Characterization of aggressiveness and vegetative compatibility diversity of *Fusarium oxysporum* associated with crown and root rot of birdsfoot trefoil

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Abstract

Birdsfoot trefoil (*Lotus corniculatus*) fields were selected in a 9-site-matrix of three locations and three stand ages, and surveyed twice a year during two successive years. Twenty-five plants in each of 12 permanent quadrats were sampled at each site and date. Samples of infected crown and root tissues were used for fungal isolation. *Fusarium oxysporum* was the primary pathogen associated with diseased plants (40% of isolations). Vegetative compatibility assessed using nitrate non-utilizing mutants was used as a measure of genetic relatedness of *F. oxysporum* isolates. No complementation was found among 18 isolates (630 pairings of 36 *nit* complementary mutants), indicating a high degree of genetic diversity in the pathogen population. A culture plate method was used to characterize isolate aggressiveness to birdsfoot trefoil seeds and seedlings, based on a 5-class scale: 1 = healthy seedling, 5 = dead seed. Most *F. oxysporum* isolates (36 out of 44) were pathogenic to birdsfoot trefoil, and were highly variable in aggressiveness (range: 1.44-3.85). The variability observed in the pathogen population needs to be considered when selecting isolates for resistance screening.
Additional keywords: crown and root diseases, forage legumes.

Introduction

Crown and root diseases are a major cause of premature stand decline and reduced productivity in birdsfoot trefoil pastures (Altier, 1997; Altier *et al.*, 2000; Bergstrom *et al.*, 1995; Berkenkamp *et al.*, 1972; Beuselinck, 1988; Henson, 1962; Hill and Zeiders, 1987; Kainski, 1960; Miller *et al.*, 1964; Pettit *et al.*, 1966; Taylor *et al.*, 1973). *Fusarium* species make up the largest number of pathogens causing crown and root diseases of birdsfoot trefoil (Berkenkamp *et al.*, 1972; Beuselinck, 1988; Drake, 1961; Henson, 1962; Kainski, 1960). The species of *Fusarium* most frequently associated with crown and root rots of forage legumes is *F. oxysporum*, followed by *F. avenaceum*, *F. solani*, *F. acuminatum*, *F. tricinctum*, and *F. moniliforme* (Grau, 1996; Leath, 1989; Leath *et al.*, 1971). More recently, Bergstrom and Kalb (1995) described a wilt organism of birdsfoot trefoil as a specific pathogen of this species, for which they proposed a new taxon, *F. oxysporum* f.sp. *loti*. In

Uruguay, Altier (1994, 1997) studied the fungi associated with diseased birdsfoot trefoil plants in a space-planted nursery and found that the majority of fungi isolated from crown and root tissues were *Fusarium* spp. (72%), with the two most frequently isolated species being *F. oxysporum* (54% of total fungi) and *F. solani* (9% of total fungi).

Authors disagree on the role that *Fusarium* spp. have in the development of the root and crown disease complex of forage legumes (Grau, 1996). Some contend that pathogenic forms only invade root tissues damaged or killed by other causes; others assert that the fungus plays a major role in root and crown disease development (Leath *et al.*, 1971). The pathogenicity of *Fusarium* species varies considerably among and within species depending on their ability to penetrate roots directly, their degree of host specificity, and their interaction with plant stress factors (Fezer, 1961; Leath, 1989; Leath and Kendall, 1978; Pederson *et al.*, 1980; Stutz and Leath, 1983; Venuto *et al.*, 1995). A number of environmental factors including soil moisture, drainage, air and soil temperature, nutrients, stand density, plant age, frequency and height of cutting, crop rotation, insect injury, and previous invasion by viruses and nematodes, have an effect on the expression of the disease and on plant susceptibility (Leath *et al.*, 1971).

Root rotting *Fusaria* may penetrate unwounded roots directly, but most species have limited ability to initiate root rot on their own (Chi *et al.*, 1964; Stutz *et al.*, 1985). The wounding of roots increases the frequency of penetration by disrupting the mechanical barrier imposed by the epidermis. Furthermore, Stutz *et al.* (1985) asserted that wounding alters the host-pathogen interaction to favor fungal development in the root. Wounding can be the result of insect damage or mechanical injuries by soil heaving, harvesting machinery, and trampling and soil compaction caused by animals.

Kommedahl and Windels (1979) asserted that *F. oxysporum* is mainly a wilt-inducing pathogen. However, it is so frequently isolated from necrotic roots that it is regarded as a root rot, wound-associated, pathogen (Kalb *et al.*, 1994; Leath, 1989; Leath and Hower, 1993). *F. oxysporum* has been described as an aggressive, pioneer colonizer of moribund tissues and it can readily invade roots (Leath, 1989).

An understanding of the evolutionary basis for the pathogenicity of *F. oxysporum* and the genetic diversity of the fungal population is critical and would help to develop, or improve, the effectiveness of strategies for disease management (Gordon and Martyn, 1997; Kistler, 2001). Classical genetics based on segregation and recombination is not possible with this anamorphic fungus, since it lacks a known perithecial state (Kistler, 1997). Among diverse approaches in genetic techniques, heterokaryosis or vegetative compatibility analysis provides the opportunity to study the genetics of *F. oxysporum* with greater precision than before (Bosland, 1988; Klein and Correll, 2001). The mechanisms available for genetic exchange in this species are still largely unknown, but numerous possibilities exist beyond simple sexual or clonal reproduction. Genetic evidence consistent with horizontal genetic transfer (transposable elements) and past genetic hybridization between lineages indicate that means for recombination and production of novel genotypes are effectively available (Kistler, 1997). Heterokaryosis and parasexual recombination have been postulated as mechanisms that play a role in explaining the diverse pathogenic potential of *F. oxysporum*

(Bosland, 1988). Heterokaryon formation is favored by vegetative compatibility, which is mediated by nuclear loci, called *het* or *vic* genes (Leslie, 1993). Two strains are vegetatively compatible if they have the same allele at each incompatibility locus. In an asexual population (as in *F. oxysporum*), differences at the *vic* loci are assumed to effectively limit the exchange of genetic information to those individuals that belong to the same vegetative compatibility group (VCG). Since sexual recombination does not occur, members of each VCG will form a genetically isolated subpopulation that will be subjected to standard population genetic forces such as selection, mutation, migration, and drift (Leslie, 1993). Vegetative compatibility can serve as a natural means to further subdivide closely related fungal populations and has been used to estimate genetic diversity within and among these same populations (Correll, 1991; Klein and Correll, 2001; Leslie, 1993).

Puhalla (1985) initiated the idea of grouping isolates of *F. oxysporum* into VCGs based on the use of nitrate nonutilizing (*nit*) mutants generated on medium containing potassium chlorate. Mutants resistant to the salt are usually also *nit* mutants. On a minimal medium with nitrate as the sole nitrogen source, mutants have a radial growth comparable to wild type, but their colonies are very thin. If mutants produce a dense, wild-type growth when paired, they are vegetatively compatible (Puhalla, 1985). This author reported evidence for a correlation between VCG and forma specialis, and proposed an evolutionary model to explain the origin of formae speciales, races and VCGs. He asserted that vegetative compatibility may be a fast and easy way to distinguish pathotypes of *F. oxysporum* with unique virulence capabilities (Puhalla, 1985). Recently, Klein and Correll (2001) asserted that molecular markers and VCG are usually not independently associated, which may mean that VCGs in *F. oxysporum* represent clones, or closely related strains descended from a common ancestor (lineage). However, when pathotypes have been considered, there often has been no clear-cut association between these and molecular genotypes (Kistler, 1997).

Soil populations of some phytopathogenic fungi are extremely diverse with respect to VCG (Correll *et al.*, 1986; Gordon and Martyn, 1997; Gordon and Okamoto, 1991; 1992). Studies that have used *nit* mutants to differentiate strains in the nonpathogenic portion of a *F. oxysporum* population revealed a large number of distinct VCGs (Correll *et al.*, 1986; Gordon and Okamoto, 1991; Steinberg *et al.*, 1997). Moreover, most of the strains associated with a given host within the same geographic area, or even isolated from a single field in two consecutive years, belonged to different VCGs (Correll *et al.*, 1986; Gordon and Okamoto, 1991). Thus, the frequency with which anastomosis occurs within such populations is likely to be low.

Despite many references concerning VCGs in diverse formae speciales, few references are found concerning *F. oxysporum* that cause crown and root diseases of forage legumes. Venuto *et al.* (1995) studied the virulence, legume host specificity and genetic relatedness of *F. oxysporum* isolates from red clover, and reported that VCGs were not useful in predicting host reaction because isolates from distinct groupings elicited similar host reactions. Their results indicate that the number of genes controlling compatibility seems to be higher than the number of virulence genes (Venuto *et al.*, 1995).

Information on the pathogenicity and genetic diversity of *F. oxysporum* isolates from

birdsfoot trefoil is required if breeding for resistance is to be explored as a means of managing the Fusarium crown and root disease complex (Altier *et al.*, 2000). The major objective of our study was to characterize the *F. oxysporum* population associated with diseased plants of birdsfoot trefoil, in terms of aggressiveness and genetic relatedness.

Materials and methods

Fungal isolations

Birdsfoot trefoil fields were selected in a 9-site-matrix of three locations (Colonia, Tacuarembó and Treinta y Tres, Uruguay) and three stand ages (1-, 2-, and 3-yr-old), and surveyed during September of 1994 and 1995. A stratified sampling design was employed using 12 permanent 5x5 m quadrats per site and sample size was 25 plants per quadrat. At the laboratory, subsamples of five diseased roots from each quadrat of each site were used for fungal isolation. Pieces of 0.5-1.0 cm^2 from different areas of the root and crown (primarily from the interface of infected and non-symptomatic tissues) were washed under flowing tap water overnight, surface-disinfested by soaking in 95% ethanol for 1 min, then soaking in 1% sodium hypochlorite for 3 min, followed by a rinse in sterile distilled water, and finally plated on PDA. Two and five pieces were plated per quadrat, for roots sampled in September of 1994 and September of 1995, respectively. The intention was to obtain at least one fungal isolate per quadrat per site (12 quadrats x 3 locations x 3 stand ages = 108 isolates). Hyphal tips from each fungal colony (except for easily identified genera) were transferred to PDA plates and tubes for further identification and storage. Each year (1994 and 1995) a collection of *Fusarium* spp. isolates was maintained on PDA slants at 4 C during the identification process (four months). Subsequently, selected isolates were stored on silica gel crystals at 5 C until needed (Windels, 1992). Isolates were identified as *F. oxysporum* using the procedures outlined by Nelson *et al.* (1983). Three randomly selected isolates were sent to the International Mycological Institute (IMI-CAB International, UK) for confirmation of identification (IMI No. 368015, 368016, 368017, report from Dr. D. Brayford).

Two core collections of *F. oxysporum* isolates (composed of 15 out of 64 isolates of the 1994 *Fusarium* spp. collection, and 36 out of 208 isolates of the 1995 *Fusarium* spp. collection) were used to perform aggressiveness and vegetative compatibility tests. Isolates to compose the core collections were selected as follows: 1. the three geographical locations were represented; 2. different stand ages within locations were represented; 3. different quadrats within sites were represented, and if there were more than one isolate per quadrat within a site, one isolate was randomly selected.

Aggressiveness of isolates

A culture plate method was used to characterize *F. oxysporum* isolates for aggressiveness to seeds and seedlings of birdsfoot trefoil. The seedling test was not aimed to parallel the development of the Fusarium crown and root rot in the field, which primarily occurs in mature plants, but rather to compare the behavior of isolates on a potential host plant as

reported by Fulton and Hanson (1960), Kainski (1960), and Kilpatrick *et al.* (1954). The method is similar to one used to select alfalfa germplasm for resistance to *Pythium* seedling diseases (Altier and Thies, 1995).

Fungal inoculum, consisting of mycelia and conidia, was produced in a 9 cm-diameter petri plate containing PDA. A 3 mm-diameter disc of inoculum was removed from the periphery of the resulting 4 to 5-day-old colony, placed in the center of a 9 cm-diameter petri plate containing 1.5% water agar (WA) and incubated at 22 C for 7 days. Using a vacuum template, 25 surface-disinfested birdsfoot trefoil seeds were placed equidistantly to the inoculum disc in a radiate pattern on the agar surface. The plates were incubated in growth chambers at 22 C for 7 days under cool-white light (12-h photoperiod; 330 FT. candles). Noninoculated plates of WA containing 25 surface-disinfested seeds were used as controls to determine seed germination, and expected percentage of dead and hard seed.

Disease severity was used as a measurement of isolate aggressiveness and was rated using a five-class scale, in which 1 = healthy seedling, primary root free of necrosis or with slight discoloration; 2 = infected seedling, primary root tip necrotic but firm, cotyledons free of disease; 3 = severely infected seedling, primary root tip and/or cotyledons rotted and soft, seedling will die as infection progresses; 4 = dead seedling, germinated seed with emerged radicle rotted; 5 = dead seed, nongerminated seed rotted. Aggressiveness was expressed as disease severity index (DSI), calculated as the numerical value of each class times the number of individuals in the class, divided by the number of seeds expected to germinate as determined in the noninoculated control, and percentage of surviving plants (PSP), calculated as the total of classes 1 and 2 divided by the number of seeds expected to germinate as determined in the noninoculated control.

Eight *F. oxysporum* isolates from the 1994 core collection were tested against San Gabriel and Estanzuela Ganador birdsfoot trefoil cultivars in June 1995, and 36 *F. oxysporum* isolates from the 1995 core collection plus one isolate from 1994 (used as a control) were tested against San Gabriel birdsfoot trefoil in June 1996. The experimental design was a randomized complete block with four replications over time totaling 100 seeds per treatment, with a factorial arrangement of treatments (isolates x birdsfoot trefoil cultivars). For both experiments, data on DSI and PSP were subjected to analysis of variance (general linear model procedure, SAS Institute) and means were separated using Fisher's protected LSD test ($P<0.05$).

Genetic relatedness of isolates

Vegetative compatibility was used as a measure of genetic relatedness using the methodology developed by Puhalla (1985). Nitrate-nonutilizing (*nit*) mutants were recovered by plating the *F. oxysporum* isolates on a chlorate-containing medium (KPS), and complementation tests were performed on a minimal agar medium (MM) that contained sodium nitrate as the sole source of nitrogen (Puhalla, 1985).

For the recovery of *nit* mutants, each isolate of *F. oxysporum* was grown on MM at 22 C for 3-4 days. Four 3 mm-diameter mycelial plugs were taken from each colony and spaced well

apart on each plate of KPS. The KPS plates were incubated at 22 C for 14 days, during which time they were inspected for fast-growing, chlorate-resistant sectors. Different sectors from the same isolate were then transferred to MM. Very thin, but normally expansive growth on MM indicated that the sectors were also unable to reduce nitrate (*nit* mutants). Different *nit* mutants of a given isolate were plated on MM as follows: 1 mm^3 mycelial block of one of them, arbitrarily designated *nitA*, was placed at the center of a plate of MM, and five of the other *nit* mutants of that isolate were spaced in a circle of radius 15 mm around *nitA*. Plates were incubated at 22 C for 7 days and then examined. Any outer *nit* mutant that developed a line of dense growth where it contacted the central *nitA* colony was designated *nitB*. Based on Puhalla's results concerning efficiency of recovery of *nit* mutants (number of *nit* mutants per inoculum plug = 0.58, number of *nitB* mutants per inoculum plug = 0.13), we estimated that for each isolate, at least 16 wild type inoculum plugs should be plated on KPS (four KPS plates per isolate), to obtain nine *nit* sectors and two *nitB* mutants (Puhalla, 1985). The number of *nit* mutants tested for each isolate ranged from 12 to 24.

The number of isolates from which *nit* mutants were tested, the number of isolates from which *nitA* and *nitB* complementary mutants were obtained, and the number of isolates from which no complementary mutants were obtained, were recorded. Complementary *nitA* and *nitB* mutants from each of 18 *F. oxysporum* isolates were then paired on MM in all possible combinations to perform complementation tests among isolates (Puhalla, 1985). Nine isolates from the 1994 core collection and eight isolates from the 1995 core collection, plus the isolate 067NY-94 of *F.o.* f.sp. *loti* provided by Dr. G.C. Bergstrom (Dept. of Plant Pathology, Cornell University, Ithaca, NY 14853) were characterized for vegetative compatibility. Complementation tests for the 18 isolates (630 pairings of 36 *nit* complementary mutants) were repeated once during 1995 and 1996.

Results

Fungal isolations

Fungal colonies were recovered from root and crown pieces of plants sampled at the three locations. Independent of the location, root and crown pieces of 1-yr-old plants yielded few fungal colonies, but they were isolated readily from root and crown pieces of 2- and 3-yr-old plants.

The majority of fungi isolated from diseased crown and root tissues of birdsfoot trefoil were *Fusarium* spp., with the most frequently and consistently isolated species being *F. oxysporum* (66% and 70% of isolates, n=42 and n=146, in 1994 and 1995, respectively). Taxonomic identification was not done for the other *Fusarium* spp. Additional frequently isolated fungi included presumed saprophytic genera, *Penicillium*, *Aspergillus*, *Gliocladium*, *Epicoccum*, *Cladosporium*, *Rhizopus*, and *Mucor*. Unknown fungi (that were recovered in relatively low frequencies) included sterile hyphomycetes and coenocytic, nonsporulating species. One fungal isolate recovered in September 1994, identified tentatively as *Mycoleptodiscus* spp., and two isolates recovered in September 1994, identified tentatively as *Rhizoctonia solani*, were counted as unknown fungi.

Sixty-four isolates of *Fusarium* spp. were recovered from roots sampled in 1994, and 208 isolates from roots sampled in 1995, and composed the two *Fusarium* spp. collections. Fifteen *F. oxysporum* isolates from 1994, and 36 isolates from 1995, were selected to compose the two *F. oxysporum* core collections.

Aggressiveness of isolates

The eight *F. oxysporum* isolates tested from the 1994 core collection were pathogenic to seed and seedlings of birdsfoot trefoil, but significant differences in aggressiveness were observed among the isolates (Table 1). The effect of host cultivar was significant, with Estanzuela Ganador being more susceptible than San Gabriel (data not shown). Results could be due to differences in the germplasm reaction and/or to differences in the seed vigor of the seed lots. However, there was no interaction between isolates and cultivars and therefore, data on DSI and PSP were averaged over the two cultivars. The ranges for average DSI and PSP among all isolates were 1.44-2.25 and 58.7-85.7, respectively (Table 1). One isolate from Treinta y Tres (TT1C8) was significantly more aggressive than all the rest, as determined by the highest DSI and the lowest PSP. The other isolates showed a continuous range in variation for aggressiveness.

Table 1. Aggressiveness on birdsfoot trefoil of *Fusarium oxysporum* isolates from the 1994 core collection, as determined by disease severity and percentage of surviving plants.

Isolate[1]	Disease severity[2] (DSI)	Percentage of surviving plants[3] (PSP)
TT1C8	2.25[4]	58.6[4]
C2C1	1.73	76.9
T2C1	1.68	79.5
C2C2	1.64	80.9
T3C1	1.61	80.1
T3C8	1.57	83.8
TT3C8	1.46	85.7
T2C6	1.44	85.6
LSD (0.05)	0.30	9.8
CV (%)	17.28	12.1

[1] The first letter(s) refer(s) to the geographic location of an isolate; TT= Treinta y Tres, C= Colonia, T= Tacuarembó. The first number refers to the age in years of the diseased plant from which an isolate was derived; the second number refers to the quadrat in the field from which the diseased plant was sampled (1-12).
[2] DSI, disease severity index based on a 5-class scoring system of individual seedlings: 1= healthy seedling, 2= primary root tip necrotic and firm, 3= primary root tip and cotyledons rotted and soft, 4= dead seedling, 5= dead seed.
[3] PSP, percentage of surviving plants: percentage of seedlings in classes 1 and 2.
[4] Values were averaged over birdsfoot trefoil cultivars San Gabriel and Estanzuela Ganador.

Since the results from the first aggressiveness test (1994 isolates) indicated that the interaction between isolates and cultivars was not significant, and the number of isolates to be tested from the 1995 core collection was large, the second aggressiveness test (1995 isolates) included only birdsfoot trefoil cv. San Gabriel.

All of the 36 *F. oxysporum* isolates tested from the 1995 core collection were pathogenic to seed and seedlings of birdsfoot trefoil, but significant differences in aggressiveness were observed among the isolates (Table 2). The range for average DSI was 1.69-3.85 and for average PSP, it was 4.8-78.9. With few exceptions, isolates from Colonia were more aggressive than isolates from the other two locations. One isolate from Colonia (C2C7) was significantly less aggressive than all other isolates, as determined by the lowest DSI and the highest PSP. Isolates from Tacuarembó and Treinta y Tres showed a large variability in aggressiveness (DSI = 2.44-3.76). There was a tendency for isolates from different quadrats from the same site to have similar DSI and PSP values. The control isolate TT1C8, from the 1994 core collection, had low aggressiveness as compared to isolates from the 1995 core collection (DSI = 2.61, PSP = 48.9). However, these values are similar to those obtained from the previous test (DSI = 2.25, PSP = 58.7) (Table 2).

Genetic relatedness of isolates

Nit mutants were obtained for 45 out of 52 isolates: 15 of 15 isolates that composed the 1994 core collection, 29 of 36 isolates of the 1995 core collection, and for the isolate of *F.o.* f.sp. *loti* provided by Dr. Bergstrom. The number of *nit* mutants tested for each of the 45 isolates ranged from 12 to 24. Complementary *nitA* and *nitB* mutants were obtained for only 17 out of 45 isolates: 9 of 15 isolates of the 1994 core collection, 8 of 29 isolates of the 1995 core collection and for the *F.o.* f.sp. *loti* isolate (Figure 1). No complementary mutants were obtained from the rest of the isolates.

Figure 1. Complementary *nitA* and *nitB* mutants derived from *Fusarium oxysporum* isolates on minimal medium. The plate in the upper left corner is used as an example: one mutant, arbitrarily designated *nitA*, was placed in the center of the plate; then five other *nit* mutants obtained from the same isolate were spaced in a circle of radius 15mm around the *nitA* block. In this case the five outer *nit* mutants were complementary with the *nitA* mutant and were designated *nitB* mutants.

Table 2. Aggressiveness on birdsfoot trefoil cv. San Gabriel of *Fusarium oxysporum* isolates from the 1995 core collection, as determined by disease severity and percentage of surviving plants.

| Isolate[1]	Disease severity[2] (DSI)	Exp. Surv. Plants[3] (%)	Isolate[1]	Disease severity[2] (DSI)	Perc. surv. plants[3] (PSP)
C2C11	3.85	4.8	C2C10	3.53	14.7
C2C2	3.82	7.6	TT1C9	3.48	15.6
C3C1	3.77	7.4	TT3C1	3.48	16.1
TT3C3	3.76	7.5	T2C9	3.44	14.5
C3C5	3.75	11.4	T3C5	3.44	18.4
TT3C10	3.73	8.8	T3C4	3.43	16.6
C3C4	3.73	9.9	T3C7	3.35	19.3
C3C10	3.73	10.5	TT1C10	3.29	21.3
C3C11	3.73	12.3	T3C9	3.25	24.2
C2C9	3.71	8.6	T3C12	3.25	25.4
T3C10	3.70	14.3	TT3C4	3.17	25.8
TT3C12	3.67	11.5	TT3C5	3.15	19.0
TT2C9	3.67	12.5	C3C2	3.14	26.4
T3C8	3.64	12.2	TT3C8	3.11	25.5
C3C8	3.64	12.7	T1C2	3.10	35.8
TT1C4	3.63	14.2	TT2C3	3.03	30.2
TT2C6	3.58	12.1	TT2C11	2.44	53.4
T3C2	3.58	12.3	C2C7	1.69	78.9
			TT1C8/94[4]	2.61	48.9
LSD (0.05)	0.42	16.1	LSD (0.05)	0.42	16.1
CV (%)	8.77	58.9	CV (%)	8.77	58.9

[1] The first letter(s) refer(s) to the geographic location of an isolate; C= Colonia, TT= Treinta y Tres, T= Tacuarembó. The first number refers to the age in years of the diseased plant from which an isolate was derived; the second number refers to the quadrat in the field from which the diseased plant was sampled (1-12).
[2] DSI, disease severity index based on a 5-class scoring system of individual seedlings: 1= healthy seedling, 2= primary root tip necrotic and firm, 3= primary root tip and cotyledons rotted and soft, 4= dead seedling, 5= dead seed.
[3] PSP, percentage of surviving plants: percentage of seedlings in classes 1 and 2.
[4] Control isolate from the 1994 core collection.

No complementation was found either among the nine *F. oxysporum* isolates from the 1994 core collection or among the eight isolates from the 1995 core collection. Pairings among the nine 1994 isolates and the eight 1995 isolates also resulted in no complementation (Fig. 2). When the 17 isolates from Uruguay were tested with the *F.o.* f.sp. *loti* isolate, no complementation was obtained. Based on these results, the 17 isolates should be assigned to different VCGs. Since each plate contained two complementary *nit* mutants for at least one isolate, we were assured that the methodology was adequate to detect compatibility among isolates if it did exist (Fig. 2). In these tests, complementation always and only occurred between complementary *nitA* and *nitB* mutants from the same isolate, used as controls.
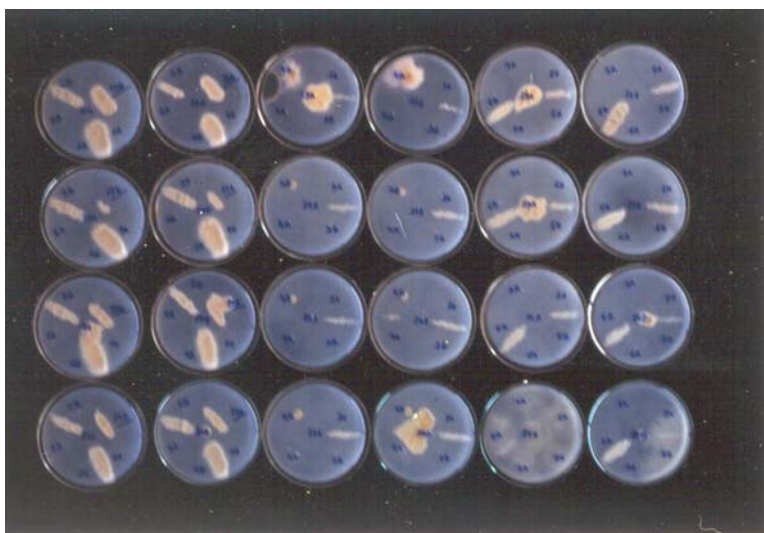
Figure 2. Complementation tests among *Fusarium oxysporum* isolates from which complementary *nitA* and *nitB* mutants were obtained, on minimal medium. No complementation occurred among different isolates. The observed complementation only and always occurred between complementary *nitA* and *nitB* mutants of the same isolate.

Discussion

The repeated isolation of *F. oxysporum* from diseased roots of birdsfoot trefoil suggests this species is primarily responsible for crown and root rot and stand decline, consistent with previous findings (Altier, 1994; 1997; Berkenkamp *et al.*, 1972; Beuselinck, 1988; Henson, 1962; Kainski, 1960). In addition, *F. oxysporum* has been reported as the causal organism of Fusarium wilt on birdsfoot trefoil (Bergstrom and Kalb, 1995). No other known pathogen that is alone capable of causing these disease symptoms was isolated from diseased crown and root tissues.

All the examined isolates of *F. oxysporum* incited a host reaction in birdsfoot trefoil. This means they may have similar genetic factors that determine pathogenicity and host disease reaction. However, isolates showed a continuous range in aggressiveness to birdsfoot trefoil seeds and seedlings. The observed variability was expected, and was consistent with previous reports (Leath and Kendall, 1978; Venuto *et al.*, 1995). Aggressiveness is defined as a property of the pathogen reflecting the relative amount of damage caused to the host without regard to resistance genes (Shanner *et al.*, 1992). With few exceptions, the highest aggressiveness was expressed by isolates from sites with long legume pasture history (e.g., Colonia as compared with the other two locations). There was a tendency for isolates from

the same site to have similar values for DSI and PSP; however, the reason for this is unknown. Results in our tests were similar to those of Fulton and Hanson (1960), Kainski (1960), and Kilpatrick *et al.* (1954), who used seedling tests to compare aggressiveness of *Fusarium* spp. isolates causing crown and root rot on forage legume hosts, and reported variability in the pathogen. Kainski (1960), while studying the fungi involved in root rots and seedling diseases of birdsfoot trefoil, showed that most of the fungi that were pathogenic to seeds and seedlings were also pathogenic to established plants but differed in their relative aggressiveness. Kilpatrick *et al.* (1954) studied the pathogenicity of 72 isolates of *F. oxysporum* associated with root rots of red clover and observed a wide variation in disease severity as measured by percentage dead plants (range among isolates: 26-95%). This variable is the complement of the one we used, percentage of surviving plants (PSP).

Despite obtaining *nit* mutants for 45 out of 52 isolates, complementary *nitB* mutants were obtained for only 17 out of 45 isolates. There are two plausible explanations for these results. First, the efficiency of recovering *nitB* complementary mutants is low and isolate dependent (average 0.13 per inoculum plug, range 0-0.25; Puhalla, 1985). Therefore, a large number of inoculum plugs per isolate should have been plated on KPS to recover more *nitB* mutants. Secondly, some isolates could have been heterokaryon self-incompatible (HSI), as defined by Correll *et al.* (1987), though HSI strains usually occur at low frequency in *F. oxysporum* populations (1-2%, J.F. Leslie, Kansas State University, U.S.A., 1996, pers. comm.; 4%, Steinberg *et al.*, 1997).

The lack of complementation among the isolates that compose the *F. oxysporum* population associated with birdsfoot trefoil indicates a large genetic diversity, as measured by vegetative compatibility. Isolates do not share genes for complementation and thus, they belong to different VCGs. However, they may share genetic factors that induce host disease reaction, since all of them have the ability to cause disease symptoms in birdsfoot trefoil seeds and seedlings. We may conclude that similarity or dissimilarity in *vic* genes does not reflect pathogenicity and aggressiveness capabilities. Our results agreed with those of Venuto *et al.* (1995) who showed that VCGs were not useful in predicting host reaction of red clover to isolates of *F. oxysporum*, because isolates from distinct groupings elicited similar host reactions. This indicates that the number of genes controlling compatibility seems to be greater than the number of virulence genes. Correll (1991) reported that over 46 distinct VCGs have been identified among a collection of *F. o.* f.sp. *asparagi* isolates pathogenic to asparagus in greenhouse pathogenicity tests, and that race 1 isolates of *F. o.* f.sp. *lycopersici* were found to belong to at least 41 VCGs.

Some genetic diversity studies have been consistent with Puhalla's initial generalization and have stated a correlation between pathogenic phenotype and genotype (Puhalla, 1985). However, the examination of numerous formae speciales of *F. oxysporum* has revealed that the relationship between host specialization (formae speciales), virulence capabilities (races) and VCGs can vary from simple to complex (Correll, 1991; Gordon and Martyn, 1997; Kistler, 1997; 2001; Klein and Correll, 2001). Based on pieces of evidence, authors assume that virulence and VCG phenotypes change independently of one another and at different rates (Correll, 1991; Klein and Correll, 2001). Kistler (2001) asserted that horizontal gene transfer could explain the partitioning of host specificity into genetically distant lineages.

From this hypothesis, the prediction is that genes for host specificity (as determined by pathogenic phenotype) may be more closely related than can be accounted for by the underlying phylogeny (as determined by molecular markers or VCGs). He concluded that strains pathogenic to a given host may emerge rapidly in a genetic background preadapted for fitness on any plant species currently colonized by *F. oxysporum* (Kistler, 2001). Several working models have been proposed to help explain the degree of VCG diversity thus far observed in *F. oxysporum* (Correll, 1991; Gordon and Martyn, 1997; Klein and Correll, 2001). It has been suggested that the parasitic, but nonpathogenic portion of the population may represent some primitive or basal population structure of this species and a largely unexplored reservoir of genetic diversity. From this primitive population, which has a high degree of VCG diversity, mutations to virulence may occur among isolates of the various VCGs. If selection of existing variants or a mutation occurred in isolates that are brought into proximity with a susceptible host (e.g., the roots), then they may proliferate and lead to an epidemic. This will likely result as a consequence of the intense selection pressure imposed by agricultural practices.

Soils in Uruguay support the growth of a wide range of leguminous species, either introduced crops, native species (including woody trees and shrubs) or weeds. In areas of intensive livestock production, forage legumes are used in short rotations with cereals and grasses. Under this situation, in most agricultural soils, *F. oxysporum* populations do find conditions conducive to development of host specialization, and survive, at least in part, by colonizing leguminous-host plants. Furthermore, results from a study to characterize soil populations of *F. oxysporum* under different rotation systems have demonstrated that even soil under continuous agriculture without any legume crop supports high population densities of this fungus (Altier, 2003). We could speculate that this population may represent what Correll (1991) designates the primitive or basal population structure of this species. Given the global distribution of *F. oxysporum* and its pervasive association with plants, this gives reason for concern. Future work should focus especially on the role of alternate hosts in maintaining pathogen populations in soil and in the study of factors that influence the dynamics of isolate competition within a heterogeneous *F. oxysporum* population.

Characterization of the pathogen population has practical implications for the success of breeding for resistance to Fusarium crown and root rot in birdsfoot trefoil. The high degree of variability for aggressiveness among *F. oxysporum* isolates supports using isolates from different locations in the inoculation and selection procedures of a breeding program for cultivar development for the region. If the primary variation in the reaction of the host is due to variation in the pathogen, selection for resistance to one or a few isolates would not result in resistance to other isolates (Pederson *et al.*, 1980; Venuto *et al.*, 1995). Developing germplasm with increased resistance to *F. oxysporum* should involve the screening of birdsfoot trefoil against several genetically divergent isolates of the pathogen, if the resistant cultivars are to be widely used.

Acknowledgements

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