

Relationships between *Fusarium* population structure, soil nutrient status and disease incidence in field-grown asparagus

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Abstract

Fusarium species cause important diseases in many crops. Lack of knowledge on how *Fusarium* species and strains interact with their environment hampers growth management strategies to control root diseases. A field experiment involving asparagus as host plant and three phosphorus fertilization levels was designed to examine the seasonal changes and ecological relationships between *Fusarium* populations and their soil and plant environments. *Fusarium* taxa were identified and assessed using PCR-denaturing gradient electrophoresis of the EF1- α gene. Resulting profiles were analyzed with respect to 17 ecological parameters measured during the three main asparagus phenological phases across a growing season. Multivariate statistical analysis showed that *Fusarium* population structure was strongly influenced by soil P level while seasonal variation was less important. A significant relationship between *Fusarium* population composition and *Fusarium* crown and root rot incidence was also found in September. Canonical analysis further revealed significant relationships between *Fusarium* population structure, and plant manganese and iron contents, soil dehydrogenase activity and soil calcium concentration. If higher *Fusarium* crown and root rot incidence is related to the *Fusarium* community structure, strategies to reduce the incidence in asparagus plantations may be found through manipulation of the soil fertility.

Introduction

Fusarium species are the causal agents of several plant diseases of global importance. Thus development of management strategies designed to reduce *Fusarium*-related diseases has become a high priority. However, a better understanding of *Fusarium* ecology is necessary before targeted management strategies can be conceived. Previous knowledge on *Fusarium* ecology has been summarized by Nelson *et al.* (1981) and updated by Summerell *et al.* (2001). However, knowledge on the ecology of *Fusarium* has until recently been hampered by a lack of reliable and reproducible approaches examining *Fusarium* population structure in large numbers of samples. As with other microorganisms, molecular tools are now becoming available to study the ecology of *Fusarium* at various levels (Nicholson, 2001; Pasquali *et al.*, 2003; Kowalchuk & Smit, 2004; Mulè *et al.*, 2004; Yergeau *et al.*, 2005).

Asparagus (*Asparagus officinalis*) could be a valuable model to study microbial ecology and biocontrol of pathogens, as it is a low-input perennial crop heavily affected by a *Fusarium*-related disease. Many *Fusarium* taxa are involved and the major species causing *Fusarium* crown and root rot of asparagus (FCRR) differ in various parts of the world (Schreuder *et al.*, 1995; Elmer *et al.*, 1996; Blok & Bollen, 1997). Despite the ubiquitous presence of pathogenic *Fusarium* species involved in FCRR in soils, disease expression remains unpredictable. This suggests that some ecological conditions may be conducive or suppressive to FCRR expression. The effects of different ecological factors on *Fusarium* populations are poorly known, but it appears that judicious management of the soil and plant conditions could reduce FCRR incidence and improve the profitability of asparagus fields. Recently, *Fusarium* populations in asparagus fields have been shown to be widely diverse, and to vary with growing areas, edaphic and climatic conditions,

and even between plant parts (Vujanovic *et al.*, 2006). Variations in *Fusarium* and arbuscular mycorrhizal fungi population structures were also related with FCRR incidence, harvesting, plantation age, sampling site, and sampling date (Hamel *et al.*, 2005a; Yergeau *et al.*, 2006). Moreover, disease incidence in asparagus fields was related to manganese concentration in soil and planting depth (Hamel *et al.*, 2005b). Still, many ecological questions are yet to be addressed, such as the effects of fertilization on *Fusarium* and potential antagonistic populations. Defining the impact of fertilization and soil conditions on microbial communities may provide key information on the development of simple and effective ways to delay and reduce outbreaks of *Fusarium*-related diseases.

Phosphorus fertilization affects soil microbes such as arbuscular mycorrhizal fungi (Treseder & Allen, 2002; Treseder, 2004), but the impact of P fertility on *Fusarium* taxa and relation with disease development is complex and poorly defined. In one of the few studies addressing this issue, the effect of P fertilization on *Fusarium* abundance in UK wheat fields varied with the form of the fertilizer and the season (Bateman & Coskun, 1995). Working in soybean fields, Sanogo & Yang (2001) reported that P addition increased *Fusarium* disease incidence and *Fusarium* growth *in vitro*, and that the level of these increases depended on the form of the fertilizer used.

The objective of this study was therefore to assess the effect of different levels of P fertilization on the *Fusarium* population structure in an asparagus field. *Fusarium* population structure was determined from genomic DNA extracted directly from asparagus plants using a specific PCR-denaturing gradient electrophoresis (DGGE) analysis (Yergeau *et al.*, 2005). The relationship between *Fusarium* taxa and the plant and soil environment was then examined using multivariate analyses.

Materials and methods

Experimental design

The study was conducted in a commercial asparagus field at St Liguori, QC, Canada (45°48' N; 73°26' W). The soil was a fine, frigid Typic Endoaquent (USDA, 2003) of the St Bernard series (Lamontagne & Nolin, 1997), pH 6.2, with 797 g sand kg⁻¹, 145 g silt kg⁻¹ and 58 g clay kg⁻¹ containing 23.9 g organic C kg⁻¹. In June 2001, ammonium nitrate and potash fertilizers were broadcast and incorporated to supply 100 kg N ha⁻¹ and 155 kg K₂O ha⁻¹ at planting. One-year-old asparagus (*Asparagus officinalis* L. cv. Guelph Millennium) crowns were then hand-planted 30 cm apart in 15 cm-deep trenches. Triple superphosphate was banded along the bottom of the trench, approximately 3–5 cm from the crowns, at rates described below, and the trenches were

gradually filled in during the summer. The experimental design was a randomized complete block design (three blocks) with three P fertilizer treatments within each block, making a total of nine plots. Each plot contained a row of 20 asparagus plants, and treatment plots were separated by guard rows, with 175 cm between rows. At planting, the P fertilizer treatments supplied the equivalent of 0, 50 and 100 kg P₂O₅ ha⁻¹. These treatments supplied 0%, 100% and 200% of the recommended P fertilizer rate for the site, based on a soil test showing 146 mg Mehlich III P kg⁻¹ (CRAAQ, 2003). In the spring of 2002, about 1 week after the shoots emerged (May 19, 2002), fertilizers were applied in a 10 cm deep trench located about 5 cm from the row. The equivalent of 60 kg N ha⁻¹ and 55 kg K₂O ha⁻¹ was applied to all rows, and P fertilizers were applied at rates of 0, 55 and 110 kg P₂O₅ ha⁻¹. Thus, no P fertilizer was applied to the 0% P treatment during this study, while the 100% P treatment received about 105 kg P₂O₅ ha⁻¹ and the 200% P treatment received equivalent to 210 kg P₂O₅ ha⁻¹. A band application of 40 kg N ha⁻¹ was also carried out on August 5, 2002.

Data collection and sample analysis

Three asparagus plants per treatment per block were sampled on May 22, July 23 and September 6, 2002. Plant shoots, roots and crowns were separated, washed under tap water and frozen at –80 °C within a day for DNA extraction, as described below. At each sampling date, the proportion of plants showing FCRR symptoms in each plot was recorded. This FCRR incidence measure was preferred over traditional root infection scores as it is nondestructive and directly related to productivity losses in each plot. As soon as any of the many stems of the plant was expressing stunting, chlorosis, brown necrosis or reddish lesions on its base, the whole plant was considered symptomatic (Elmer, 2001). A severe rust infestation weakened the plants in August 2002, so the producer felt the plantation was too weak to harvest, and should have a year without harvesting stress to recover. Therefore, no estimate of asparagus biomass was made and tissue analysis was used as an indicator of nutrient assimilation by the plants. Plant tissue samples were collected from three randomly selected plants per plot at full fern stage on June 21, 2002. The third, fifth, eighth and 11th leaves from the apex of the dominant stem were combined and used for plant tissue analysis as described below. All foliage from the three plants in each plot was combined, dried at 60 °C, ground (< 1 mm mesh) and digested in H₂SO₄/H₂O₂ (Robarge & Fernandez, 1986). Total N and P concentrations were determined on a Lachat Quick-Chem flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI). The K, Ca, Mg, Fe, Zn and Mn concentrations in the plant digests were analyzed on an atomic absorption spectrophotometer.

Root subsamples from the plants sampled in July were cut into 1 cm pieces, placed in small plastic cartridges and cleared for 25 min in a 10% (w/v) KOH solution in the autoclave. After clearing, roots were rinsed with tap water, stained with 0.02% (v/v) acid fuchsin and the percentage of root length bearing arbuscular mycorrhizal fungal colonization was determined using the gridline intersect method (Giovannetti & Mosse, 1980).

Soil samples were collected in mid-August 2002 and consisted of seven cores (3 cm diameter by 15 cm depth) collected between plants within each treatment row. Soil samples were sieved (5 mm mesh) and split in two parts. One part was kept at 4 °C and used within a few days to determine enzyme activities. Soil monophosphatase, β -D-glucosidase and dehydrogenase activities were assessed on soils from each experimental plot. Triplicate subsamples of soil were used for each enzyme assay, with one subsample serving as a control (no substrate was added). For monophosphatase activity, 1 g of field-moist soil was incubated in test tubes for 1 h at 37 °C with 1 mL of 0.05 M *p*-nitrophenyl phosphate and 4 mL modified universal buffer [pH 6.5, containing 0.02 M tris(hydroxymethyl) aminomethane, 0.02 M maleic acid, 0.01 M citric acid and 0.02 M boric acid]. The reaction was stopped by placing the tubes in a ice-cold water bath and color was developed by adding 4 mL of 0.5 M NaOH and 1 mL of 0.5 M CaCl₂. Tubes were mixed on a vortex mixer for 30 s and the contents were filtered through Whatman No. 2 filter paper. The absorbance of the reaction product in filtered extracts was measured at 420 nm (Tabatabai, 1994). For β -D-glucosidase activity, 1 g of field-moist soil was incubated in test tubes for 1 h at 37 °C with 1 mL of 0.05 M *p*-nitrophenyl- β -D-glucoside and 4 mL of modified universal buffer (pH 6.0, described above). The reaction was stopped by placing the tubes in a cold water bath and color was developed by adding 4 mL of 0.1 M tris(hydroxymethyl)aminomethane (pH 12) and 1 mL of CaCl₂. Tubes were mixed on a vortex mixer for 30 s and the contents were filtered through Whatman No. 2 filter paper. The absorbance of the reaction product in filtered extracts was measured at 420 nm (Tabatabai, 1994). The dehydrogenase assay was modified from Casida *et al.* (1964). Briefly, 1 g of field-moist soil was incubated for 6 h at 40 °C with 1 mL of 0.5% 2,4,5-triphenyl tetrazolium chloride and 1 mL of 0.5 M tris(hydroxymethyl)aminomethane (pH 7.6). To stop the reaction, samples were placed in a freezer at -10 °C. Samples were thawed, extracted with 10 mL of methanol for 1 h and then placed overnight in the fridge to allow sediments to settle. The supernatant was then analyzed colorimetrically at 480 nm.

The other series of soil subsamples were dried at 60 °C for 48 h, and finely ground (2 mm mesh sieve). Soil-available P, K, Ca, Mg, Mn, and Al were extracted with the Mehlich III solution (1:10 soil:solution) after shaking for 5 min at

130 r.p.m. (Tran & Simard, 1993), and analyzed colorimetrically within 2 h on a Lachat Quick-Chem flow injection autoanalyzer.

Nucleic acid extraction and PCR amplification

Plant roots, shoots and crowns from each sample were ground together in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted from 200 mg of plant tissue per sample using a DNAeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified DNA was eluted in 100 μ L of elution buffer and stored at -20 °C until used.

A nested PCR procedure was used to amplify the gene encoding elongation factor-1 α (EF-1 α) from *Fusarium* DNA extracted from plant samples. The first PCR step was performed using the EF-1-EF-2 primers (O'Donnell *et al.*, 1998). The amplicons were subsequently diluted (1:1000) and reamplified using the Alfie1-GC-Alfie2 primers, as described in Yergeau *et al.* (2005). All reactions were carried out in 50 μ L volumes containing 5 μ L of 10 $\times$ PCR buffer, 5 μ L of each primer (5 μ M), 1 μ L of 10 mM dNTP mix, and 1.25 U of *Taq* polymerase (QIAGEN). Reaction mixtures were overlaid with 40 μ L sterile mineral oil prior to PCR in a PTC-100 thermal cycler (MJ-Research Inc., Waltham, MA).

DGGE profiling of *Fusarium* population

DGGE analyses were performed with a 40–65% denaturant gradient [100% is defined as 40% (v/v) formamide and 7 M urea] using a D-Code Universal Mutation Detection System (BioRad, Hercules, CA), as described in Yergeau *et al.* (2005). Bands were identified by comparison with migration position of known *Fusarium* taxa (Yergeau *et al.*, 2005) or of bands whose sequences were previously determined (Yergeau *et al.*, 2006). As migration identity is not necessarily equal to sequence identity, several bands were excised from DGGE gels, reamplified using the Alfie1-Alfie2 primers and commercially sequenced (Montreal Genomic Centre, Montreal, QC, Canada). Sequenced bands were identified by similarity search in GenBank. The resulting sequences were deposited in GenBank under accession number DQ363539–DQ363559. DGGE gels were digitized using a Gel Doc imager (BioRad). Pictures were visually inspected and banding patterns converted to a binary code for presence or absence of a band at each migration position observed. As each migration position generally corresponds to a different *Fusarium* sequence variant of different nucleotide composition, the matrix obtained was used as a sequence variant presence-absence matrix for statistical analyses. Counting the number of bands visible for each sample assessed the richness of the sequence variants.

Statistical data analyses

Canonical and noncanonical correspondence analyses were carried out in CANOCO for Windows version 4.5 (ter Braak & Šmilauer, 2002) to analyze the relationships between *Fusarium* sequence variants and environmental variables. Experimental blocks were used as covariables in both analyses. Outlier sequence variants were eliminated from all correspondence analyses by following the empirical method described by D. Borcard (<http://biol10.biol.umontreal.ca/BIO6077/outliers.html>). To reveal the influence of all significant environmental factors in a background of overriding P fertilization effects, P treatment was used as a covariable in canonical correspondence analysis. The environmental variables were submitted to a forward selection with a 5% baseline. Testing the effect of fertilization treatments, sampling dates or disease incidence on the multivariate sequence variants matrix was done by successively including each of these variables (sometimes recoded as dummy binary variables) as the only explicative variable in the canonical correspondence analysis. The whole canonical model test, as well as first and second axes signification tests, was performed with 999 permutations. Sequence variants clustering analyses were carried out in the R package (Casgrain & Legendre, 2001), available at <http://www.fas.umontreal.ca/biol/casgrain/fr/labo/R/index.html>. Jaccard's community coefficient between sequence variants was calculated and six clustering methods (weighted centroid, unweighted centroid, weighted arithmetic average, unweighted arithmetic average, single linkage, complete linkage) were compared using Pearson's r and Kendall's τ_b . The similarity matrix was then exported, and the final figure was redrawn in STATISTICA 6.0 (StatSoft Inc., Tulsa, OK) using the most effective clustering method (unweighted arithmetic average, UPGMA). Multiple linear regression analyses were carried out in STATISTICA 6.0 using forward-stepwise selection. Analyses involving environmental factors were carried out using *Fusarium* population data from plants sampled in September, as this sampling date immediately followed the assessment of the soil environmental factors (mid-August). The effects of fertilization treatments on plant and soil nutrient and on root mycorrhizal colonization were analyzed using Kruskal–Wallis nonparametrical tests in STATISTICA 6.0.

Results

Fusarium-specific DGGE patterns

As reported previously (Yergeau *et al.*, 2005, 2006), DGGE bands representing *Fusarium oxysporum*, *Fusarium proliferatum* and *Fusarium solani* were easy to discriminate from the others just by looking at the DGGE gels, whereas other *Fusarium* species were more difficult to identify directly. Thus, a good identification was possible for the *Fusarium*

taxa to which the main asparagus pathogens (*F. oxysporum* and *F. proliferatum*) belong. Four bands were identified to the Red *Fusarium* complex without being able to be more specific (Table 1). BLAST results invariably gave *Fusarium lateritium* as the closest match, but with low identity. However, five species from this group were recently isolated from asparagus plants in Quebec (Vujanovic *et al.*, 2006). Seventeen different migration positions were detected in DGGE gels. Occurrence of bands at a specific migration position ranged from 1 (taxa FT1 and FT2; excluded from analysis) to 33 (FT6). One to eight different bands were found per plant samples, with an average per treatment ranging from 2.7 (0 kg P₂O₅ ha⁻¹, September) to 4.4 (110 kg P₂O₅ ha⁻¹, September). No *Fusarium* sequences were detected in several of the May samples, even with a detection limit of 10 pg of *Fusarium* DNA with 100 ng of competing asparagus DNA (data not shown). Therefore, only the data from the July and September samplings were used in the statistical analyses.

Influence of P fertilization and sampling dates on the *Fusarium* population

Phosphorus fertilization significantly influenced ($P = 0.001$) *Fusarium* population composition, according to a canonical correspondence analysis. A correspondence analysis ordination showed that *Fusarium* sequence variants were mainly spread along two opposed vectors associated with the 105 and 210 kg P₂O₅ ha⁻¹ treatments (Fig. 1 and Table 1). This result may indicate the occurrence of different ecological preferences among the *Fusarium* taxa detected. *Fusarium* populations also differed significantly ($P = 0.031$) across sampling dates, according to the canonical correspondence analysis tests. Sequence variants located in the left part of the ordination were detected more frequently in September, whereas those in the right part of the ordination were observed more frequently in July. The influence of the sampling date, however, was small compared with that of P fertilization, as indicated by the relative length of the corresponding vectors in the correspondence analysis ordination (Fig. 1).

Environmental soil and plant variables

Root length of asparagus plants showing arbuscular mycorrhizal colonization did not differ significantly between the 0 and 105 kg P₂O₅ ha⁻¹ treatments, with a mean of respectively 3.2% and 3.4%; plants which received 210 kg P₂O₅ ha⁻¹ were not colonized. There were no significant differences ($P > 0.05$) in the concentration of elements measured in plant tissues at the three P fertilization rates. Mean concentrations across all such rates were: P, 2.42; N, 26.6; K, 22.9; Ca, 4.78; Mg, 0.99; Fe, 0.13; Mn, 0.5; Zn, 0.03 (all mg g⁻¹). Similarly, there were no significant effects of P

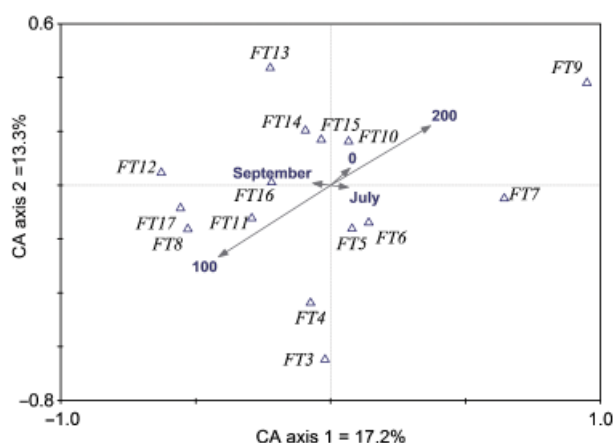
Table 1. Closest match and relative abundance of each *Fusarium* sequence variants, expressed as *Fusarium* taxa (FT) observed in denaturing gradient gel electrophoresis gels

Band	Match*	Relative abundance (%) [†]		
		0 kg P ₂ O ₅ ha ⁻¹ (n = 52)	105 kg P ₂ O ₅ ha ⁻¹ (n = 74)	210 kg P ₂ O ₅ ha ⁻¹ (n = 56)
FT3	<i>Fusarium oxysporum</i>	0.0	5.5	0.0
FT4	<i>Fusarium oxysporum</i>	3.9	8.2	3.6
FT5	<i>Fusarium oxysporum</i>	23.1	9.6	14.6
FT6	<i>Fusarium oxysporum</i>	21.2	15.1	20.0
FT7	<i>Fusarium oxysporum</i>	0.0	0.0	10.9
FT8	<i>Fusarium oxysporum</i>	1.9	4.1	0.0
FT9	<i>Fusarium oxysporum</i>	1.9	1.4	5.5
FT10	<i>Fusarium oxysporum</i>	3.9	8.2	0.0
FT11	<i>Fusarium oxysporum</i>	1.9	6.9	0.0
FT12	Red <i>Fusarium</i> [‡]	0.0	2.7	1.8
FT13	Red <i>Fusarium</i>	3.9	5.5	10.9
FT14	Red <i>Fusarium</i>	9.6	8.2	16.4
FT15	<i>Fusarium proliferatum</i>	28.9	11.0	14.6
FT16	Red <i>Fusarium</i>	0.0	8.2	1.8
FT17	<i>Fusarium solani</i>	0.0	5.5	0.0

*Closest match as determined by comparison with migrating position of known organisms or with bands sequenced previously. See Materials and methods for details.

[†]Relative abundance was calculated as follows: $100 \times \text{number of bands observed at this position} / \text{total number of bands observed for all samples for that treatment (n)}$.

[‡]*Fusarium* strains that belong to the Red *Fusarium* complex of species producing Carmine red pigments on potato dextrose agar. BLAST search results and migration position comparison were not significant enough to enable us to identify the members of this complex to the species level. BLAST results invariably gave *Fusarium lateritium* as the closest match, but with low identity. A very low number of EF-1 α sequences for *Fusarium* ssp. belonging to this group were available in GenBank databases. See Vujanovic et al. (2006) for an enumeration of the species of this complex found in asparagus fields of Québec.

**Fig. 1.** Correspondence analysis showing *Fusarium* sequence variants repartition with regard to P fertilization treatments (0%, 100%, 200% the recommended dose) and sampling date. Sequence variant closest matches are given in Table 1.

fertilization rates on measured elemental concentrations in soil. Mean concentrations were: P, 150; K, 185; Ca, 2307; Mg, 139; Al, 957; Mn, 10.2 (all mg kg⁻¹). Furthermore, enzyme activities were also not significantly affected by P fertilization rate. Mean activities were β -D-glucosidase, 12.3; mono-

phosphatase, 114; dehydrogenase, 0.99 (all $\mu\text{g g}^{-1} \text{h}^{-1}$). A dataset including all soil and plant environmental variables and the *Fusarium* sequence variants dataset from the September sampling were included in a canonical correspondence analysis. Soil P content was the most significant variable in the analysis and, when included, little variation was left to be explained by other variables (data not shown). To see the effect of the remaining factors, another canonical correspondence analysis was performed using P treatments as covariables, thereby mathematically taking out the dominating effect of the P treatments (Fig. 2). This analysis showed that, in addition to P treatments, the Fe and Mn concentrations in plant tissues, as well as Ca concentration and dehydrogenase activity in soil, were also significantly correlated with *Fusarium* populations. A model using these variables significantly explained ($P=0.001$) the sequence variants–environment relationships. The first and second canonical axes were significant (respectively, $P=0.001$ and $P=0.006$) and explained 44.4% and 26.7% of the sequence variants–environment relationships, respectively. A multiple linear regression analysis between *Fusarium* sequence variant richness (number of bands in DGGE for September samples) and environmental variables also resulted in significant relationships (R^2 of 0.908, $P=0.0016$). This analysis

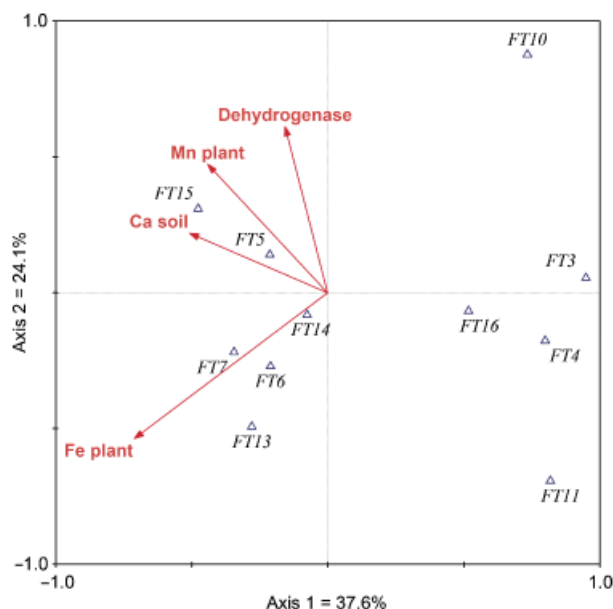


Fig. 2. Canonical correspondence analysis of the relations between all environmental variables and *Fusarium* population structure in September. Variables represented were chosen by forward selection with a 5% baseline. Sequence variants closest matches are given in Table 1.

is complementary to canonical correspondence analysis as it selects the variables related to the sequence variant richness without any reference to population structure, as is the case with canonical correspondence analysis. The variables chosen by forward-stepwise selection were plant Ca concentration ($b = 2.44$), soil available P ($b = 0.02$) and dehydrogenase activity ($b = -3.34$), which indicates that *Fusarium* sequence variant richness increased with plant Ca content or soil available P, and decreased with increasing dehydrogenase activity.

Fusarium crown and root rot incidence

Canonical correspondence analysis revealed a significant ($P = 0.007$) relationship between *Fusarium* population composition and FCRR incidence in September, but not with July samples. The corresponding correspondence analysis ordination is presented in Fig. 3. The *Fusarium* sequence variants more strongly associated with higher disease incidence were, in decreasing order, FT 10, 4, 3, 16 and 11 (Fig. 3). Sequence variant clustering, which was achieved using an unweighted arithmetic average approach (UPGMA; Fig. 4), showed a grouping pattern similar to Fig. 3. FT3 was closely clustered with FT4 (linkage distance = 0.60) and FT11 clustered with FT16 (linkage distance = 0.43). These sequence variants were most strongly related to high disease incidence in Fig. 3. Clusters FT3 and FT4 and FT11 and FT16 only met at a high linkage distance (0.87), showing two distinct groups of sequence variants related to FCRR

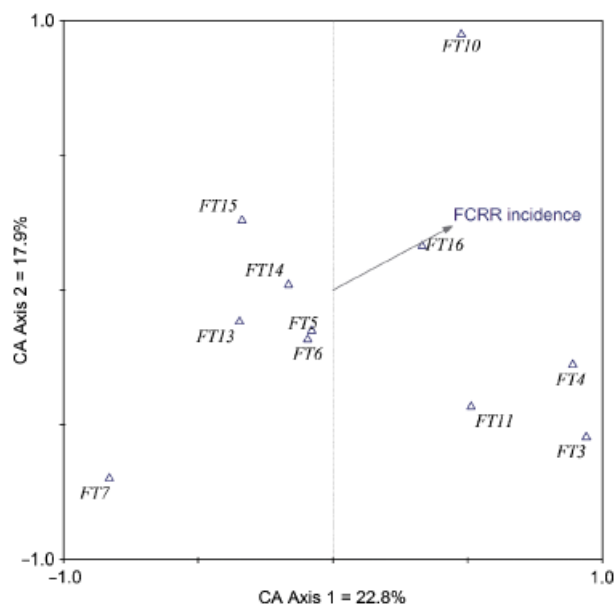


Fig. 3. Correspondence analysis showing *Fusarium* sequence variants repartition with regard to Fusarium crown and root rot incidence in September. Sequence variants closest matches are given in Table 1.

incidence. On the other hand, FT 5, 6 and 15 clustered together (Fig. 4; linkage distance = 0.631). These sequence variants were the most abundant (Table 1) and were also related to lower FCRR incidence (Fig. 3). FT 13 and 14 were less frequent, both related to low FCRR incidence, and also clustered together (Fig. 4; linkage distance = 0.55).

Discussion

In this study, P fertilization had a strong influence on *Fusarium* population composition in asparagus plants. This

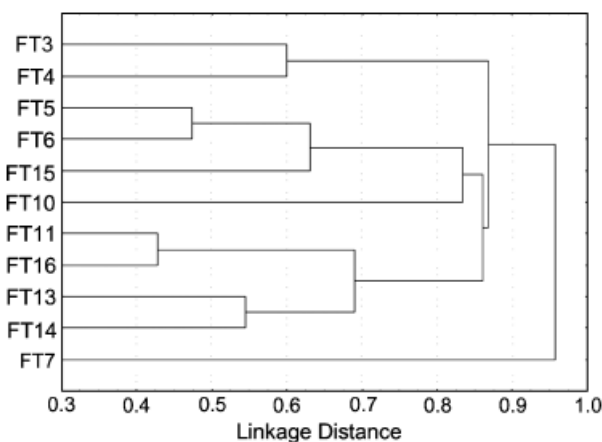


Fig. 4. Unweighted arithmetic average clustering (UPGMA) between *Fusarium* sequence variants detected in asparagus plants. The distance used is 1-Jaccard's community coefficient. Sequence variants closest matches are given in Table 1.

suggests that this parameter may be an important key in managing FCRR. *Fusarium* sequence variants associated with symptomatic asparagus plants (Fig. 3) were also more frequently found below the recommended P fertilization level of 105 kg P₂O₅ ha⁻¹ (Table 1). On the other hand, sequence variants related to low FCRR incidence were more commonly found in plots with no P fertilization or with twice the recommended P rate. This novel information may call into question the actual fertilization recommendation to the asparagus growers, as the recommended P fertilization level seems to favor the *Fusarium* population structure associated with high disease levels.

The lack of effect of P fertilization on soil and plant nutrient levels suggests that small differences, which were undetected by the statistical analysis, were biologically significant. When analyzed by canonical correspondence analysis, the data revealed that soil P content was the most significant variable. The lack of effect of P fertilization on plant P concentration suggests either that plants receiving more P grew bigger and that, although they contained more P, they had similar tissue P concentration, or that the influential P fertilization effect was not a nutritional effect. Triple superphosphate, which was banded at 0%, 100% or 200% of the recommended rate, is produced by the reaction of fluoroapatite with phosphoric acid and has an acidifying effect in soil (Parnes, 1990). Thus, lower soil pH in the zone of the fertilizer band may be involved in the fertilization effect.

The other ecological parameters with the greatest influence after P fertilization were Ca concentration and dehydrogenase activity in soil, as well as Fe and Mn concentration in plant tissues. The Ca present in the soil solution reacts with phosphate under acid conditions (Prasad & Power, 1997; Havlin et al., 1999). Thus, lower available soil Ca may be related to P fertilization and soil pH reduction. It has also been shown that some *Fusarium* species grow faster at pH 8.2 than at pH 5.7 (Sanogo & Yang, 2001). The most frequent sequence variants in this study (FT 5, 6, 13, 14 and 15; Table 1) were frequently found associated with medium and high calcium concentrations (Fig. 2). Sequence variants found in strongly FCRR-affected plots (FT 10, 11 and 16; Fig. 3) were also associated with reduced Ca (Fig. 2) and possibly low soil pH. These observations concur with earlier reports of more severe FCRR in low pH soils (Nonnecke, 1989). Indirect effects on *Fusarium* populations through modification of phosphorus availability could also occur, as Ca ions precipitate with phosphate ions (Prasad & Power, 1997).

Dehydrogenase activity is an indicator of microbial activity in soil (Dick & Tabatabai, 1993). Thus, higher dehydrogenase activity expresses increased competition between microorganisms for soil resources. Some of the sequence variants associated with high FCRR incidence (FT 11 and 16; Fig. 3) were related to low dehydrogenase activity, suggesting that

lower microbial competition in soil might be conducive to the proliferation of the *Fusarium* strains involved in FCRR.

Mn and Fe in plant tissues were other factors influencing *Fusarium* population structure in plants in the present experiment. Mn may directly affect *Fusarium*, but no records of such effects have been reported to date. Arbuscular mycorrhizal fungi can influence Fe and Mn uptake by plants. Mycorrhizal plants generally have lower Mn content than nonarbuscular mycorrhizal plants, although cases of enhanced Mn uptake have also been reported (Al-Karaki & Clark 1999; Pirazzi et al., 1999). Mycorrhizal fungi have enhanced plant acquisition of Fe in alkaline soil, where Fe availability is low, but not in acid soil, where Fe is more available (Clark & Zeto, 1996). Arbuscular mycorrhizal fungi were also shown to reduce severity of FCRR in asparagus and inhibit pathogenic soil fungi (Linderman, 2000; Matsubara et al., 2001; Elmer, 2002; St-Arnaud & Elsen, 2005), but the association of low FCRR expression with high plant Mn and Fe suggest that this effect may depend on soil pH. Effects may also be indirect via the action of Mn-reducing bacteria, which increase Mn bioavailability. Several of these bacteria have a biocontrol activity (Elmer, 1995). In addition, high Mn concentrations in soil and plant roots have been associated with lower FCRR incidence in asparagus (Elmer, 1995, 2003; Hamel et al., 2005b). Fe has no known direct effects on *Fusarium*, but many siderophore-producing bacteria, which increase iron availability for plants (Sharma & Johri, 2003a, b), have been shown to inhibit *Fusarium* populations by effectively competing for iron and by releasing antibiotics (de Boer et al., 2003).

Species respond to ecological parameters in a unimodal way rather than linearly (i.e. with an optimum growth at a certain level of a parameter and a reduced growth at lower and higher levels of this parameter). It is possible that asparagus plants, beneficial soil microbes and pathogenic *Fusarium* strains have dissimilar optima in response to P fertilization. Overall, the optimal P level for asparagus might be different than the recommended fertilization levels as they were formulated without considering their effect on beneficial and pathogenic soil microbial inhabitants.

Factors other than P fertilization also influenced *Fusarium* species dynamics and composition. Significant temporal variation was also recorded in relation to the *Fusarium* populations. *Fusarium* seasonal variability was reported by Schreuder et al. (1995) in asparagus fields in South Africa and by Bateman & Murray (2001) in U.K. wheat fields. *Fusarium* is a large genus, and ecological preferences at the species or even strain level have been observed (Burgess, 1981; Elmer et al., 1997; Backhouse et al., 2001; Vujanovic et al., 2006; Yergeau et al., 2006). The monthly variations in air temperature, soil temperature and precipitation that occur in temperate climates have been shown to influence *Fusarium* populations significantly, and could explain a part

of the variation observed here. The physiological and developmental changes occurring in asparagus throughout the growing season might also contribute to this variation by modifying soil and plant environments.

FCRR incidence was a significant factor influencing the population structure. The plant may have a direct influence on the *Fusarium* sequence variants that can proliferate in the roots or a particular *Fusarium* population may be required for disease onset. We found that five *Fusarium* sequence variants were associated with high FCRR incidence (Fig. 3). Four of these were closely related to *F. oxysporum* and probably included at least a pathogenic strain responsible for the high FCRR incidence. Not all *F. oxysporum* are pathogenic on asparagus, many being nonvirulent or specific to other hosts, and some may even protect plants from diseases (Blok *et al.*, 1997; Edel *et al.*, 1997; Skovgaard *et al.*, 2002; Fravel *et al.*, 2003). On the other hand, many of the sequence variants found associated with relatively low FCRR incidence were also linked to plants rich in Mn or Fe, or soils showing a high dehydrogenase activity (FT5, 6, 13, 14 and 15; Figs 2 and 3). Healthy plants containing high levels of Mn and Fe may represent a more favorable environment for these *Fusarium* sequence variants. Additionally, as discussed above, Mn, Fe and dehydrogenase are potential indicators of other antagonistic microbial communities that could compete with, and modify, the *Fusarium* populations infecting healthy plants. The presence of pathogenic *Fusarium* in soil seems therefore to be a necessary but not sufficient condition for the development of FCRR.

The competitiveness and ecological preferences of *Fusarium* strains may be important factors in the development of FCRR. This disease was recently associated with a cultivar-specific reorganization of the soil microbial community (Hamel *et al.*, 2005a). Both edaphic and climatic factors influence *Fusarium* species distribution (Vujanovic *et al.*, 2006). It had also been reported that *Fusarium* taxa are cosmopolitan and omnipresent in asparagus fields, but that disease develops in a characteristic patchy pattern (Hamel *et al.*, 2005b; Vujanovic *et al.*, 2006). Efficient control of root diseases is difficult to achieve and there is actually no effective way to control FCRR in asparagus fields. The control of this important disease could come from the adoption of cultural practices that lead to a soil environment unfavorable to the proliferation of pathogenic *Fusarium*. Here, we found that P fertilization largely influences *Fusarium* population structure. Mn, Fe, Ca and dehydrogenase were also factors modifying the structure of *Fusarium* populations in asparagus fields. This supports that antagonistic microbial groups could be important factors in the control of FCRR. In conclusion, our results suggest that nutrient availability influences the structure of *Fusarium* populations and may provide means to manage and lower *Fusarium* disease outbreaks in asparagus and other crops.

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