VCGs among 21 pathogenic isolates of *F. oxysporum* representing 12 formae speciales (Puhalla, 1985). In another study involving non-pathogenic isolates of *F. oxysporum* from celery, 14 VCGs were represented within 28 randomly selected isolates and subsequent analysis showed that these 14 VCGs accounted for only 50 out of the total of 110 nonpathogenic isolates studied (Correll and Puhalla, 1985). These findings suggest that there may be greater VCG diversity within *F. oxysporum* than has demonstrated in other fungal species (Puhalla and Hummel, 1983).

One vegetatively self-incompatible isolate was found in my study. The #4 isolate of FOL was heterokaryon self-incompatible. This phenomenon has been reported in *F.*

oxysporum (Jacobson and Gordon, 1988), *F. moniliforme* (Correll *et al.*, 1989), *Rhizoctonia solani* (Hyakumachi and Ui, 1987), *Verticillium albo-atrum* (Correll *et al.*, 1988), *Verticillium dahliae* (Puhalla and Hummel, 1983), and probably in *Aspergillus flavus* (Papa, 1986). The occurrence of self-incompatibility within a sample of only 13 isolates is somewhat surprising, however, since the frequency of self-incompatibility in other studies has usually been very low (Correll *et al.*, 1989; Jacobson and Gordon, 1988). The mechanism of vegetative self-incompatibility in *F. oxysporum* is unknown. However, Brooker *et al.* (1991) suggested that these strains can be very useful in biological control work because of their ability to form a heterokaryon with any other strain in the population, thus transmuting genetic material. It may be important to study and get a better understanding of these self-compatible strains of *F. oxysporum* from both a basic and applied perspectives (Brooker, 1991).

In my preliminary screening, no complementation occurred between any *Nit* mutants of isolate #3 (FO) or isolate #4 (FOL), even after repeated attempts, and no complementation was observed when the *Nit*1 or *Nit*M mutants were paired among themselves, respectively. Surprisingly, when the #3 isolate was tested again using two different *Nit* mutants, which had been stored on filter paper as previously described, a positive reaction was seen. In the

DNA analysis study (see chapter 3), a dsRNA was found in this isolate. The possibility that dsRNA might be related in some way to the inconsistent results regarding self compatibility of isolate #3 merits further investigation.

In the study of RFLP in restriction digests of PCR amplified mitochondrial rDNA using ML1-ML4 primers for 10 isolates of *F. oxysporum*, no variation was revealed in the FOL and FORL isolates. The variation in mitochondrial DNA that has been used to assess diversity in *F. oxysporum* is most commonly due to length mutations, although nucleotide substitution also can contribute to the variability (Jacobson and Gordon, 1990). Gordon and Okamoto (1992) reported no variation of mitochondrial DNA in *F. oxysporum* isolates within the same VCG, but variation between isolates in different VCGs was observed. In contrast, to these findings mitochondrial DNA did not distinguish between the FOL and FORL isolates examined in my study. If the lack of variation in mitochondrial DNA is interpreted to suggest that isolates of FOL and FORL are closely related, this might indicate that FORL is a recent derivative of FOL as compared with other formae speciales (Katan *et al.*, 1991).

In contrast, comparisons by RFLP analysis of PCR-amplified nuclear rDNA (NTS1-NTS2) of 10 isolates of *F. oxysporum* revealed considerable genetic variation and a strong correlation between VCG and RFLP. With *AluI* digests

of PCR amplified intergenic rDNA, FOL and FORL could be consistently differentiated from each other. The use of primers NTS1 and NTS2 for PCR amplification of nuclear rDNA and digestion with four restriction enzymes always gave clear bands and revealed distinct patterns with different restriction enzymes. Thus, primers NTS1 and NTS2 appear to be very useful for detecting genetic variation within FOL and FORL, and may prove to be useful for differentiation of FOL and FORL isolates via PCR amplification and RFLP analysis.

In the studies of RFLP of PCR-amplified mitochondrial rDNA, among the 10 isolates of *F. oxysporum*, isolate #12 always yielded additional bands which were not common to and of the other isolates of *F. oxysporum*. The cause of this unique banding pattern in isolate #12 is not known, but it could be due to microbial contamination or a plamid. I obtained no evidence for either of these possibilities, however, and further work is needed to explain the unique RFLP characteristics of this isolate.

During this research, no information was obtained regarding the function of dsRNA in isolate #3 of FO, but it is important to mention that isolate #3 was one of the strains of *F. oxysporum* described by Brammall and Higgins (1987) which gave biological control of tomato crown and

root rot (pers communication, R.J. Copeman). Most known mycoviruses contain dsRNA (Bozarth, 1972). Fungal virulence may be modified by mycovirus infection in some cases (Moffitt and Lister, 1975). For example, the hypovirulent strains of *Endothia parasitica* first described by Grente in 1965 were later shown to contain virus-like RNA (Van Alfen and Hansen, 1983). Further work is needed to confirm whether or not there is a relationship between dsRNA and the biological control potential of isolate #3.

Based on the work described in this thesis, it appear possible that RFLP analysis of PCR amplified DNA or a probe containing a base sequence complementary to a portion of the 0.39 kb fragment produced in AluI digests of amplified NTS1-NTS2 rDNA, can be used to separate FORL from FOL without recourse to pathogenicity tests. These two laboratory tests could provide a useful and easy method for FORL identification.

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LITERATURE CITED

Armstrong, G.M., and Armstrong, J.K. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399 *In*: *Fusarium*: Diseases, Biology, and Taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., *eds*. Pennsylvania State University Press, University Park. 457pp.

Benhamou, N., Charest, P.M., and Jarvis, W.R. 1988. Biology and host-parasite relations of *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Pages 95-105 *In*: Vascular Wilt Diseases of Plants. Tjamos, E.C., and Beckman, C.H. *eds*. Springer-Verlag, Berlin. 590pp.

Biles, C.L., and Martyn, R.D. 1988. Isozyme analysis of *Fusarium oxysporum* f.sp. *niveum* races and selected *Fusarium* sp. (Abstr.). Phytopathology 78:625.

Booth, C. 1971. The genus *Fusarium*. CMI, Kew, Surrey, England. 237pp.

Bosland, B.W., and Williams, P.H. 1987. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 65:2067-2073.

Bozarth, R.F. 1972. Mycoviruses: a new dimension in microbiology. Environ. Health Perspect. 2:23-39.

Brammall, R.A., and Higgins, V.J. 1987. The control of fusarium crown and root rot disease of tomato by root inoculation with alien pathogenic fungi (Abstr.). Can. J. Plant Pathol. 9:274

Brooker, N.L., Leslie, J.F., and Dickman, M.B. 1991. Nitrate non-utilizing mutants of *Colletotrichum* and their use in studies of vegetative compatibility and genetic relatedness. Phytopathology 81:672-677.

Bruns, T.D., White, T.J., and Taylor, J.W. 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. 22:525-564.

Coddington, A., Matthews, P.M., Cullis, C., and Smith, K.H. 1987. Restriction digest patterns of total DNA from different races of *Fusarium oxysporum* f.sp. *pisi* an improved method for race classification. J. Phytopathology 118:9-20.

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

Correll, J.C., Gordon, T.R., and McCain, A.H. 1988. Vegetative compatibility and pathogenicity of *Verticillium albo-atrum*. Phytopathology 78:1017-1021.

Correll, J.C., Klittich, C.J.R., and Leslie, J.F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640-1646.

Correll, J.C., Klittich, C.J.R., and Leslie, J.F. 1989. Heterokaryon self-incompatibility in *Gibberella fujikuroi* (*Fusarium moniliforme*). Mycol. Res. 93:21-27.

Correll, J.C., Puhalla, J.E., and Schneider, R.W. 1986*a*. Identification of *Fusarium oxysporum* f.sp. *apii* on the basis of colony size, virulence, and vegetative compatibility. Phytopathology 76:396-400.

Correll, J.C., Puhalla, J.E., and Schneider, R.W. 1986*b*. Vegetative compatibility groups among nonpathogenic root colonizing strains of *Fusarium oxysporum*. Can. J. Bot. 64:2358-2361.

Croft, J.H., and Jinks, J.L. 1977. Aspects of population genetics of *Aspergillus ridulans*. Page 339-360 *In*: Genetics and Physiology of *Aspergillus nidulans*. Smith, J.E. and Paterman, J.A., *eds*. Academic Press. New York. 552pp.

Cubeta, M.A., Echandi, E., Abernethy, T., and Vigalys, R. 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of an amplified ribosomal RNA gene. Phytopathology 81:1395-1400.

Curran J., McClure, M.A., and Webster, J.M. 1986. Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length difference in total DNA. Journal of Nematology 18:83-86.

Elias, K.S., and Schneider, R.W. 1991. Vegetative compatibility groups in *Fusarium oxysporum* f.sp. *lycopersici*. Phytopathology 81:159-162.

Elmer, W.H., 1991. Vegetative compatibility groups of *Fusarium proliferatum* from asparagus and comparisons of virulence, growth rates, and colonization of

asparagus residues among groups. Phytopathology 81:852-857.

Elmer, W.H., and Stephens, C.T. 1989. Classification of *Fusarium oxysporum* f.sp. *asparagi* into vegetatively compatible groups. Phytopathology 79:88-93.

Farley, J.D., Oades, G., and Jaberg, C. 1975. A new greenhouse tomato root-rot disease caused by *Fusarium oxysporum*: a preliminary report. Ohio agric. Res. Dev, Cent. Res. Summ. 82, pp. 27-29.

Gordon, T.R., and Okamoto, D. 1991. Vegetative compatibility groupings in a local population of *Fusarium oxysporum*. Can. J. Bot. 69:168-172.

Gordon, T.R., and Okamoto, D. 1992. Variation within and between populations of *Fusarium oxysporum* based on vegetative compatibility and mitochondrial DNA. Can. J. Bot. 70:1211-1217.

Gordon, W.L. 1965. Pathogenic strains of *Fusarium oxysporum*. Can. J. Bot. 43:1309-1318.

Grente, M.J. 1965. Les formes hypovirulentes d' *Endothia parasitica* et les espoirs de lutte contre le chancre du chataignier. C.R. Seances Acad. Agric. Fr. 51:1033-1037.

Guadet, J., Julien, J., Lafay, J.F., and Brygoo, Y. 1989. Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. Mol. Biol. Evol. 6:227-242.

Hadar, E., Katan, J., and Katan, T. 1989. The use of nitrate-nonutilizing mutants and a selective medium for studies of pathogenic strains of *Fusarium oxysporum*. Plant Dis. 73:800-803.

Jacobson, D.J., and Gordon, T.R. 1988. Vegetative compatibility and self-incompatibility within *Fusarium oxysporum* f.sp. *melonis*. Phytopathology 78:668-672.

Jacobson, D.J., and Gordon, T.R. 1990. Further investigations of vegetative compatibility within *Fusarium oxysporum* f.sp. *melonis*. Can. J. Bot. 68:1245-1248.

Jarvis, W.R. 1988. Fusarium crown and root rot of tomatoes. Phytoprotection 69:49-64.

Jarvis, W.R., and Shoemaker, R.A. 1978. Taxonomic status of *Fusarium oxysporum* causing foot and root rot of tomato. Phytopathology 68:1679-1680

Jarvis, W.R., Thorp, H.J., and MacNeill, B.H. 1975. A foot and root rot disease of tomato caused by *Fusarium oxysporum*. Can.Plant Dis. Sruv. 55:25-26.

Joaquim, T.R., and Rowe, R.C. 1991. Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plants. Phytopathology 81:552-558.

Jones, J.R., and Woltz, S.S. 1981. Fusarium-incited diseases of tomato and potato and their control. Pages 157-168 *In*: Fusarium: Diseases, Biology, and Taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., *eds*. Pennsylvania State University Press, University Park. 457pp.

Katan, T., and Katan, J. 1988. Vegetative-compatibility grouping of *Fusarium oxysporum* f.sp. *vasinfectum* from tissue and the rhizosphere of cotton plants. Phytopathology 78:852-855.

Katan,T., Zamir, D., Sarfatti, M., and Katan, J. 1991. Vegetative compatibility groups and subgroups in *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Phytopathology 81:255-262.

Kim, D.H., Martyn, R.D., and Magill, C.W. 1990. Preliminary investigation of geographic isolates and races of *F. oxysporum* f.sp. *niveum* using mitochondrial DNA restriction fragment length polymorphisms (Abstr.). Phytopathology 80:435.

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.

Kistler, H.C., Bosland, P.W., Benny, U., Leong, S., and Williams, P.H. 1987. Relatedness of strains of *Fusarium oxysporum* from crucifers measured by examination of mitochondral and ribosomal DNA. Phytopathology 77:1289-1293.

Kuninaga, S., and Yokosawa, R. 1991. Determination of the taxonomic status of tomato crown and root rot isolates in *Fusarium oxysporum* by DNA homology. Ann. Phytopath. Soc. Japan 57:9-16.

MacHardy, W.E. and Beckman, C.H. 1981. Vascular wilt fusaria: infection and pathogenesis. Page 365-390. *In*: *Fusarium*: Diseases, Biology, and Taxonomy. Nelson, P.E., Toussoun, T.A., and Cook, R.J., *eds*. Pennsylvania State University Press, University Park. 457pp.

Manicom, B.Q., Bar-Joseph, M., Kotze, J.M., and Becker, M.M. 1990. A restriction fragment length polymorphism probe relating vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f.sp. *dianthi*. Phytopathology 80:336-339.

Menzies, J.G., Koch, C., and Seywerd, F. 1990. Additions to the host range of *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Plant Dis. 74:569-572.

Messiaen, C.M., and Cassini, R. 1968. Recherches sur les Fusarioses. IV. La Systematique des *Fusarium*. Ann. Epiphyties. 19:387-454.

Michelmore, R.W., and Hulbert, S.H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. 25:383-404.

Mills, D., and McCluskey, K. 1990. Electrophoretic karyotypes of fungi: the new cytology. Mol. Plant-Microbe Interact. 3:351-357.

Moffitt, E.M., and Lister, R.M. 1975. Application of a serological screening test for detecting double-stranded RNA mycoviruses. Phytopathology 65:851-859.

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.

Nash, S.M., and Snyder, W.C. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567-572.

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. Physiological and Molecular Plant Pathology. 39:1-11.

Nelson, P.E. 1981. Life cycle and epidemiology of *Fusarium oxysporum*. Pages 51-80 *In*: Fungal Wilt Diseases of Plants. Mace, M.E., Bell, A.A., and Beckman, C.H., *eds*. Academic Press, New York. 640pp.

Ploetz, R.C., and Correll, J.C. 1988. Vegetative compatibility among races of *Fusarium oxysporum* f.sp. *cubense*. Plant Dis. 72:325-328.

Puhalla, J.E. 1985. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 63:179-183.

Puhalla, J.E. and Hummel, M. 1983. Vegetative compatibility groups within *Verticillium dahliae*. Phytopathology 73:1305-1308.

Puhalla, J.E., and Spieth, G. 1985. A comparison of heterokaryosis and vegetative compatibility in *Gibberella fujikuroi* (*Fusarium moniliforme*). Exp. Mycol. 9:39-47.

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.

Rowe, R.C., and Farley, J.D. 1981. Strategies for controlling Fusarium crown and root rot in greenhouse tomatoes. Plant Dis. 65:107-112.

Saiki, R.K., Sharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnhelm, N. 1985. Enzyme amplification of B-globin genomic sequences and restriction site analysis for diagnosis sickle cell anaemia. Science 233:1350-1354.

Snyder, W.C., and Hansen, H.N. 1940. The species concept in *Fusarium*. Amer. J. Bot. 27:64-47.

Snyder, W.C. and Toussoun, T.A. 1965. Current status of taxonomy in *Fusarium* species and their perfect stages. Phytopathology 55:833-837.

Thibodeau, P.O., and Simard, M. 1978. Evalution de la resistance de cultivars de tomate de serre a la fusariose. Research Report Canadian Hoticultural Council 1978. Page 172 (Abstr.).

Toussoun, T.A. 1975. Fusarium-suppressive soils. Pages 145-151 *In*: Biology and control of soil-borne plant pathogens. Bruehl, G.W. ed. American Phytopathological Society. St. Paul, MN. 216pp.

Toussoun, T.A. and Nelson, P.E. 1968. A pictorial guide to the identification of *Fusarium* species according to the taxonomic system of snyder and Hansen. Pa. State Univ. Park. Pa. 51p.

Van Alfen, N.K., and Hansen, D.R. 1983. Hypovirulence: A unique phenotypic expression of dsRNA in a pathogenic fungus. Page 463-469 *In*: Double-Stranded RNA Viruses. Compans, R.W., and Bishop, D.H.L. *eds*. Elseviver Biomedical. New York. 505pp.

Venter, S.L., Theron, D.J., Steyn, P.J., Ferreira, D.I., and Eicker, A. 1992. Relationship between vegetative compatibility and pathogenicity of isolates of *Fusarium oxysporum* f.sp. *tuberosi* from potato. Phytopathology 82:858-862.

Walker, J.C. 1971. Fusarium wilt of tomato. Monograph *No*.6 Amer. Phytopathol. Soc. St. Paul, MN 55104. 56pp.

Wensley, R.N., and McKeen, C.D. 1962. A soil suspension-plants method of estimating populations of *Fusarium oxysporum* f.sp. *menlonis* in muskmelon wilt soils. Can. J. Microbiol. 8:57-64.

White, T.J., Bruns, T.D., Lee, S., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 38:315-322. In *PCR Protocols*: A Guide To Methods and Applications, Innis, M.A., Gelfand, D.H., Sninsky, J.S., and White, T.J. *eds*. Academic Press. New York. 482pp.

Wollenweber, H.W. 1913. Studies on the *Fusarium* problem. Ibid. 3:24-50.

Yamamoto, I., Komada, H., Kuniyasu, K., Saito, M., and Ezuda, A. 1974. A new race of *Fusarium oxysporum* f.sp. *lycopersici* inducing root rot of tomato. Proc. Kansai Plant Prot. Soc. (Jpn.) 16:17-29.