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Caractérisation moléculaire de la biodiversité des *Fusarium* associés à la fusariose de l'asperge (*Asparagus officinalis* L.) au Québec

par

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Caractérisation moléculaire de la biodiversité des *Fusarium* associés à la fusariose de l'asperge (*Asparagus officinalis* L.) au Québec

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Résumé

L'asperge (*Asparagus officinalis* L.) est une plante pérenne (15- 20 ans) et de grande valeur commerciale. Mais, la longévité et la rentabilité de cette culture sont souvent diminuées en raison d'un phénomène connu sous le nom de déclin de l'asperge et qui est en partie due à la fusariose (« Fusarium crown and root rot »). Une méthode moléculaire permettant d'identifier rapidement et facilement plusieurs espèces de *Fusarium* dans un plant d'asperge a donc été mise au point et est présentée au Chapitre 2. Cet outil a permis la discrimination de la majorité des 39 isolats de *Fusarium* testés et l'identification *in planta* de *Fusarium* impliqués dans la fusariose. Cette méthode a par la suite été utilisée pour observer l'effet à court et à long terme de la récolte de l'asperge sur la biodiversité de *Fusarium* (Chapitre 3). Des changements sont observés rapidement dans les communautés de *Fusarium* suite à la première récolte et il est discuté que ces changements sont probablement reliés à la physiologie de l'asperge. Ces changements semblent aussi se prolonger à long terme comme constaté lors de la comparaison des communautés de *Fusarium* de champs jamais récoltés avec celles de champs récoltés depuis quelques années. Les champignons mycorhiziens à arbuscules sont aussi influencés par la récolte, ce qui indique que la récolte provoque un changement en profondeur dans les communautés microbiennes associées à l'asperge.

Mots-clés : *Fusarium*, *Asparagus officinalis*, champignons mycorhiziens à arbuscules, DGGE (denaturing gradient gel electrophoresis), biodiversité, récolte, fusariose, pourriture racinaire, phytopathologie, écologie.

Abstract

Asparagus (*Asparagus officinalis* L.) is a perennial crop (15-20 years) with a high market value. However, the longevity and profitability of this crop is often diminished by a problem called asparagus decline that is partly caused by Fusarium crown and root rot. A molecular method allowing to rapidly and easily identify numerous *Fusarium* species in asparagus plant samples was therefore designed and is presented in Chapter 2. This tool permitted the discrimination of the majority of the 39 *Fusarium* isolates tested and the *in planta* identification of pathogenic *Fusarium* species. This method was subsequently used to observe the short- and long-term effects of asparagus harvest on *Fusarium* biodiversity (Chapter 3). Changes in *Fusarium* community are occurring rapidly following the first harvest and it is discussed that these changes are probably related to asparagus physiology. These shifts seem to continue in a long-term fashion as observed when comparing *Fusarium* community in fields never harvested to harvested fields. Arbuscular mycorrhizal fungi were also influenced by the harvest, indicating that harvest is profoundly affecting the microbial communities associated to asparagus.

Keywords : *Fusarium*, *Asparagus officinalis*, arbuscular mycorrhizal fungi, DGGE (denaturing gradient gel electrophoresis), biodiversity, harvest, Fusarium crown and root rot, phytopathology, ecology, asparagus decline.

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Liste des sigles et des abréviations

ADN: Acide désoxyribonucléique (DNA)

AFLP: Amplified fragment length polymorphism

AMF: Arbuscular-mycorrhizal fungi

ARN: Acide ribonucléique (RNA)

BLAST: Basic local alignment search tool

CMA: Champignons mycorhiziens à arbuscules

DGGE: Denaturing gradient gel electrophoresis

dNTPs: Desoxyribonucléotides tri-phosphate

EF-1 alpha: Translation elongation factor 1 alpha

FCRR: Fusarium crown and root rot (fusariose)

f. sp.: forma specialis

ITS: Internal transcribe spacer

PCR: Polymerase chain reaction

RFLP: Restriction fragment length polymorphism

RT-PCR: Reverse-transcriptase polymerase chain reaction

rRNA: Acide ribonucléique ribosomal

SSCP: Single strand conformation polymorphism

***Taq* polymerase:** *Thermophilus aquaticus* DNA polymerase

T-RFLP: Terminal restriction fragment length pattern

tRNA: Acide ribonucléique de transfert

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Chapitre

1 Introduction générale

1.1 La culture de l'asperge

Contrairement à la plupart des légumes, l'asperge (*Asparagus officinalis* L.) est monocotylédone et pérenne. L'établissement des asperges dans les champs du Québec se fait majoritairement par l'utilisation de rhizomes âgés d'un an produits en pépinière. Au Québec, la récolte est quotidienne et commence habituellement dès la troisième année pour une durée de 7 à 10 jours si la plantation est suffisamment vigoureuse (Tessier 2003). À la quatrième année, la récolte dure de 3 à 4 semaines et les années subséquentes, elle dure 5 semaines (environ du 10 mai au 24 juin). Au Canada en 2003, la récolte a été de 3 200 Mt (tonne métrique) avec une productivité de 2 263 kg/ha sur une superficie totale de 1 414 ha (United Nations 2003). Au Québec, 117 producteurs d'asperges exploitent une superficie de 300 ha (MAPAQ 2002). En 2003, les plus gros producteurs à l'échelle mondiale étaient la Chine (1 101 430 ha; 4 092 kg/ha, pour une production de 4 506 954 Mt), le Pérou (19 000 ha; 9 736 kg/ha; production de 185 000 Mt) et les États-Unis (26 000 ha; 3 307 kg/ha; production de 86 000 Mt) (United Nations 2003).

Malgré la rentabilité et la longévité potentielle de l'asperge, la plupart des plantations sont détruites par les producteurs après une dizaine d'années de croissance. La cause de cette destruction est la baisse de la productivité du champ engendrée par la mortalité et le manque de vigueur des plants. Cette baisse de productivité est connue sous le nom de « déclin de l'asperge » ou « asparagus

decline », en anglais, et ce phénomène est répertorié depuis près de cinquante ans (Grogan and Kimble 1959). Le déclin de l'asperge est associé à une combinaison de plusieurs facteurs, tant abiotiques que biotiques (Elmer et al. 1996).

L'un des stress abiotiques parmi les plus importants provient de l'asperge elle-même. Les débris de plants d'asperges libèrent des substances autotoxiques qui retardent la germination, diminuent la longueur des racines et des tiges et augmentent la ramification secondaire des racines (Yang 1982). La nature exacte de ces substances est inconnue, mais les observations démontrent qu'elles sont stables, persistent dans les tissus morts, s'accumulent avec l'âge de la plantation et que leurs effets augmentent avec leur concentration (Yang 1982). Le déclin de l'asperge est d'ailleurs beaucoup plus rapide et sévère lors de l'établissement d'une plantation dans un champ où la culture précédente était l'asperge (Blok and Bollen 1993; Elmer 2002; Schofield 1991). Ce problème est connu sous le nom de « replant problem » et il n'est donc pas recommandé aux producteurs d'installer une plantation dans un champ où des asperges ont été cultivées récemment (Tessier 2003). De plus, certaines études ont montré un effet synergique entre les substances autotoxiques relachées par les racines et les infections dues aux *Fusarium* spp., causant une augmentation des symptômes de la fusariose chez les plantes (Hartung and Stephens 1983; Peirce and Colby 1987; Peirce and Miller 1990). Par contre, Blok and Bollen (1996) ont observé l'inverse en utilisant des sols non-stériles et des quantités de tissus d'asperge plus près des biomasses retrouvées en champ. Quoiqu'il en soit, les extraits de plantes semblent affecter différentiellement les organismes composant la microflore du sol, inhibant ainsi certains micro-organismes du sol, mais pas les *Fusarium* spp. (Blok

and Bollen 1996; Hartung and Stephens 1983). Cette inhibition pourrait entraîner une modification de la microflore du sol de la rhizosphère de l'asperge, ce qui pourrait permettre à des micro-organismes moins affectés par ces substances (ex. : *Fusarium*) de proliférer plus facilement ou avec une inhibition moins grande.

Une autre source de stress abiotique important pour l'asperge est la pression induite par la récolte. Une aspergeraie mature (5 ans) est récoltée quotidiennement pendant 5 semaines au Québec (Tessier 2003). Il est reconnu que la récolte induit une baisse de la concentration de sucres à l'intérieur de la plante. La sévérité de cette baisse, le temps requis pour que la plante retrouve un niveau de sucre normal et la baisse de productivité durant les années subséquentes augmentent tous avec la durée de la récolte (Sanders 1985; Shelton and Lacy 1980; Takatori et al. 1970). Le métabolisme des sucres n'est pas le même pour tous les cultivars (Guo et al. 2002a; Guo et al. 2002b) et l'effet de la récolte ne devrait pas être le même non plus. Une variation saisonnière dans le métabolisme du sucre a aussi été notée (Bhowmik et al. 2001; Guo et al. 2002b).

Les facteurs biotiques qui causent le déclin des aspergeraies sont principalement liés à l'augmentation des infections par des champignons pathogènes du sol, l'augmentation des insectes ravageurs et la prolifération des mauvaises herbes. Les plus importants et les plus dommageables de ces facteurs biotiques sont les champignons appartenant au genre *Fusarium*.

1.2 *Fusarium*

Fusarium est un genre fongique qui se retrouve dans tous les types d'environnements et dans toutes les régions du globe (Burgess 1981). Ce genre cause d'importantes maladies dans de nombreux agro-écosystèmes (maïs, riz, blé, etc.) (Nelson et al. 1981). Par conséquent, *Fusarium* est considéré comme l'un des genres fongiques les plus importants, économiquement parlant. Malgré son importance, l'écologie du genre et les espèces le composant sont souvent mal connues et mal caractérisées. Cette méconnaissance est en partie due aux difficultés rencontrées lors de l'identification morphologique précise d'une espèce. En effet, une connaissance taxonomique qui exige souvent de nombreuses années de pratique et de longs travaux d'isolation et de culture sont essentiels à cette fin. Les critères retenus pour l'identification se basent pour la plupart sur la couleur de la colonie, la production de certains types de spores ainsi que la taille et la forme de ces spores (Nelson et al. 1983). Certains de ces critères exigent une longue expérience et peuvent être difficiles à maîtriser. De plus, plusieurs de ces critères sont clairement polyphylétiques tandis que d'autres sont reliés au milieu sur lequel le champignon pousse.

Mondialement, 12 espèces de *Fusarium* sont isolées des aspergeraies (Elmer 2001). Les espèces les plus fréquemment isolées sont les *F. proliferatum*, *F. oxysporum*, *F. solani* et *F. culmorum* (Blok and Bollen 1995; Elmer et al. 1997; Fantino 1990; Schreuder et al. 1995; Tello et al. 1985; Vujanovic et al. 2004). Les champs d'asperges de l'Afrique du Sud, du Canada et de l'Australie ont des communautés dominées par les *F. oxysporum* et *F. proliferatum*, avec le *F. solani*

troisième en importance et plusieurs autres espèces en faible quantité (Elmer et al. 1997; Schreuder et al. 1995; Vujanovic et al. 2004). Par contre, les communautés des champs de Nouvelle-Zélande et des Pays-Bas sont nettement dominées par le *F. oxysporum*, avec comme deuxième espèce en importance, soit le *F. solani* ou le *F. culmorum*, tandis que le *F. proliferatum* est absent des isolations (Blok and Bollen 1995; Elmer et al. 1997). Les auteurs ont émis l'hypothèse que les climats plus froids de ces deux pays empêchaient la persistance du *F. proliferatum* dans les champs d'asperges. Cette hypothèse est cependant en contradiction avec les études menées au Québec, où l'on retrouve régulièrement le *F. proliferatum* dans les champs d'asperges (Caron et al. 1985; Plante et al. 1983; Vujanovic et al. 2004). En Italie, le *F. oxysporum* et le *F. proliferatum* dominent les communautés et sont accompagnés du *F. culmorum* et du *F. solani* en moindre quantité (Fantino 1990). En Espagne, le *F. oxysporum* est aussi l'espèce la plus isolée suivie de près par le *F. culmorum* puis le *F. proliferatum* (Tello et al. 1985).

La maladie la plus fréquente causée par les membres du genre *Fusarium* dans les champs d'asperges est la fusariose de l'asperge, nommée « Fusarium crown and root rot (FCRR) » en anglais (Elmer et al. 1996). En Amérique du Nord, deux espèces sont reconnues comme étant les agents causaux principaux de cette maladie : le *F. oxysporum* f.sp. *asparagi* et le *F. proliferatum*, à cause de leur abondances et de leur pathogénicités élevées (Damicone and Manning 1985; Elmer 2001). Ces deux agents pathogènes sont omniprésents dans le sol de champs d'asperges et peuvent coloniser les tissus racinaires des plantes sans provoquer de symptômes. Le *F. oxysporum* est observé plus fréquemment dans les jeunes racines alors que le *F.*

proliferatum est dominant dans presque toutes les autres parties de la plante (Gilbertson and Manning 1983; LaMondia and Elmer 1989). Le rôle des autres espèces, telles les *F. solani* et *F. culmorum*, dans la fusariose est mal caractérisé. Cependant, il est généralement admis qu'elles ne sont pas des agents pathogènes majeurs, compte tenu de leur faible virulence et de leur faible abondance. Néanmoins, ces espèces pourraient jouer un rôle non négligeable dans le développement de la maladie en étant en concurrence avec les espèces pathogènes dans le sol et dans la plante.

Aucun moyen de contrôle de la fusariose, totalement efficace et économique à utiliser, n'est connu. Il n'existe pas pour le moment de cultivar d'asperge résistant à la fusariose (Stephens et al. 1989; Tessier 2003). La lutte chimique à l'aide de fongicides est peu efficace ou seulement utile à court terme (Damicone and Manning 1982; Lacy 1979). Le sel de table (NaCl) semble efficace pour contrôler la fusariose dans une certaine mesure (Elmer 1992). Le mode d'action du sel semble passer par une modification des communautés bactériennes du sol, particulièrement des bactéries réductrices de manganèse dont certaines ont un effet antagoniste sur les *Fusarium* (Elmer 1995a; Elmer 2003). Le *F. solani* et des isolats avirulents du *F. oxysporum* ont aussi été efficacement utilisés pour réduire la sévérité des symptômes associés à la fusariose (Blok et al. 1997; Damicone and Manning 1982). Le mécanisme d'action de cette protection semble être relié à une activation des voies de défense de la plante (He et al. 2002). Comme cela sera mentionné dans la section suivante (section 1.3), les champignons mycorhiziens à arbuscules (CMA) peuvent aussi protéger les plants d'asperges de la fusariose. Le mécanisme sous-jacent à ce

type de protection n'est pas encore clairement determiné, mais plusieurs sont proposés : modification de la nutrition de la plante, stimulation des mécanismes de résistance de la plante, modification de la microflore du sol ou encore interaction directe entre les CMA et le pathogène (St-Arnaud et al. 1995). D'autres champignons, appartenant au genre *Trichoderma*, ont montré une capacité à réduire les symptômes de la maladie (Arriola 1997), mais Rousseau et al. (1996) ont observé que ces champignons pouvaient parasiter les CMA en conditions *in vitro*. Par contre, d'autres travaux ont présenté des interactions positives ou neutres entre les *Trichoderma* et les CMA (St-Arnaud and Elsen 2004).

La variété et la sévérité des maladies causées par les membres du genre *Fusarium* combinées aux difficultés rencontrées lors de leur identification fait en sorte qu'il existe un grand besoin pour des méthodes innovatrices d'étude de leur diversité. En connaissant mieux la dynamique et la diversité des communautés de *Fusarium*, il sera possible de mieux combattre les maladies qu'ils provoquent, autant dans les champs d'asperges que dans les autres cultures. Cela sera possible par la modification de certains paramètres biotiques ou abiotiques du sol, c'est-à-dire en introduisant des micro-organismes ou en portant une attention particulière à la régie culturale.

1.3 Champignons mycorhiziens à arbuscules

L'asperge est une plante qui est colonisée par des champignons endomycorhiziens à arbuscules (CMA; Embranchement: Glomeromycota). Compte tenu que l'asperge est une plante vivace non-arborescente, cela en fait un bon modèle pour étudier à long terme la succession d'espèces et le biocontrôle relatifs à la mycorhization chez les plantes. Les CMA augmentent la croissance (nombre de tiges, hauteur des tiges, masse des tiges et des racines) et réduisent la sévérité des infections causées par les *Fusarium* spp. chez les asperges (Arriola 1997; Burrows et al. 1990; Chang 1985; Hussey et al. 1984; Matsubara et al. 2001; Pedersen et al. 1991). Par contre, la stimulation de la croissance de la plante et la protection contre les maladies n'ont pas été les mêmes pour toutes les espèces de CMA testées (Chang 1985; Matsubara et al. 2001; Pedersen et al. 1991). De plus, Wacker et al. (1990c) ont démontré qu'il y avait une succession d'espèces de CMA dans les champs d'asperges en relation avec l'âge. Le phosphore disponible dans les champs d'asperges diminue avec l'âge de la plantation et, compte tenu que certaines espèces de CMA sont inhibées par une haute teneur en phosphore, il semble possible que le changement observé dans les communautés soit causé par cette diminution (Burrows et al. 1990; Hussey et al. 1984; Pedersen et al. 1991; Wacker et al. 1990a). Également, les plants d'asperges infectés par des CMA ont toléré de plus grands stress thermiques et ce degré de résistance a varié selon l'espèce mycorhizienne présente (Matsubara et al. 2000). Il semble donc que les mycorhizes augmentent la résistance des plants d'asperges aux stress biotiques et abiotiques et que cette résistance varie selon les espèces de CMA présentes.

1.4 Diversité des micro-organismes

Afin d'avoir une idée plus précise de la diversité taxonomique des micro-organismes, voici un tableau qui résume de nombreuses études qui avaient pour but d'estimer la diversité de différents groupes de micro-organismes:

TABLEAU I: Nombre d'espèces décrites et estimées pour quatre groupes de micro-organismes*

	Champignons	Nématodes	Bactéries
Nombre d'espèces décrites (en milliers)	72	25	4
Nombre d'espèces estimées (en milliers)	200-2700	100-1000	50-3000
Pourcentage connu	2,7-36 %	2,5-25 %	0,13-8 %

* adapté de Heywood and Watson (1995)

Les micro-organismes sont donc extrêmement méconnus et cela devient encore plus évident lorsqu'il est question de la connaissance estimée que nous avons des plantes (54-90% de connues) et des chordés (82-90% de connus) (Heywood and Watson 1995). L'impossibilité de détecter certains de ces organismes avec les méthodes traditionnelles d'isolation sur milieux sélectifs n'est certainement pas étrangère à cette méconnaissance. En effet, on estime que seulement 17 % des champignons (Hawksworth 1991) et de 0,1 à 10 % des bactéries sont cultivables (Head et al. 1998). Les raisons de cette non-cultivabilité sont diverses : méconnaissance des conditions de croissance appropriées, manque de compétitivité de l'organisme ou impossibilité pour l'organisme de croître hors son habitat naturel.

Les méthodes moléculaires, biochimiques ou physiologiques permettent souvent de contourner ces impasses en visant la totalité des molécules présentes dans le sol et en ne se basant pas sur la capacité de croître sur un milieu de croissance. Paradoxalement, une partie de la biodiversité restera à tout jamais inconnue, car pour certains micro-organismes, seulement des séquences ou des molécules d'intérêt seront connues et les organismes d'origine ne pourront être isolés. La mise au point de nouveaux milieux de croissance plus efficaces pour les micro-organismes permettra peut-être de cultiver les organismes que l'on croyait auparavant non-cultivables. De plus, l'observation *in situ* des structures permet déjà d'identifier certains organismes sans passer par l'isolation.

De nombreuses méthodes moléculaires sont disponibles et efficaces pour étudier la biodiversité des micro-organismes dans des échantillons environnementaux: le « single strand conformation polymorphism » (SSCP), le « denaturing gradient gel electrophoresis » (DGGE), le « terminal restriction fragment length polymorphism » (T-RFLP), le « amplified fragment length polymorphism » (AFLP) et le clonage-séquençage (Bridge and Spooner 2001; Morgan and Winstanley 1997). Le DGGE est une méthode pouvant traiter beaucoup d'échantillons simultanément et est relativement simple à utiliser, permettant d'avoir un patron de biodiversité reproductible (Heuer et al. 2001; Muyzer 1999). Cette méthode comporte les principaux avantages d'être moins dispendieuse, plus rapide et permet d'avoir un patron de diversité en même temps que l'information supplémentaire sur ce patron (séquences des bandes du patron). De plus, cette méthode peut détecter des micro-organismes qui ne contribuent que pour 1 % de

l'ADN génomique total de la communauté (Muyzer et al. 1993). Lors du DGGE, les produits de PCR sont séparés par migration électrophorétique dans un gel de polyacrylamide contenant un gradient d'agents dénaturants chimiques qui augmente de façon linéaire. L'ADN double brin migre jusqu'à un point dans le gel où il se dénature partiellement et arrête pratiquement sa progression. L'endroit où s'arrête la migration est déterminé majoritairement par le pourcentage de GC (guanine et cytosine) dans la molécule. On peut ainsi séparer les fragments d'ADN de même longueur de différents organismes selon leur séquence. Le DGGE permet théoriquement de détecter de petites différences (jusqu'à une paire de base) dans les séquences (Myers et al. 1985; Myers et al. 1987). Chaque échantillon composite génèrera donc un patron de bandes qui correspondra aux organismes qui le composent.

1.5 Objectifs

L'objectif général de cette maîtrise est d'augmenter la connaissance de la biodiversité et de l'écologie des champignons du genre *Fusarium* dans les champs d'asperges du Québec afin de mieux comprendre et possiblement de contrôler la fusariose de l'asperge. Pour ce faire, deux objectifs spécifiques ont été poursuivis. Le premier objectif spécifique (Chapitre 2) était de mettre au point une méthode d'étude rapide et précise de la biodiversité des *Fusarium* basée sur l'ADN. Compte tenu des difficultés d'identification des espèces du genre *Fusarium* et de celles reliées à l'isolation des micro-organismes, cette méthode a été essentielle à la réalisation du deuxième objectif. Le second objectif spécifique (Chapitre 3) était d'étudier les changements survenant à court et à long terme dans la composition des communautés de *Fusarium* et mycorhiziennes associées aux plants d'asperge, à la suite de la récolte. L'hypothèse sous-jacente à ce second objectif est que la récolte induit un changement dans les communautés fongiques associées aux plantes, ce qui cause l'augmentation de l'incidence de la fusariose observée dans les champs âgés. En effet, il existe une compétition entre les espèces et les isolats de *Fusarium* pathogènes et ceux non pathogènes. De plus, les espèces de champignons mycorhiziens à arbuscules ne possèdent pas toutes les mêmes capacités à protéger les plantes contre les champignons pathogènes.

Chapitre

2 A PCR-denaturing gradient gel electrophoresis (DGGE) approach to assess *Fusarium* diversity in asparagus

Le manuscrit qui suit porte sur le développement d'un outil moléculaire pour caractériser la diversité des champignons du genre *Fusarium* dans des échantillons environnementaux. C'est la première mention d'un outil spécifique au genre *Fusarium* qui permet d'identifier rapidement les espèces présentes dans un échantillon environnemental sans passer par les étapes de culture. Ce manuscrit a été soumis à la revue « Journal of Microbiological Methods » le 8 avril 2004. Les auteurs sont : Étienne Yergeau, Martin Filion, Vladimir Vujanovic et Marc St-Arnaud. Afin de ne pas se répéter, les références relatives à ce manuscrit sont à la fin du mémoire et non pas immédiatement après le manuscrit.

2.1 Abstract

In North America, asparagus (*Asparagus officinalis*) production suffers from a crown and root rot disease mainly caused by *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*. Many other *Fusarium* species are also found in asparagus fields, whereas accurate detection and identification of these organisms, especially when processing numerous samples, is usually difficult and extremely time consuming. In this study, a PCR-denaturing gradient gel electrophoresis (DGGE) method was developed to assess *Fusarium* species diversity in asparagus plant samples. *Fusarium*-specific PCR primers targeting a partial region of the translation elongation factor-1 alpha (EF-1 alpha) gene were designed, and their specificity was tested against genomic DNA extracted from a large collection of closely and distantly related organisms isolated from multiple environments. Amplicons of 450 bp were obtained from all *Fusarium* isolates, while no PCR product was obtained from non-Fusarium organisms. The ability of DGGE to discriminate between *Fusarium* taxa was tested with 19 different *Fusarium* species represented by 39 isolates, including most species previously reported from asparagus fields worlwide. The technique was effective to visually discriminate between the majority of *Fusarium* species and/or isolates tested in pure culture, while a further sequencing step permitted to distinguish between the few species showing similar migration patterns. Total genomic DNA was extracted from field-grown asparagus plants naturally infested with different *Fusarium* species, submitted to PCR amplification, DGGE analysis and sequencing. The two to four bands observed for each plant sample were all affiliated with *F. oxysporum*, *F. proliferatum* or *F. solani*, clearly supporting the

reliability, sensitivity and specificity of this approach for the study of *Fusarium* diversity from asparagus plant samples.

2.2 Introduction

Asparagus (*Asparagus officinalis*) is a low-input perennial crop having a high market value. Yet, asparagus fields are frequently showing a marked decline following several years of harvesting until a point were the plantation is no more profitable. Crown and root rot of asparagus is a main factor in this decline of productivity and longevity (Elmer 2001). The disease is caused by *F. oxysporum* f.sp. *asparagi* and *F. proliferatum*, but more than 12 species of *Fusarium* have been isolated from asparagus fields worldwide (Blok and Bollen 1995; Elmer 2001; Elmer et al. 1997; Fantino 1990; Schreuder et al. 1995; Tello et al. 1985; Vujanovic et al. 2002). While *F. oxysporum* and *F. proliferatum* roles in asparagus fields are quite clear, the other *Fusarium* species are often overlooked. They might as well play a significant role in asparagus production, being pathogenic in specific conditions, or competing for similar ecological niches with the pathogenic isolates. More information is therefore needed on the diversity and dynamics of these low-occurring but potentially important species, if we want to reach the full potential concerning the use and management of disease-suppressive microbial communities in biological control of asparagus decline.

Standard methods to assess *Fusarium* diversity in asparagus fields are based on isolation and enumeration of species growing on selective media (Vujanovic et al. 2002). However, this procedure is time-consuming and *Fusarium* species identification based on morphological characters is a difficult task that requires unusual skills. Molecular methods, that are easier to master, will permit efficient and low-cost processing of the large number of samples required in large-scale ecological

studies. PCR primers specific to *F. oxysporum* and *F. proliferatum* in asparagus plants have been published recently (Mulè et al. 2004), but no method is currently available to analyse the whole genus diversity using a simple, time efficient and reliable approach.

Denaturing gradient gel electrophoresis (DGGE) separates PCR amplicons of similar length with dissimilar nucleotide compositions on a denaturing gradient gel (Muyzer et al. 1993). PCR-DGGE is a high-resolution technique (Myers et al. 1985; Myers et al. 1987) where a large number of samples can be rapidly compared to reveal microbial communities dynamics (Muyzer and Smalla 1998). So far, most PCR-DGGE studies that focused on fungal diversity have targeted either portions of the 18S gene (Kowalchuk et al. 1997; Smit et al. 1999; van Elsas et al. 2000) or the rDNA ITS region (Landeweert et al. 2003). In a recent study, O'Donnell et al. (1998b) indicated that for the genus *Fusarium*, these ribosomal regions contain less interspecific variation than the translation elongation factor 1 alpha gene (EF-1 alpha). Due to its high discriminating power at the species level, the EF-1 alpha gene has also been used as a genetic marker for phylogenetic studies, allowing the accurate discrimination of *formae speciales* or strains for specific *Fusarium* species such as *F. oxysporum* and *F. solani* (O'Donnell 2000; O'Donnell et al. 1998b).

The aims of the present study were therefore: 1) to design a *Fusarium* specific PCR primer pair targeting a partial region of the EF-1 alpha gene, 2) to evaluate the sensitivity of these primers to discriminate between the different *Fusarium* species isolated from asparagus fields using PCR-DGGE assays, and 3) to detect and identify

Fusarium species directly from asparagus plants without prior isolation on selective media.

2.3 Materials and methods

2.3.1. Fungal, bacterial and plant materials

Thirty-nine *Fusarium* isolates belonging to 19 species, and 35 other heterogeneous organisms (25 fungi, 6 bacteria, and 4 plant species) were previously isolated from asparagus fields (Vujanovic et al. 2002), obtained from culture collections or received from other researchers (Table I). For plants (except asparagus) and bacteria, already extracted DNA samples stored at -80°C were obtained from Dr. S.H. Jabaji-Hare, McGill University. Fungi were grown on potato dextrose agar for 7 d at 25°C in the dark, and one agar plug was then transferred into 50 ml of potato dextrose broth. Mycelial mats were recovered by vacuum filtration after 7-10 days incubation at 20°C on a rotatory shaker (200 rpm). All isolates used in this study are available upon request.

Seeds of *Asparagus officinalis* cv Guelph Millenium were surface sterilized in 2.5% sodium hypochlorite solution for 5 minutes and then rinsed twice in sterile distilled water. After 14 days of growth in a sterile Petri dish containing a moist filter paper, seedlings were collected and frozen at –80°C until processed.

2.3.2. Environmental asparagus plant samples

Three asymptomatic and three *Fusarium* root rot symptomatic asparagus plants (*Asparagus officinalis*) were harvested in September 2003 from a commercial asparagus field located at St-Liguori (30 km N-E of Montreal), Qc, Canada. Within a

day from harvest, roots were removed, washed under tap water, and stored at –80°C prior to DNA extraction.

2.3.3. DNA isolation

Mycelial mats and asparagus plant material were freeze-dried and ground in liquid nitrogen with a mortar and pestle. Total genomic DNA was extracted from 200 mg fungal or plant tissues sub-samples using the DNAeasy plant mini kit (Qiagen, Valencia, CA.) following manufacturer's instructions. The purified DNA was resuspended in 100 µl of elution buffer and stored at -20°C until PCR amplification.

2.3.4. Primer design

EF-1 alpha gene sequences from 50 *Fusarium* strains (30 different species) and 15 sequences from both closely and distantly related fungal genera were recovered from GenBank and aligned using Clustal X version 1.82 (Thompson et al. 1997). Following primer design guidelines (Mitsuhashi 1996), highly conserved DNA strings unique to *Fusarium* bordering a ~500 bp region (optimal length for DGGE analysis) containing a high level of inter-specific variation were identified. These strings were then submitted to the GenBank BLAST algorithm (Altschul et al. 1997) to test for specificity. A GC-clamp (underlined) was added to the 5' end of the forward primer, as described in Muyzer et al. (1993) for the purpose of DGGE analysis. Primers Alfie1-GC (forward, 5'-<u>CGC CCG CCG CGC GCG GCG GGC</u>

GGG GCG GGG GCA CGG GGG GTC GTC ATC GGC CAC GTC GAC TC-3') and Alfie2 (reverse, 5'-CCT TAC CGA GCT CRG CGG CTT C-3') were commercially synthesized (Alpha DNA, Montreal, Canada). NS1-NS2 primers, amplifying the fungal and plants partial 18S rRNA gene (White et al. 1990), and 968f-1401r primers, amplifying the bacterial partial 16S rRNA gene (Nübel et al. 1996), were used as universal primers.

2.3.5. PCR amplification

PCR were carried out in 50 µl volumes containing 5 µl of 10× PCR buffer, 5 µl of each primer (5 µM), 1 µl of 10 mM dNTPs mix, and 1.25 U of *Taq* polymerase (QIAgen). Reaction mixtures were overlaid with 40 µl sterile mineral oil prior to PCR on a PTC-100 thermalcycler (MJ-Research, inc. Waltham, MA). For DNA amplification from pure fungal cultures with Alfie1 (with or without the GC-clamp) and Alfie2 primers, the following cycling protocol was used: one cycle of initial denaturation (94°C for 5 min), 40 cycles of denaturation (94°C for 1 min), annealing (67°C for 1 min) and extension (72°C for 1 min), followed by a final cycle of extension (72°C for 10 min). For DNA amplifications from asparagus plant tissue samples, a nested PCR procedure was applied using the EF-1-EF-2 primers (O'Donnell et al. 1998b) in the first amplification round (same cycling protocol as above but for 30 cycles with annealing at 50°C). The amplicons were subsequently diluted (1:1000) and reamplified (35 cycles) using Alfie1-GC-Alfie2 primers. For DNA amplifications with universal primers, the cycling protocols were: one cycle of

initial denaturation (94°C for 3 min), 35 cycles of denaturation (94°C for 1 min), annealing (55°C, NS1-NS2; 62°C, 968f-1401 for 1 min) and extension (72°C for 2 min), and a final cycle of extension (72°C for 10 min). Ten-fold serial dilutions of all DNA samples were first prepared and amplified with the appropriate universal primers. Amplified products were visualized by 1.5% agarose gel electrophoresis in 1×TAE buffer after staining with ethidium bromide (10 $\mu g\ ml^{-1}$), and the dilution showing the strongest amplification was then used for PCR amplification using the Alfie1-Alfie2 primer pair in order to test for specificity.

2.3.6. DGGE

DGGE analyses were performed using a DCode Universal Mutation Detection System (BioRad, Hercules, CA). Electrophoreses were run for 16 hours at 80 V on a 6% acrylamide/bis-acrylamide (37.5:1) gel with a 40%-60% denaturant gradient (100% denaturant corresponding to 7 M urea and 40% (v/v) formamide). Electrophoresis gels were stained with ethidium bromide (10 $\mu g\ ml^{-1}$), digitized using a Chemi-Doc apparatus (BioRad) and analysed using the Quantity One 4.0 software (BioRad). A molecular marker composed of known species (Fusarium oxysporum f. sp. asparagi FOA50, F. subglutinans UG #6, F. sambucinum MT-F148, F. solani MT-F240) was loaded on both sides of each gel to facilitate gel-to-gel comparisons. When sequence informations were needed, DGGE bands were excised using a sterile scalpel blade, mixed with 50 µl of Tris-HCl (10 mM, pH 8.0), incubated at 50°C for 10 min, crushed with a microcentrifuge tube pestle and centrifuged at 10,000 g for 1

min. Two microliters of the resulting supernatant were used for subsequent PCR. All excised bands were re-amplified and submitted to DGGE, to confirm the excision of a single band. To test the reliability of the excision method, bands of the molecular marker composed of known organisms were also excised, sequenced and compared with sequences obtained from pure cultures.

2.3.7. Investigation of multiple banding patterns

In order to investigate the cause of multiple banding patterns, the Alfie1-Alfie2 amplicons of a *F. proliferatum* strain (N-7268-84) showing a clear multiple banding pattern were purified on agarose gel using the QIAquick gel extraction kit and cloned (shotgun cloning) using the TOPO-TA cloning kit (Invitrogen, Burlington, Canada) following the manufacturer's instructions. Eleven colonies were randomly selected and the plasmids were recovered using the QIAprep spin miniprep kit (QIAgen). Inserts were amplified using Alfie1-GC/Alfie2 primer pair, screened with DGGE and six clones covering each migration position within the initial multiple banding pattern were then chosen and sequenced.

2.3.8. DNA sequencing and distance analyses

PCR amplicons were purified on agarose gels using the QIAquick gel extraction kit (Qiagen) and commercially sequenced (Montreal Genomic Centre, Montreal, Canada). Similarity searches were performed in the GenBank database using the

BLAST search algorithm. Sequences were aligned using Clustal X, and distance trees were produced with the PAUP* program (Swofford 2000) using a neighbor-joining approach. Statistical support for groups in the tree was assessed via bootstrap analysis (1000 reps).

2.3.9. Nucleotide sequence accession numbers

All EF-1 alpha sequences generated in this study for pure *Fusarium* isolates were deposited in GenBank under accession numbers AY337415 to AY337454.

2.4 Results

2.4.1. Primers specificity

BLAST searches for the Alfie1 binding site resulted in 387 significant hits, with 84.0% belonging to *Fusarium* species or known *Fusarium* teleomorphs and 96.0% of those showing 100.0% similarity. For *F. solani* f. sp. *glycines* and some unidentified *Fusarium* species, primer Alfie1 showed an imperfect match with a C→G mutation at the second position of the 5'-end of the primer. All other hits were EF-1 alpha genes from diverse organisms having imperfect similarity with the primer sequence. BLAST searches for the primer Alfie2 binding site resulted in 434 significant hits, 98.6% being sequences for EF-1 alpha of *Fusarium* species or their teleomorphs, and all showing 100.0% similarity. *Neurospora crassa* and *Aspergillus oryzae* were the only other hits showing perfect similarity with the totality of the Alfie2 primer sequence. Another database search was conducted by retrieving one representative of every *Fusarium* species and teleomorphs for which the EF-1 alpha gene had been sequenced, i.e. approximately 80 different accessions. For every species, primers Alfie1 and Alfie2 were perfectly matching.

Heterogeneous fungi, plants and bacteria were successfully amplified with the appropriate universal primers, confirming the proper quality and quantity of genomic DNA for PCR amplification (data not shown). Amplification products of approximatively 450 bp were obtained using Alfie1 and Alfie2 primers with all *Fusarium* isolates tested, and no amplification was observed with any of the non-*Fusarium* species (data not shown).

2.4.2. PCR-DGGE analyses of pure Fusarium isolates

The migration positions of amplicons obtained using the Alfie1-GC/Alfie2 primer pair directly discriminated eleven *Fusarium* species, including all major species found in asparagus fields: *F. fujikuroi*, *F. lateritium*, *F. nygamai*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. sambucinum*, *F. semitectum*, *F. solani*, *F. subglutinans* and *F. verticillioides* (Fig. 1a, b, c). Within the eight other *Fusarium* species tested, some isolates migrated to similar positions but were always clearly differentiated after excision and sequencing of the bands: group 1) *F. sacchari* and *F. sporotrichioides* (UG#6)(Fig. 1a, lanes 11 and 14); group 2) *F. chlamydosporum* and *F. sporotrichioides* (FRC T-0572)(Fig. 1a, lanes 13 and 15); group 3) *F. globosum* isolates and *F. sulphureum* (Fig. 1b, lanes 5-7); and group 4) *F. acuminatum*, *F. avenaceum* and *F. equiseti* (Fig. 1c, lanes 2-4).

A variation in migration positions was observed within five *Fusarium* species. Within those species, *formae speciales* or isolates were mostly grouped together, but showed a slight variation in migration position: *F. oxysporum* (Fig. 1a, lanes 2, 3, 5, 8, 9, 12), *F. sporotrichioides* (Fig. 1a, lanes 14-15), *F. solani* (Fig. 1c, lanes 12-14), *F. subglutinans* (Fig. 1a, lane 10, and Fig.1b, lanes 3, 8) and *F. verticillioides* (Fig. 1b, lane 14, and Fig 1c lane 10). In contrast, in the four following species that were also represented by different isolates, all isolates had homogenous migration positions: *F. fujikuroi* (Fig. 1a, lanes 6-7), *F. globosum* (Fig. 1b, lanes 5-6), *F.*

proliferatum (Fig. 1b, lanes 9-13; Fig1c, lanes 5 and 8) and *F. sambucinum* (Fig. 1c, lanes 6-7).

F. oxysporum f. sp. *dianthi* (Fig. 1a, lane 5) and all *F. proliferatum* isolates (Fig. 1b, lanes 9-13 and Fig 1c, lanes 5 and 8) showed multiple banding patterns. Shotgun cloning of *F. proliferatum* (N-7268-84) amplicons and subsequent PCR-DGGE analysis of the amplicons from 11 clones revealed two fragments showing slightly different electrophoretic mobility (Fig. 2). Six clones were sequenced and a distance analysis revealed that they clustered in two clearly distinct groups closely related to *F. proliferatum* (99.3-100.0% similarity, data not shown).

2.4.3. Sequence analysis

The partial EF-1 alpha sequence of the 39 *Fusarium* isolates contained enough variation to allow differentiation of every species, following a distance analysis (Fig. 3). There was no correlation between the genetic distances between species, as shown on the phyllogram, and the migration distance in DGGE gel, as expected with this approach. For example, species showing large differences in partial EF-1 alpha sequences, like *F. sacchari* and both *F. sporotrichioides* isolates (72.6 and 71.8% similarity), or *F. sulphureum* and *F. globosum* (73.4 % similarity), had similar migration behavior. Inversely, the three *F. subglutinans* isolates showed high sequences similarity (97.2-98.5%) but had slightly dissimilar migration patterns.

2.4.4. PCR-DGGE analyses of asparagus plant samples from the field

Both symptomatic and asymptomatic asparagus plant samples produced DGGE banding patterns showing two to four bands per sample (Fig. 4). Sequences analyses of all bands excised from the gel showed 98.0-99.0% similarity with sequences of three different *Fusarium* species from our collection of isolates from asparagus fields, and 97.0-99.0% similarity with various *Fusarium* sequences in GenBank (accession numbers of the closest match for, respectively, bands a-g: AF008484, AF362167, AF246887, AF433304, AF160280, AF336913, AF178337). Sequences retrieved from the bands were always equally or more similar to the isolates from our culture collection than to the Genbank sequences, and therefore only those former similarities were thus retained for further analyses. Sequences from bands a-d were related to different *F. oxysporum* isolates (respectively 18131, L6886#44, MT#147, MT-F142), while bands e-f were closer to one *F. proliferatum* isolate (MT-F147), and band g was related to a *F. solani* isolate (MT-F116). The migration behavior of the bands obtained from environmental samples were identical to those observed from pure cultures of *F. proliferatum*, *F. oxysporum* and *F. solani* (Fig.1). Bands recovered from the molecular marker had also sequences identical to those obtained from pure cultures of the four *Fusarium* species used for this marker, confirming the efficiency of the recovery method used.

2.5 Discussion

The PCR-DGGE approach described in this study allowed to differentiate easily and rapidly between 19 *Fusarium* spp., including most species isolated from asparagus fields worldwide. Moreover, this technique has permitted to detect and identify *Fusarium* species directly from asparagus plant samples from the field, without a first isolation step. By contrast, available approaches require prior cultivation of *Fusarium* isolates (Donaldson et al. 1995; Edel et al. 1997; Lodolo et al. 1993; Pasquali et al. 2003), or were restrained to a limited number of species (Hue et al. 1999; Mishra et al. 2003; Mulè et al. 2004).

The primers developed in this study both showed a high specificity to the genus *Fusarium*. Primer Alfie1 was showing a perfect match only with *Fusarium* species. Similarly, primer Alfie2 matched perfectly with all *Fusarium* species available in GenBank, and only with *Neurospora crassa* and *Aspergillus oryzae* outside the genus *Fusarium*. Nevertheless, in combination, these primers amplified only *Fusarium* spp., despite the presence of *Neurospora* and *Aspergillus* species among the tested fungal species. Primers specificity tests using a large collection of closely and distantly related organisms are needed until enough reference sequences will be available in public databases. This is especially important for the fungal EF-1 alpha gene since only a limited number of sequences are available in databases, as compared to many well-studied bacteria, plant and animal genes.

Using DGGE analysis of PCR amplified partial EF-1 alpha sequences, species-level discrimination was visually possible for most studied *Fusarium* taxa, and for all species tested after sequencing of the excised bands. The main *Fusarium* species

found on asparagus in North America (*F. proliferatum*, *F. oxysporum* and *F. solani*) were all clearly distinguishable on DGGE gels, without need of further characterization. In addition, the sensitivity of this method permitted the separation of isolates or *formae speciales* in five tested species. The intraspecific variation within *F. oxysporum* and *F. solani* isolates studied was expected since previous studies recognized numerous cryptic species embedded in *F. oxysporum* and *F. solani* complexes (O'Donnell 2000; O'Donnell et al. 1998b). Nevertheless, no phylogenetic related information can be inferred from the migration position since there is no correlation between sequence similarity and bands proximity, as shown by the results.

As multiple banding patterns were visibles for all *F. proliferatum* isolates, as well as for *F. oxysporum* f. sp. *dianthi*, this feature, when present, might be used in addition to band position to help differentiate species on the gel. Previous DGGE studies also reported multiple banding patterns and it was hypothesized that heteroduplexes, multiple gene copies, primer degeneracy or multiple operons were the potential causes (Nicolaisen and Ramsing 2002; Salles et al. 2002). Since most *Fusarium* isolates used in this study showed only a single band on the DGGE gels, it is unlikely that the multiple banding pattern observed in some isolates was due to degenerate primer Alfie2. On the other hand, multiples copies of the EF-1 alpha gene have been reported in fungi (Hovemann et al. 1988; Schillberg et al. 1995; Schirmaier and Philippsen 1984). In this study, analysis of a *F. proliferatum* isolate showed that the observed multiple banding pattern was due to the presence of several slightly dissimilar EF-1 alpha sequences in a single strain.

Despite the high sensitivity of this method, some distinct species showed similar band migration position, as previously reported in other PCR-DGGE studies (Kowalchuk et al. 2002; Salles et al. 2002). Indeed, DGGE has a theoretical resolution of 1 bp difference between two amplicons (Myers et al. 1985; Myers et al. 1987), but this separation power will largely depend on the length and sequence of the products. In fact, it has been shown that multiple nucleotide differences do not always produce differences in electrophoretic mobility (Jackson et al. 2000). Moreover, amplicons obtained from two different species can migrate at a similar position even if their sequences are largely dissimilar (Muyzer et al. 1993; Sekiguchi et al. 2001). In the present study, DGGE and sequence analyses demonstrated that the latter case is the one applying to the *Fusarium* species having similar migration behaviour. To overcome these limitations, bands migrating at positions that are known to be common to more than one species potentially present in a sample, should always be excised and sequenced. If the resulting sequence data seem to be mixed (visualised by superimposed peaks in chromatograms), then the excised band should be cloned and multiple cloned fragments should consequently be sequenced.

In this study, all sequences of excised bands obtained from asparagus plants sampled in a commercial field were exclusively related to the three main *Fusarium* species found in asparagus fields (*F. oxysporum*, *F. proliferatum* and *F. solani*), supporting the specificity of the primer-pair used. Bands observed among these samples were mostly related to *F. oxysporum* and *F. proliferatum*, that are the most abundant *Fusarium* species usually isolated from asparagus fields in northeastern America (Caron et al. 1985; Damicone and Manning 1985; Elmer 2001). It is

interesting to note that while *F. oxysporum* shows variation in genetic diversity, vegetative compatibility, and pathogenecity in fields (Alves-Santos et al. 1999; Elmer and Stephens 1989; LaMondia and Elmer 1989), band migratory positions and sequences retrieved were also variables. In contrast, Elmer (1995b) isolated only one mating population of *F. proliferatum* from asparagus fields in Connecticut, Massachusetts and Michigan, and likewise, *F. proliferatum*-associated bands from different isolates, including asparagus samples, migrated to a single position and sequences showed little variation.

Undoubtedly, the method described here was highly efficient to distinguish and identify *Fusarium* isolates in pure culture, as well as to assess *Fusarium* species present in asparagus plant samples. However, using this approach in a different ecological system will need first an exploratory step where all bands should be excised and sequenced. Thereafter, species differentiation should be visually possible for most bands, except when a migration position is known to be common to more than a single species. Pure isolates of the dominant *Fusarium* species expected to be present in the samples should also be tested in a DGGE assay, in order to point out potential biases. With other unknown environmental samples from a similar environment, the approach described here should then permit to visualise the *Fusarium* taxa diversity and to process rapidly and at a low cost large numbers of samples. Previously published PCR-DGGE studies targeting the whole fungal kingdom were unable to detect the presence of *Fusarium* species in environmental samples where they were expected to be present (Kowalchuk et al. 1997; Smit et al. 1999). Non-detection of *Fusarium* species with fungal primers is problematic as they

are among the most economically important pathogenic species, almost ubiquitous, and play an important role in ecosystem equilibrium.

In conclusion, the molecular-based approach developed in this study permitted to clearly differentiate the 19 *Fusarium* species tested, and to identify *in planta* different species of *Fusarium*. This technique could therefore be used to assess *Fusarium* community composition directly from asparagus plant samples, without the need for isolation and culture.

2.6 Acknowledgments

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2.7 Table

TABLE I. *Fusarium* isolates, heterogeneous fungi, bacteria and plant species used in this study

Taxon	Isolate/ Cultivar	Host/Substrate (Origin)	Source[1]
Fusarium			
F. acuminatum	MT-F155	Asparagus (Canada)	IRBV
F. avenaceum	MT-F150	Asparagus (Canada)	IRBV
F. chlamydosporum	T-0773	Chickpea seeds (USA)	FRC
F. equiseti	MT-F151	Asparagus (Canada)	IRBV
F. fujikuroi (C tester)	M-1149	Rice (Taiwan)	FRC
F. fujikuroi (C tester)	M-6884	Rice (Taiwan)	FRC
F. globosum (type)	M-8014	Corn kernels (S. Africa)	FRC
F. globosum	M-7543	Corn kernels (S. Africa)	FRC
F. lateritum	MT-F140	Asparagus (Canada)	IRBV
F. nygamai	M-1374	Sorghum (Australia)	FRC
F. oxysporum	L-6886-44	NA	U.G.
F. *o.* f. sp. *asparagi*	MT-#147	Asparagus (Canada)	IRBV
F. o. f. sp. *asparagi*	MT-F142	Asparagus (Canada)	IRBV
F. o. f. sp. *asparagi*	FOA50	Asparagus (USA)	CAES
F. o. f. sp. *dianthi*	64922	Carnation (The Netherlands)	ATCC
F. o. f. sp. *phaseoli*	18131	Bean (USA)	ATCC
F. poae	T-0919	Corn (USA)	FRC
F. proliferatum	MT-F141	Asparagus (Canada)	IRBV
F. proliferatum	MT-F147	Asparagus (Canada)	IRBV
F. proliferatum	MT-F145	Asparagus (Canada)	IRBV
F. proliferatum	MT-F153	Asparagus (Canada)	IRBV
F. proliferatum	N-7268-84	NA	U.G.
F. proliferatum	95WT	Asparagus (USA)	CAES
F. proliferatum	53WT	Asparagus (USA)	CAES
F. sacchari	M-8399	*Musa sapientum* (Honduras)	FRC
F. sambucinum	MT-F148	Asparagus (Canada)	IRBV
F. sambucinum	MT-F149	Asparagus (Canada)	IRBV
F. semitectum	MT-F144	Asparagus (Canada)	IRBV
F. solani	MT-F240	Asparagus (Canada)	IRBV
F. solani	MT-F116	Asparagus (Canada)	IRBV
F. solani	UG-31 b.3	NA	U. G.
F. sporotrichioides	UG #6	NA	U. G.
F. sporotrichioides	T-0572	Winter wheat (Canada)	FRC
F. subglutinans	M-1351	Corn (Zambia)	FRC
F. subglutinans (E tester)	M-3696	Corn (USA)	FRC
F. subglutinans	UG #3	NA	U.G.
F. sulphureum	MT-F146	Asparagus (Canada)	IRBV
F. verticillioides	M-5331	Rice stem (China)	FRC

F. verticillioides	M-1325	Corn (S. Africa)	FRC
Fusarium sp.	MT-F115	Asparagus (Canada)	IRBV
Other fungi			
Alternaria alternata	F-005	NA (Canada)	McGill
Aspergillus niger	F-204	NA (Canada)	McGill
Botrytis cinerea	F-014	NA (Canada)	McGill
Ceuthospora sp.	MT-F235	*Pinus* sp.(Canada)	IRBV
Chaetomium globosum	MT-F243	*Thuja occidentalis* (Canada)	IRBV
Colletotrichum gloeosporioides	TB 44313	French bean	McGill
Coniothyrium sp.	MT-F241	Soil (Canada)	IRBV
Cylindrocladium floridanum	MT-F101	Soil (Canada)	IRBV
Epicoccum nigrum	MT-F234	*Pinus* sp. seeds (Canada)	IRBV
Geniculosporium sp.	MT-F245	Soil (Canada)	IRBV
Mucor sp.	MT-F114	Soil (Canada)	IRBV
Neurospora crassa	MT-F248	Soil (Canada)	IRBV
Penicillium sp.	MT-F236	Soil (Canada)	IRBV
Pestalotiopsis funurea	MT-F246	*Picea mariana* (Canada)	IRBV
Phialocephalia victorinii	MT-F238	Soil (Canada)	IRBV
Phytophthora nicotianae	13196	Tomato	ATCC
Pseudorobillarda monica	MT-F233	Soil (Canada)	IRBV
Pythium ultimum	N1	Grassland soil (USA)	McGill
Rhizoctonia solani	76107	Soil (USA)	ATCC
Sphaeropsis sapinea	MT-F237	*Pinus* sp. (Canada)	IRBV
Stachybotrys elegans	F-013	NA	McGill
Stemphylium vesicarium	MT-F247	Asparagus (Canada)	IRBV
Trichoderma harzianum	35433	(Canada)	CBS
Trichoderma viride	MT-F239	Soil (Canada)	IRBV
Ulocladium sp.	MT-F111	Airborne (Canada)	IRBV
Bacteria			
Agrobacterium sp.	A3	NA	McGill
Enterobacter cloacae	ECL-2	NA	McGill
Pseudomonas aureofaciens	63-28	NA	McGill
Rhizobium leguminosarum	ON1-2	NA	McGill
Serratia plymuthica	R1GC4	NA	McGill
Streptomyces coelicolor	A3(2)	NA	McGill
Plants			
Asparagus officinalis	Guelph Millenium	-	IRBV
Cucumis sativus	NA	-	McGill
Phaseolus vulgaris	UT 15	-	McGill
Solanum tuberosum	Contender Black Burban	-	McGill

Zea mays	Pionneer (hybrid 3921)	-	McGill

[1] **IRBV** : Institut de recherche en biologie végétale, Université de Montréal, Montréal, Qc, Canada. **FRC**: Fusarium Research Center, Pennsylvania State University, Philadelphia, PA, U.S.A. **CAES**: Dr. W.H. Elmer, Connecticut Agricultural Experimental Station, New Haven, CT, U.S.A. **U. G.**: Dr. L. Tamburic-Ilincic, University of Guelph, Guelph, On., Canada. **ATCC**: American Type Culture Collection, Manassas, VA., U.S.A., **McGill**: Dr. S.H. Jabaji-Hare, McGill University, Montreal, Quebec, Canada. **CBS**: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. **NA**: not available.

2.8 Legend of figures

FIG. 1: DGGE separation of amplicons of a portion of the EF-1 gene for 39 *Fusarium* isolates. **A**) Lanes 1 and 16—Molecular markers composed of (from top to bottom) *F. oxysporum* f. sp. *asparagi* (FOA50), *F. subglutinans* (UG #6), *F. sambucinum* (MT-F148), *F. solani* (MT-F240); 2—*F. oxysporum* f. sp. *asparagi* (FOA50); 3—*F. oxysporum* f. sp. *phaseoli* (18131); 4—*F. poae* (T-0919); 5—*F. oxysporum* f. sp. *dianthi* (64922); 6—*F. fujikuroi* (M-1149); 7—*F. fujikuroi* (M-6884); 8—*F. oxysporum* (L-6886-44); 9—*F. oxysporum* f. sp. *asparagi* (MT-147); 10—*F. subglutinans* (UG #3); 11—*F. sacchari* (M-8399); 12—*F. oxysporum* f. sp. *asparagi* (MT-F142); 13—*F. chlamydosporum* (T-0773); 14—*F. sporotrichioides* (UG #6); 15—*F. sporotrichioides* (T-0572). **B**) Lanes 1 and 15: Molecular markers; 2—*F. nygamai* (M-1376); 3—*F. subglutinans* (M-1351); 4—*F. lateritium* (MT-F140); 5—*F. globosum* (M-8014); 6—*F. globosum* (M-7543); 7—*F. sulphureum* (MT-146); 8—*F. subglutinans* (M-3696); 9—*F. proliferatum* (MT-F141); 10—*F. proliferatum* (MT-F145); 11—*F. proliferatum* (95WT); 12—*F. proliferatum* (53WT); 13—*F. proliferatum* (MT-F147); 14—*F. verticillioides* (M-1325). **C**) Lanes 1 and 15: Molecular markers; 2—*F. avenaceum* (MT-F150); 3—*F. acuminatum* (MT-F155); 4—*F. equiseti* (MT-F151); 5—*F. proliferatum* (N-7268-84); 6—*F. sambucinum* (MT-F148); 7—*F. sambucinum* (MT-F149); 8—*F. proliferatum* (MT-F153); 9—*F. semitectum* (MT-F144); 10—*F. verticillioides* (M-5331);

11—*Fusarium* sp. (MT-F115); 12—*F. solani* (MT-F240); 13—*F. solani* (UG 31 b.3); 14—*F. solani* (MT-F116).

FIG. 2: DGGE separation of 11 cloned fragments of the EF-1 alpha gene from *F. proliferatum* (N 7268-84). Lanes 1 and 13—Molecular markers composed of (from top to bottom) *F. oxysporum* f. sp. *asparagi* (FOA50), *F. subglutinans* (UG #6), *F. sambucinum* (MT-F148), *F. solani* (MT-F240); 2-12: cloned fragments from *F. proliferatum* and amplified with Alfie1-GC/Alfie2 primers pair.

FIG. 3: Unrooted neighbor-joining tree based on sequence analysis of all *Fusarium* isolates used in this study. Values of the bootstrap analysis (1000 repetitions) are given at the nodes.

FIG. 4: DGGE patterns of three healthy and three *Fusarium* root rot symptomatic asparagus plants harvested in a commercial production field. Lanes 1 and 8—Molecular markers composed of (from top to bottom) *F. oxysporum* f. sp. *asparagi* (FOA50), *F. subglutinans* (UG #6), *F. sambucinum* (MT-F148), *F. solani* (MT-F240); 2-4—asymptomatic plants; 5-7—asymptomatic plants. a-g: excised and sequenced bands.

2.9 Figures

FIGURE 1.

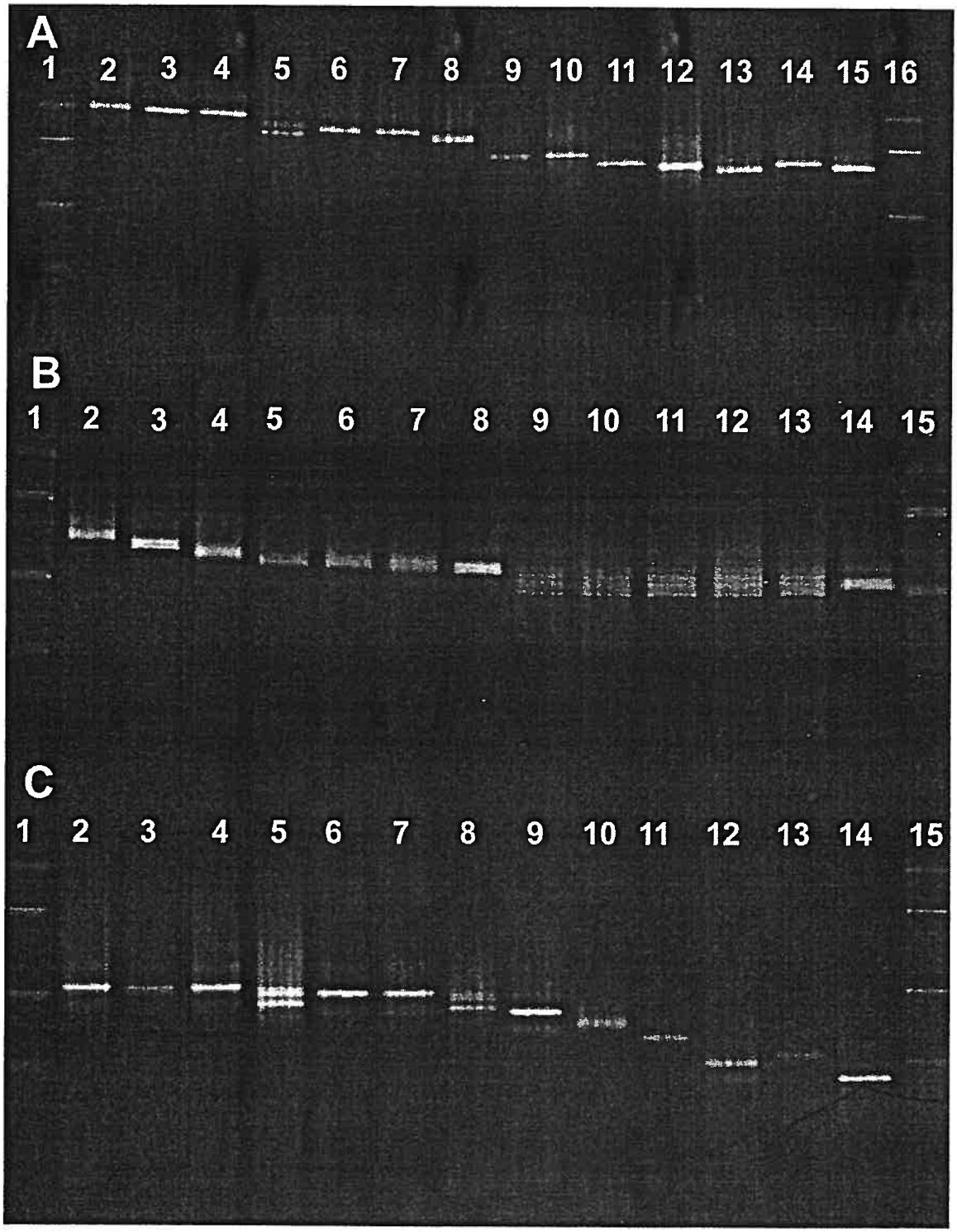


FIGURE 2.

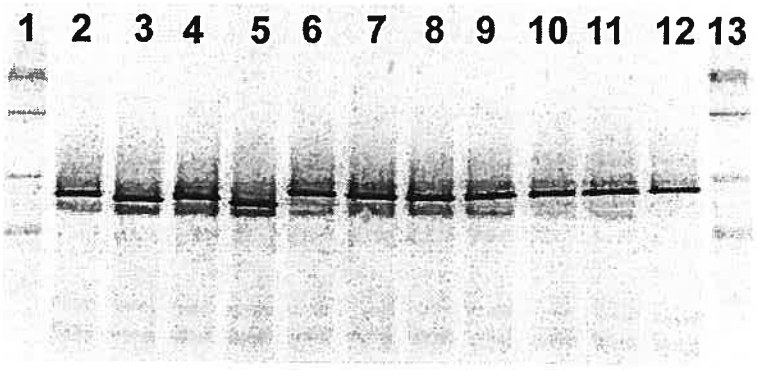


FIGURE 3.

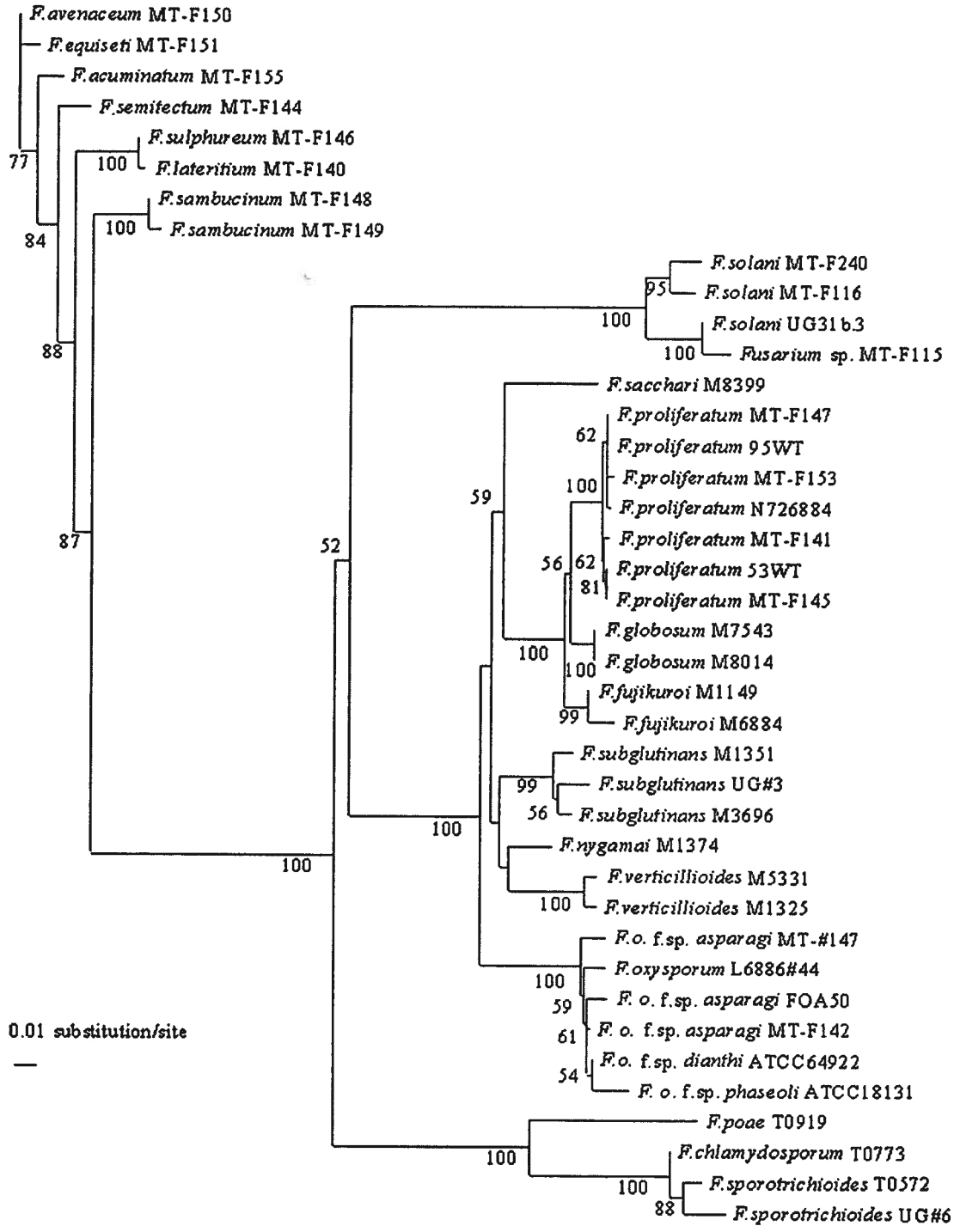
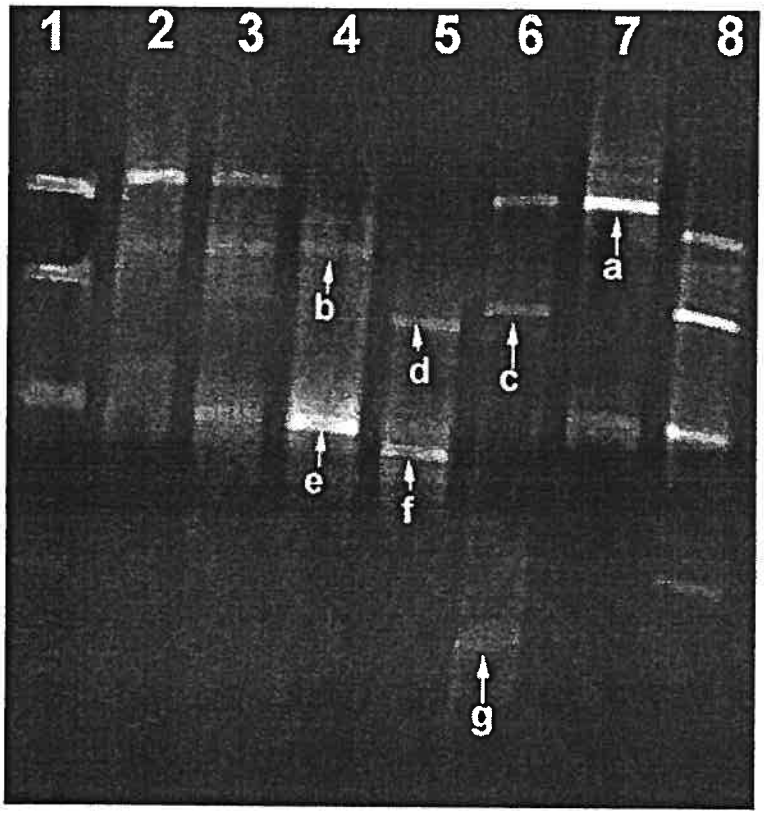

F.avenaceum MT-F150
F.equiseti MT-F151
F.acuminatum MT-F155
F.semitectum MT-F144
F.sulphureum MT-F146
F.lateritium MT-F140
F.sambucinum MT-F148
F.sambucinum MT-F149
F.solani MT-F240
F.solani MT-F116
F.solani UG31b3
Fusarium sp. MT-F115
F.sacchari M8399
F.proliferatum MT-F147
F.proliferatum 95WT
F.proliferatum MT-F153
F.proliferatum N726884
F.proliferatum MT-F141
F.proliferatum 53WT
F.proliferatum MT-F145
F.globosum M7543
F.globosum M8014
F.fujikuroi M1149
F.fujikuroi M6884
F.subglutinans M1351
F.subglutinans UG#3
F.subglutinans M3696
F.nygamai M1374
F.verticillioides M5331
F.verticillioides M1325
F.o. f.sp. asparagi MT-#147
F.oxysporum L6886#44
F. o. f.sp. asparagi FOA50
F. o. f.sp. asparagi MT-F142
F.o. f.sp. dianthi ATCC64922
F. o. f.sp. phaseoli ATCC18131
F.poae T0919
F.chlamydosporum T0773
F.sporotrichioides T0572
F.sporotrichioides UG#6
0.01 substitution/site

FIGURE 4.



Chapitre

3

Short- and long-term changes in *Fusarium* and arbuscular-mycorrhizal fungi communities in asparagus fields following harvest

Le manuscrit qui suit porte sur l'application écologique de l'outil moléculaire décrit au Chapitre 2 et d'une autre méthode de PCR-DGGE précédemment décrite pour les champignons mycorhiziens à arbuscules (CMA). Il met en évidence des fluctuations dans les communautés fongiques (*Fusarium* et CMA) suite à la récolte, au cours d'une saison et pendant plusieurs années. Ce manuscrit sera soumis à une revue avec comité de révision prochainement. Les auteurs sont : Étienne Yergeau, Vladimir Vujanovic et Marc St-Arnaud. Afin de ne pas se répéter, les références relatives à ce manuscrit sont à la fin du mémoire et non pas immédiatement après le manuscrit.

3.1 Abstract

Asparagus (*Asparagus officinalis*) is a high-value perennial vegetable crop that is showing a marked decline in productivity following many years of continuous harvesting. This decline is caused by an increase in both abiotic (autotoxicity, harvesting pressure) and biotic stresses (fungal infections, mainly Fusarium crown and root rot). In the province of Quebec, Canada, harvesting of asparagus begins when the plants are 3-4 years old and coincide with a significant increase in Fusarium crown and root rot incidence. In order to understand changes occurring in microbial communities following the beginning of harvesting, *Fusarium* and arbuscular mycorrhizal fungi species were assessed using PCR-DGGE (denaturing gradient gel electrophoresis) in both short- and long- term experiments. On one hand, a three years old asparagus field was subdivided in plots that were harvested or not, and sampled throughout the growing season to assess short-term dominant species shifts. On the other hand, six commercial fields of ages spreading from 1- to 6- years old (before and after the beginning of harvesting) were sampled to assess long-term fungal communities composition shifts. Results showed that *Fusarium* community composition is varying throughout the growing season and that changes are occurring in a short- and a long-term fashion following the first harvest. Arbuscular mycorrhizal fungi and *Fusarium* communities are showing profound reorganization in regard to the age of fields. Some species are clearly associated with plants of a specific age and almost absent on plants of other ages. Some *Fusarium* taxa are also reported as being inversely linked to various arbuscular mycorrhizal fungi taxa.

3.2 Introduction

Asparagus is a high market value and low-input perennial crop that is very profitable to growers. However, it has been reported for almost a half-century that asparagus fields are showing a decline in profitability and productivity after about ten years of harvesting (Grogan and Kimble 1959). This deterioration leads to a point where the asparagus production is no longer cost-effective and the field has to be destroyed. It is well known that this is mainly the result of increasing biotic and abiotic stresses (Elmer et al. 1996). These former stresses are caused by an intensification of the infections by soil-borne pathogens, mainly belonging to the *Fusarium* genus. In fact, the most important asparagus disease is the Fusarium crown and root rot (Elmer et al. 1996). Although more than twelve different *Fusarium* species have been reported in asparagus fields of North America (Elmer 2001; Vujanovic et al. 2004), the two major causal agents of Fusarium crown and root rot are *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* (Damicone and Manning 1985; Elmer 2001). Both pathogens are ubiquitous in asparagus field soils and colonize crowns and roots of symptomatic as well as symptomless plants (Vujanovic et al. 2004). *F. oxysporum* is more common in young roots while *F. proliferatum* is dominant in almost every other plant parts (Gilbertson and Manning 1983; LaMondia and Elmer 1989).

The main abiotic stresses on asparagus plants are allelopathic compounds (Yang 1982) and excessive harvesting pressure (Shelton and Lacy 1980). In asparagus, a prolongation of the harvesting period induces depletion in carbohydrate contents, reduction in the marketable yields and reduction of storage root dry weight

(Sanders 1985; Shelton and Lacy 1980; Takatori et al. 1970). For harvesting period ranging from 50-70 days, the severity of the depletion of carbohydrates and the time needed for a stabilization increased with longer harvest periods (Sanders 1985). In the province of Quebec, Canada, a mature asparagus plantation (5 years old) is harvested daily for 5 consecutive weeks (Tessier 2003). Despite the relative shortness of the harvest period, the beginning of the harvest increases markedly Fusarium crown and root rot in Quebec's asparagus fields (Hamel et al. 2004). However, no link between *Fusarium* communities (biotic stress) and harvesting (abiotic stress) was clearly established in previous studies.

Arbuscular mycorrhizal fungi were shown to improve growth (number, height and weight of shoots as well as weight of roots) and to reduce *Fusarium* infections in asparagus (Burrows et al. 1990; Chang 1985; Hussey et al. 1984; Matsubara et al. 2001; Pedersen et al. 1991; Wacker et al. 1990b). Growth response and disease protection varied with mycorrhizal species (Chang 1985; Matsubara et al. 2001; Pedersen et al. 1991) and evidences for a shift of these species in asparagus fields were raised (Wacker et al. 1990c). Asparagus plants colonized with arbuscular mycorrhizal fungi were also shown to tolerate greater temperature stress, and the degree of tolerance was again fungal species-dependent (Matsubara et al. 2000). Thus, mycorrhizae seem to alleviate both biotic and abiotic stresses on asparagus plants.

Fusarium and arbuscular mycorrhizal communities changes on a temporal scale have received little attention, partly because isolation and identification of numerous isolates of these organisms is a difficult and highly time consuming task.

Recently developed molecular approaches (Kowalchuk et al. 2002; Yergeau et al. 2004) facilitate such studies. On the other hand, contrasting with other ecological studies, molecular microbial ecology data are only rarely exploited with numerical ecology tools such as multivariate analysis as in Marschner et al. (2001) and in Lindström (2001). Therefore, the general objective of this study was to investigate the short- and long-term changes in *Fusarium* and arbuscular mycorrhizal fungi community composition in asparagus fields after the beginning of the harvest by using molecular fingerprinting and exploratory multivariate analysis. This paper presents the differences between *Fusarium* communities in asparagus fields after the first harvesting along one growing season (short-term), and characterizes the fluctuations of *Fusarium* and mycorrhizal fungi communities in one to six years old asparagus fields (long-term).

3.3 Material and methods

3.3.1. Plant samples: Short-term effects of harvesting

At the beginning of the 2002 growing season, an experimental setup was put in place in a 3-year-old commercial asparagus field that had never been harvested, in St-Liguori (45° 48' N; 73° 26' W). This setup was composed of three blocks that comprised two adjacent rows of about 20 plants each. Within each block, a row was harvested at three occasions (May 22, June 3 and 10) to simulate a first harvest, while the other one was left untreated. Three asparagus plants (*Asparagus officinalis* cv. Guelph Millennium) per treatment per block were sampled May 22 (before harvesting), July 23 (after harvesting) and September 6 (near the end of the growing season) 2002 to cover the whole asparagus growing season. Within a day from sampling, plant shoots, roots, and crowns were separated, washed under tap water, and frozen at –80°C. These plants were assessed for *Fusarium* community composition only. At each sampling date, *Fusarium* crown and root rot incidence was recorded by counting symptomatic and symptomless plants in each plot.

3.3.2. Plant samples: Long-term effects of harvesting

During September 2003, five asparagus fields located in the L'Assomption area (30 km NE of Montreal) and one field located on the Macdonald campus of McGill University (western Montreal island) were sampled. Two fields were 1-year-

old (one year from establishment), two were 3-year-old (just before the beginning of the harvest), one was 5-year-old (first year after harvest) and one was 6-year-old (field in full production), and all were planted with *Asparagus officinalis* cv. Guelph Millenium. In each field, three asymptomatic and three neighbouring plants showing *Fusarium* crown and root rot symptoms were randomly chosen and the root system was cut and brought back to the laboratory. Within a day from sampling, plant feeding roots were washed under tap water and frozen at –80°C. The roots were assessed for both *Fusarium* and arbuscular mycorrhizal fungi communities composition as describe further.

3.3.3. Nucleic acid extraction

Plant roots, shoots and crowns were ground in liquid nitrogen using a mortar and pestle, and total DNA was subsequently extracted from a 200 mg tissue subsample using a DNAeasy plant mini kit (Qiagen, Valencia, CA.) following manufacturer's instructions. The purified DNA was resuspended in 100 µl of elution buffer and stored at -20°C until PCR amplification.

3.3.4. PCR amplifications

All PCRs were carried out in 50 µL volumes containing 5 µL of 10× PCR buffer, 5 µL of each primer (5 µM), 1 µL of 10 mM dNTPs mix, and 1.25 U of *Taq* polymerase (QIAgen). Reaction mixtures were overlaid with 40 µL sterile mineral oil prior to PCR on a PTC-100 thermal cycler (MJ-Research, inc. Waltham, MA). A

nested PCR approach was used to efficiently amplify *Fusarium* DNA from plant samples. The first PCR step was performed using the EF-1/EF-2 primer pair (O'Donnell et al. 1998b). The results of PCR were then diluted (1:1000) and reamplified using Alfie1-GC and Alfie2, as described previously (Chapter 2;Yergeau et al. 2004). A nested approach was also used to amplify arbuscular mycorrhizal fungi DNA from root samples. The NS1 (White et al. 1990)-NS41 (Simon et al. 1992) primer pair was used in the first round of amplification (25 cycles) and the NS31-AM1 primer pair (Helgason et al. 1998) was used for the second round (35 cycles) following a 1:100 dilution.

3.3.5. Denaturing gradient gel electrophoresis (DGGE)

For *Fusarium* amplicons, DGGE analyses were performed with a 40-65% denaturant gradient (100% is defined as 40% formamide and 7 M urea) using a DCode Universal Mutation Detection System (BioRad, Hercules, CA), as described in Chapter 2 and in Yergeau et al. (2004). For arbuscular mycorrhizal fungi amplicons, DGGEs were carried as described in Kowalchuk et al. (2002), but using a 25-50% gradient. Never observed bands were excised from DGGE gels (Yergeau et al. 2004), re-amplified and commercially sequenced (Montreal Genomic Centre, Montreal, Qc, Canada). Sequenced bands were identified by similarity search in GenBank while other bands were identified by comparison to migration position of known organisms (Chapter 2; Yergeau et al., 2004).

3.3.6. Banding pattern analysis

DGGE gels (Fig. 1) were digitized using a Gel Doc imager (BioRad). Pictures were visually inspected and each migrating position was coded as a binary character (presence-absence). Since each migration position usually corresponds to a different species or isolate, the matrix obtained was considered as a species presence-absence matrix for statistical analyses. Band intensity was not accounted for since multiple quantitative biases in DNA extraction and PCR amplification are known to occur (von Wintzingerode et al. 1997). The number of bands visible for each sample assessed diversity.

3.3.7. Statistical analyses

Rare sequence types were excluded from statistical analysis due to the distortion they provoke in correspondence analyses. To analyse *Fusarium* and AMF taxa associations with harvesting, sampling date or field age, a multiple correspondence analysis was generated using the CORRESP procedure of SAS (release 6.12, SAS institute Inc., Cary, NC). In order to analyse *Fusarium* and arbuscular mycorrhizal fungi taxa relations one with another, a principal components analysis with scaling focused on inter-species correlation was run in SAS together with the computation of Pearson correlation coefficients for all pairs of *Fusarium*-AMF species. Analysis of *Fusarium* crown and root rot incidence in harvested and non-harvested asparagus plant plots were done by frequency table analysis using a log linear model in STATGRAPHICS software (STSC Inc., Rockville, MD).

3.4 Results

3.4.1. Short-term effects of harvesting on Fusarium community composition

A typical DGGE gel obtained by amplifying DNA extracted from asparagus plants with *Fusarium* specific primers is shown in Fig. 1. For the purpose of these analyses, plants sampled in May were excluded, as the PCR-DGGE method did not detect any *Fusarium* species for many samples even with a detection limit of 10 pg of *Fusarium* DNA with 100 ng of competing asparagus DNA (data not shown). Harvesting induced a change in plant-associated *Fusarium* taxa between July and September (Fig. 2). DGGE bands identities for all figures are listed in Table I. *F. oxysporum* and a "red" Fusaria complex species (FT03 and FT09) showed a close association with plant that were submitted to harvest while *F. solani* (FT15) was more closely associated with non-harvested plants. Three different isolates of *F. oxysporum* (FT02, FT06 and FT07) were related to plants sampled in September and were almost absent for the other sampling date. In contrast, *F. oxysporum*, a "red" Fusaria complex species and *F. solani* (FT05, FT08 and FT13) were more often present on plants sampled in July. The plant organ (roots or shoots) from which community composition was assessed had no strong influence on the *Fusarium* taxa repartition in the two dimensions represented in Fig 2. In further analyses, the correspondence analysis was restricted to the first axis (roughly equivalent to time) and harvested plants were separated from non-harvested plants. In this analysis, a band (FT12; identified as closely related to *F. lateritium*) was found to be closely

associated to plants sampled in July for harvested plants, but on the contrary, it was associated with plants sampled in September for non-harvested plants. All other species were associated similarly to sampling dates for harvested and non-harvested plants. *Fusarium* diversity (number of bands) in harvested and non-harvested plants was constant throughout the growing season (data not shown).

3.4.2. Short-term effects of harvesting on crown and root rot incidence

Harvesting significantly increased the number of symptomatic asparagus plants at the end of the growing season (Table III). In May, when no harvesting had occurred, *Fusarium* crown and root rot incidence is identical in all plots. In July, some differences are being visible between plots, but they are not statistically significant. However, in September, harvested plants are showing significantly more symptoms than non-harvested plants.

3.4.3. Long-term effects of harvesting on AMF and Fusarium communities composition

Asparagus plants having dissimilar ages were harbouring different *Fusarium* communities (Fig. 3). *F. oxysporum* (FT27) was more closely associated with the 1-year-old fields, while *F. proliferatum* and *F. solani* (FT29 and FT32) were associated with the 3-year-old fields, and two *F. oxysporum* isolates (FT26 and FT23) were respectively the most closely associated taxa with the 5- and 6-year-old fields. Similarly, some *Fusarium* taxa were more closely associated to asymptomatic plants

(FT29=*F. proliferatum*) or symptomatic plants (FT24=*F. oxysporum* and FT33=*F. solani*).

Arbuscular mycorrhizal fungi communities associated to asparagus roots also showed changes throughout the years (Fig. 4). In the 1-year-old fields, the most closely associated taxon was *Glomus* sp. (AT07). In the 3-year-old fields, it was *Scutellospora* sp. and a non-Glomus AMF species (AT08 and AT09; BLAST results did not allow us to unambiguously identify taxa AT09 to the genera level) and a *Glomus* sp. (AT03). For the 5-year-old field, the association was with *G. intraradices* (AT01) and with an unidentified *Glomus* sp. (AT04). The plants growing in the 6-year-old-field are harbouring communities similar to the 3-year-old fields as visualized by the small angle between those two vectors in Fig. 4. There was no strong difference between asymptomatic and symptomatic plants with regard to the associated mycorrhizal taxa.

Following a principal component analysis, it appeared that some *Fusarium* and AMF isolates were linked together positively or negatively (Fig. 5). Negative links were visualized by the opposition of the species vectors in Fig. 5 and by strong negative Pearson correlation coefficient (result not shown). The strongest opposition following both analyses were between *F. oxysporum-Glomus intraradices* (FT22-AT01), *F. oxysporum-Glomus* sp. (FT25-AT03), *F. oxysporum-Scutellospora* sp. (FT25-AT08), "red" *Fusarium* species-*Glomus fasciculatum* (FT28-AT02), *F. solani-Glomus* sp. (FT31-AT01) and *F. solani-Glomus* sp. (FT31-AT04).

3.4.4. Long-term effects of harvesting on Fusarium and AMF diversity

Fusarium species diversity (number of visible bands) showed a significant increase from the 1-year-old plants to the 3-year-old plants (Fig. 6). After the third year of asparagus growing, *Fusarium* diversity in plants declined, as the diversity observed in the 5-year-old plants was comparable to the first year diversity. In the six-year-old plants, variation between samples hampered the drawing of any conclusion, as diversity was not significantly different from any other year sampled. Arbuscular mycorrhizal taxa diversity (Fig. 6) was statistically comparable for every age. In opposition, mycorrhizal taxa diversity was significantly (α=0.05) higher in symptomatic plants when compared to asymptomatic plants for the 5-year-old fields (data not shown). No difference was observed between symptomatic and asymptomatic plants in regard to *Fusarium* diversity (data not shown). *Fusarium* and AMF diversity were only weakly correlated at 29.4%.

3.5 Discussion

Short- and long-term reorganization in the composition of *Fusarium* and arbuscular mycorrhizal fungal communities following the beginning of the harvest in asparagus fields are shown in the present paper. To the best of our knowledge this is the first observation of such shifts in fungal community composition associated to asparagus. The effects of harvest stress on asparagus plant physiology have been well studied, but the associated fungal communities have been overlooked. Still, it makes sense that changes in plant physiology will affect fungal communities inhabiting that plant.

Short-term changes were observed in field plots submitted to a first harvest as *Fusarium* taxa associated with asparagus roots, crowns and shoots were different than those associated with the non-harvested plants (Fig. 2). Harvesting not only induces a change in the physiology of the asparagus (Sanders 1985; Shelton and Lacy 1980) but it also offers a clear entryway for fungi. The initial harvest simulated here has possibly lowered the asparagus content in carbohydrates that in turn affected competition within *Fusarium* communities. The entryway created by harvesting might have permitted the presence of some *Fusarium* taxa that otherwise would have been absent. Moreover, physiologically weaker and wounded plants are less efficient to defend themselves against fungal pathogens and, in fact, a significant increase of *Fusarium* crown and root rot symptomatic plants was observed in harvested plots at the end of the growing season. The shift observed in *Fusarium* communities is possibly partly responsible for that increase or conversely, the increase in disease

incidence might be responsible for *Fusarium* communities shift by allowing some isolates to proliferate abundantly in the weakened plant.

Although not related to harvesting, a seasonal succession of *Fusarium* species was observed in asparagus fields (Fig. 2). Schreuder et al. (1995) also observed a shift in incidence of *Fusarium* species in asparagus fields throughout a growing year. *Fusarium* community in wheat fields was also fluctuating in relation to the sampling date (Bateman and Murray 2001). *Fusarium* is a large genus wherein every species and even isolates have ecological preferences (Burgess 1981; Elmer et al. 1997). Seasonal variation in precipitation and temperature that are occurring under temperate climate can also contribute to the changes in community composition observed for *Fusarium*.

On a larger time scale, harvesting also influenced *Fusarium* community composition (Fig.3). This second experiment was designed to assess long-term changes in *Fusarium* community by comparing plants before (1- and 3-year-old) and after (5- and 6-year old) the beginning of the harvest. As the incidence of *Fusarium* crown and root rot is increasing monotonically with the age of the field (Hamel et al. 2004), it is possible that the taxa observed in older fields are more detrimental to asparagus health than taxa observed in young fields. It is as well likely that this switch in community composition is at least partly related to harvest. This change in communities is adding-up with other soil problems that affect aged asparagus plants such as allelopathic compounds build-up (Yang 1982), increasing pH or decreasing available phosphorus concentration (Wacker et al. 1990c).

Arbuscular mycorrhizal community composition was also affected by the age of the field (Fig. 4). This is in line with the succession of AMF species observed in asparagus fields of Michigan (Wacker et al. 1990c). Once more, the observed increase in Fusarium crown and root rot incidence in older asparagus fields might be partly related to the variation observed here as arbuscular mycorrhizal species are showing different efficiencies to inhibit this disease (Matsubara et al. 2001). On the other hand, it is well known that yields and plant vigour are normally declining following continuous monoculture. It was suggested that a yield decline in corn and soybean is partly caused by shifts in AMF communities (Johnson et al. 1992). Asparagus cropping is quite similar to continuous monoculturing since the same crop is grown for many years in the same field and, thus, asparagus decline might as well be related to AMF succession. Since AMF community composition is quite similar in 3- and 6-years-old fields it is less likely that the shift observed here is only related to harvest. Other environmental factors such as soil characteristics and allelopathic compounds might have also influenced the succession observed.

As suggested by our results (Fig. 5), some arbuscular mycorrhizal species are rarely found in association with particular *Fusarium* isolates. Close association of these taxa with plants of different ages or other factors (ex: soil factors, other microbial communities) might cause these negative relationships. They may also indicate direct antagonistic relationships between arbuscular mycorrhizal fungi and *Fusarium* species, relationships that are known to happen (Filion et al. 1999).

For arbuscular mycorrhizal fungi, we reported a low, equal diversity for all ages of fields (Fig. 6). The equal diversity observed in our fields was also observed in

Michigan fields having contrasting ages where species showed abundance peaks at regular time intervals but without changing the total biodiversity or dominance index (Wacker et al. 1990c). Moreover, a decrease followed by an increase of AMF biomass in asparagus fields has been reported (Hamel et al. 2004). Therefore, it seems that arbuscular mycorrhizal fungi diversity in asparagus fields is stable but that species composition is varying which in turn probably affects biomass.

On the other hand, *Fusarium* diversity in asparagus plants is higher in 3-year-old plants while it is lower for other ages (Fig. 6). Interestingly, it appears that diversity in *Fusarium* is low when plants are installed in field (1st year) and it increases gradually until plants are submitted to harvest (4th year) and then decreases in the following years. Here again, community composition is different even in plants showing a similar diversity. It is also interesting to note that no short-term changes in *Fusarium* diversity were observed in asparagus fields.

Numerous *Fusarium* taxa were observed in plant samples (up to 15 different migration positions). This confirmed the diversity previously observed when sixteen species of *Fusarium* were found in asparagus fields in the province of Quebec, Canada (Vujanovic et al. 2004). In the asparagus production area under study (great Montréal region), a clear dominance of *F. oxysporum* and *F. proliferatum* with minor companion species was observed using culture-dependent methods (Vujanovic et al. 2004). Since the molecular fingerprinting method used here portrays mainly the most abundant sequences types present, the majority of the taxa detected were *F. oxysporum* and *F. proliferatum* (Table I). Interestingly, *F. oxysporum* was showing a high variability in his migration positions. High numbers of VCGs (vegetatively

compatible groups) has also been observed in *F. oxysporum* f.sp. *asparagi* but no link could been made between pathogenecity and vegetative compatibility (Elmer and Stephens 1989). Our results indicated an annual and a seasonal succession of different sequences type associated to *F. oxysporum* following harvest. Although the DGGE analyses of the EF-1 alpha gene do not permit to support such conclusion, this succession is perhaps biased toward more virulent *F. oxysporum* isolates and might be one of the causes of the short- and long-term increase in Fusarium crown and root rot incidence following harvest.

F. proliferatum was present in almost all samples from the short-term experiment but at a lower rate in the samples related to contrasting ages fields. This might be related to the size of roots from which DNA was extracted. Young feeding roots were sampled in that experiment, while samples from all plant parts were evaluated in the short-term experiment as mycorrhizal fungi are rarely detected in older storage roots. It is known that *F. oxysporum* is usually the main fungus in young asparagus roots (Elmer et al. 1996) and it may have masked *F. proliferatum* in some samples. All other samples were crowns, storage roots, and shoots where both species seem to be equally present. *F. proliferatum* showed no diversity in regard to migration position and a single mating population might be present in the sampled asparagus fields as observed by Elmer (1995b) in Connecticut, Massachusetts and Michigan. We observed an association of *F. proliferatum* with asymptomatic plants in the study related to long-term effects of harvesting (sp9, Fig. 3). This is somehow surprising but a closer look at raw data indicated that the species was present on both diseased and healthy plants and that a relatively weaker association of this species

with diseased plants (6 presences in diseased plants vs. 9 in healthy plants) caused this grouping.

F. solani was detected in numerous samples and was also showing an large variation in migration behavior. Worldwide, *F. solani* was isolated at frequencies varying between 2 and 20 % of the total isolates from asparagus fields (Elmer et al. 1997; LaMondia and Elmer 1989; Schreuder et al. 1995). *F. solani* has an unclear role in crown and root rot but it has been reported as a weak pathogen to asparagus (Schreuder et al. 1995). In contrast, some isolates were even reported to protect asparagus from pathogenic *Fusarium* spp. (Damicone and Manning 1982). The low frequency of isolation and pathogenicity of *F. solani* suggests a minor role in the crown and root rot disease. However, in the present study, an isolate of *F. solani* (FT33) was found closely associated with a *F. oxysporum* isolate (FT22) in symptomatic plants. It is therefore possible that some *F. solani* strains might be involved directly or indirectly in pathogenesis.

Fusarium species producing carmine red pigments on potato dextrose agar growth media ("*Gibberella pulicaris* complex" and allied species) were also detected in some samples. *F. culmorum*, a member of this group, is a major pathogen in asparagus fields in Europe (Blok and Bollen 1995) but it was not found in our samples, even if it is present in asparagus fields in the province of Québec (Vujanovic et al. 2004). Many bands having dissimilar migration patterns were identified as close to *F. lateritium*, also a member of this complex. Since the limits between two species based on DNA sequences is not always clear, those bands could represent closely related species. Nevertheless, a species closely related to *F.*

lateritium was present at different sampling dates depending if it was on harvested or non-harvested plants. This might indicate that non-pathogenic species are also affected by the harvest and this in turn affects competition for infection sites or soil resources.

In this paper, dynamics of two fungal communities were characterized in asparagus fields and linked with harvesting and Fusarium crown and root rot development. Community composition shifts were observed rapidly after the first harvesting and also in a long-term fashion. The increase of Fusarium crown and root rot in asparagus fields following the beginning of harvest is most probably linked to *Fusarium* community composition and might also be depending on other fungal communities such as arbuscular mycorrhizal fungi and environmental factors. Perhaps the observed shifts in species composition are not favorable to asparagus as more pathogenic *Fusarium* isolates and less effective arbuscular mycorrhizal fungi would be present in aged fields where the abiotic stresses are building-up. Others studies with a finer scale (sampling fields of every age), with older fields (up to more than 15 years old) and by comparing different cultivars should be conducted in order to verify if communities are effectively changing toward less effective or more virulent species. The molecular method used will permit such large studies as, when optimized, more than 100 samples can be assessed in a week by a single experimentator. The correlation between soil factors, crown and root rot and *Fusarium* communities is presently analysed more throughoutly for geographically distinct regions of the province of Quebec.

3.6 Acknowledgements

This work was supported by a NSERC grant to M. St-Arnaud and a CORPAQ-PAR team grant. Financial assistance to E. Yergeau was provided by a FQRNT postgraduate scholarship. Stéphane Daigle is thanked for statistical analysis of data. We also thank Stéphane Roy, Michel Cormier and Guy Champagne for letting us sample their fields and David W. Sommerville for help in the fieldwork.

3.7 Tables

TABLE I. Identity for bands observed in DGGE gels as determined by a BLAST search of sequenced bands or by comparison to migration positions of known taxa.

Taxa	Band	Identity	Determination[1]	Acc #, similarity[2]
Fusarium	FT01	NSM[3]	BLAST	-
Short-term	FT02	*F. oxysporum*	Fig. 1A, Chap 2	-
	FT03	*F. oxysporum*	BLAST	AF008484, 97%
	FT04	*F. oxysporum*	BLAST	AY337434, 93%
	FT05	*F. oxysporum*	BLAST	AY337428, 97%
	FT06	*F. oxysporum*	BLAST	AF246887, 97%
	FT07	*F. oxysporum*	BLAST	AY337437, 97%
	FT08	Red *Fusarium* complex[4]	Fig. 1A, Chap 2	-
	FT09	Red *Fusarium* complex	Fig. 1A, Chap 2	-
	FT10	*F. lateritium*	BLAST	AY337435, 98%
	FT11	*F. proliferatum*	BLAST	AY337420, 97%
	FT12	Red *Fusarium* complex	BLAST	AY337435, 82%
	FT13	*F. solani*	Fig. 1C, Chap 2	-
	FT15	*F. solani*	BLAST	AF178355, 97%
Fusarium	FT22	*F. oxysporum*	Fig. 1A, Chap 2	-
Long-term	FT23	*F. oxysporum*	BLAST	AF008484, 97%
	FT24	*F. oxysporum*	BLAST	AY337434, 93%
	FT25	*F. oxysporum*	Fig. 1A, Chap 2	-
	FT26	*F. oxysporum*	BLAST	AY337428, 97%
	FT27	*F. oxysporum*	BLAST	AF246887, 97%
	FT28	*F. oxysporum*	BLAST	AY337437, 97%
	FT29	*F. proliferatum*	BLAST	AY337420, 97%
	FT30	Red *Fusarium* complex	BLAST	AY337435, 82%
	FT31	*F. solani*	Fig. 1C, Chap 2	-
	FT32	*F. solani*	Fig. 1C, Chap 2	-
	FT33	*F. solani*	BLAST	AF178355, 97%
AMF	AT01	*Glomus intraradices*	BLAST	X58725, 99%
	AT02	*Glomus fasciculatum*	BLAST	Y17640, 99%
	AT03	*Glomus* sp.	BLAST	AY512347, 82%
	AT04	*Glomus* sp.	BLAST	AF074353, 99%
	AT07	*Glomus* sp.	BLAST	AF074370, 98%
	AT08	*Scutellospora gilmorei*	BLAST	AJ276094, 99%
	AT09	Non-*Glomus* AMF[5]	BLAST	-

1: Method (BLAST) or picture of the DGGE gel used to retrieve band identity. See materials and methods for details.

2: GenBank accession number and similarity of the closest match

3: No Significant Match. Sequencing and BLAST search of the DNA extracted from that position did not give any significant match

4: Complex of *Fusarium* species producing carmine red pigments on potato dextrose agar. BLAST search results and migration comparison did not enabled us to identify the members of this complex to the species level.

5: BLAST search results did not enabled us to unambiguously identify the sequence to the genus level

TABLE II. Frequency of observation by PCR-DGGE of *Fusarium* taxa in asparagus plants sampled in harvested or non-harvested plots for three sampling dates

Taxa[1]	May			July			September			All dates			
	NH[2]	H	All	NH	H	All	NH	H	All	NH	H	All	%[3]
FT01	0	0	0	1	0	1	1	3	4	2	3	5	1.41
FT02	0	0	0	1	0	1	5	4	9	6	4	10	2.82
FT03	1	0	1	7	9	16	8	11	19	16	20	36	10.14
FT04	2	2	4	9	8	17	9	6	15	20	16	36	10.14
FT05	1	0	1	4	7	11	2	1	3	7	8	15	4.23
FT06	0	0	0	1	0	1	11	9	20	12	9	21	5.92
FT07	0	0	0	1	0	1	10	9	19	11	9	20	5.63
FT08	3	0	3	6	1	7	1	0	1	10	1	11	3.10
FT09	2	5	7	5	9	14	5	11	16	12	25	37	10.42
FT10	1	4	5	8	10	18	11	12	23	20	26	46	12.96
FT11	2	5	7	8	10	18	17	18	35	27	33	60	16.90
FT12	1	3	4	6	11	17	11	6	17	18	20	38	10.70
FT13	0	0	0	7	5	12	1	0	1	8	5	13	3.66
FT15	0	0	0	2	1	3	2	2	4	4	3	7	1.97
Sums	13	19	32	67	72	137	94	92	186	173	182	355	100

1: Taxa are numbered according to Table 1

2: NH: Non-harvested, H: Harvested

3: Relative abundance was calculated as follows: (nb. of bands observed at this position /total nb. of bands observed for all samples in that experiment) X 100.

TABLE III. *Fusarium* crown and root rot incidence in harvested and non-harvested asparagus field plots located in St-Liguori for the three sampling dates.

Date	Treatment	Symptomatic plants	Asymptomatic plants	Z score[1]
May	No harvest	0	42	
	Harvest	0	55	0.13
July	No harvest	9	63	
	Harvest	18	59	1.70
September	No harvest	4	49	
	Harvest	14	35	2.61

1: When Z score $\geq$ 1.96, there was a statistically significant effect of harvesting on the number of asymptomatic and symptomatic plants ($P<0.05$), following a Frequency Table Analysis using a log linear model.

TABLE IV. Frequency of observation by PCR-DGGE of *Fusarium* and AMF taxa in symptomatic and asymptomatic asparagus plant roots in fields of contrasting ages

Taxa[1]	Field (age)												All fields	
	1 (1)		2 (1)		3 (3)		4 (3)		5 (5)		6 (6)		Freq.	%[2]
	S[3]	A	S	A	S	A	S	A	S	A	S	A		
FT22	1	0	2	0	0	1	1	1	0	0	3	1	10	5.95
FT23	1	0	2	0	2	3	3	1	0	0	3	2	17	10.12
FT24	2	2	3	2	2	3	3	2	3	3	3	2	30	17.86
FT25	0	1	2	1	0	1	3	2	3	2	0	2	17	10.12
FT26	1	2	0	0	2	3	0	0	2	3	0	1	14	8.33
FT27	2	2	2	2	2	2	0	0	3	3	2	0	20	11.90
FT28	1	0	0	1	1	3	0	0	0	1	0	0	7	4.17
FT29	1	0	0	3	2	3	3	2	0	1	0	0	15	8.93
FT30	0	2	1	2	0	0	1	1	0	2	2	3	14	8.33
FT31	1	0	0	0	1	1	1	2	0	0	0	3	9	5.36
FT32	0	0	0	0	2	1	0	2	0	0	0	3	8	4.76
FT33	1	0	1	1	0	0	2	1	0	0	1	0	7	4.17
Sums	11	9	13	12	14	21	17	14	11	15	14	17	168	100
AT01	0	0	0	1	0	2	0	0	2	1	0	0	6	7.89
AT02	2	3	3	3	2	2	3	2	2	3	3	3	31	40.79
AT03	0	0	0	0	2	2	0	0	0	0	2	0	6	7.89
AT04	1	1	1	0	0	0	1	0	2	0	0	0	6	7.89
AT07	0	0	2	3	0	1	0	0	0	0	0	0	6	7.89
AT08	0	0	0	0	2	0	0	0	0	0	1	0	3	3.95
AT09	2	2	1	0	1	2	3	2	0	0	3	2	18	23.68
Sums	5	6	7	7	7	9	7	4	6	4	9	5	74	100

1: Taxa are numbered according to Table 1

2: %: relative abundance was calculated as follows: (nb. of bands observed at this position /total nb. of bands observed for all samples in that experiment) X 100

3: S: Symptomatic plants, A: Asymptomatic plants

3.8 Legend of figures

FIG. 1: Typical DGGE gel obtained from asparagus plants sampled in the harvested and non-harvested plots in May and July. The far left and far right lanes contain a molecular marker composed of four *Fusarium* species: (from top to bottom) *F. oxysporum* f. sp. *asparagi* (FOA50), *F. subglutinans* (UG #6), *F. sambucinum* (MT-F148), *F. solani* (MT-F240). Band numbering is shown here as an example and the correspondences are found in Table I.

FIG. 2: Multiple correspondence analysis of *Fusarium* taxa detected by PCR-DGGE in July and September 2002 in harvested or non-harvested asparagus plants. See Table I for the meaning of species numbers. R=Roots, S=Shoots, H=Harvested, NH=Non-Harvested.

FIG. 3: Multiple correspondence analysis of *Fusarium* species detected by PCR-DGGE in roots of asparagus plants of different ages. See Table I for the meaning of species numbers.

FIG. 4: Multiple correspondence analysis of arbuscular mycorrhizal fungi detected by PCR-DGGE in roots of asparagus plants of different ages. See Table I for the meaning of species numbers.

FIG. 5: Principal components analysis of *Fusarium* (o) and AMF (•) species detected by PCR-DGGE in roots of asparagus plants of different ages. See Table I for the meaning of species numbers.

FIG. 6: Diversity (number of bands in DGGE gels) in relation with the age of the fields for *Fusarium* and AMF species detected in roots of asparagus plant samples. Errors bars represent 95 % confidence intervals.

3.9 Figures

FIGURE 1.

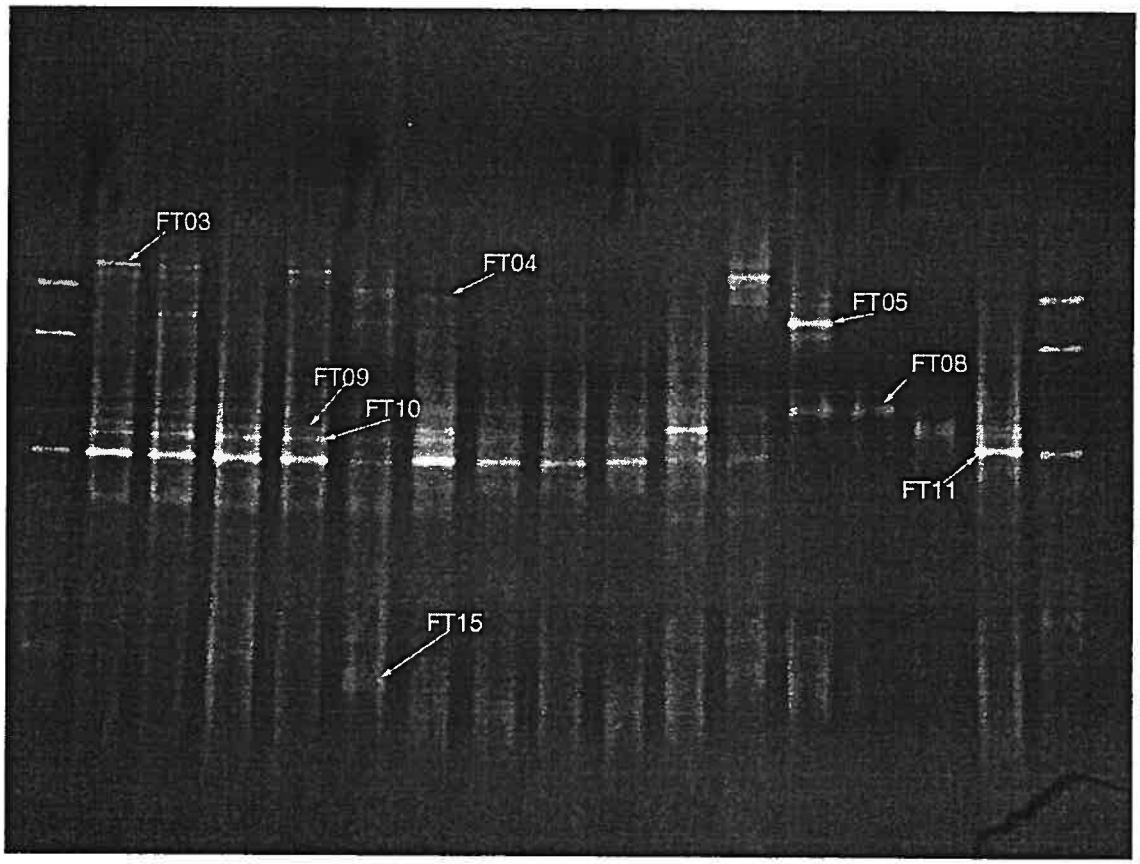


FIGURE 2.

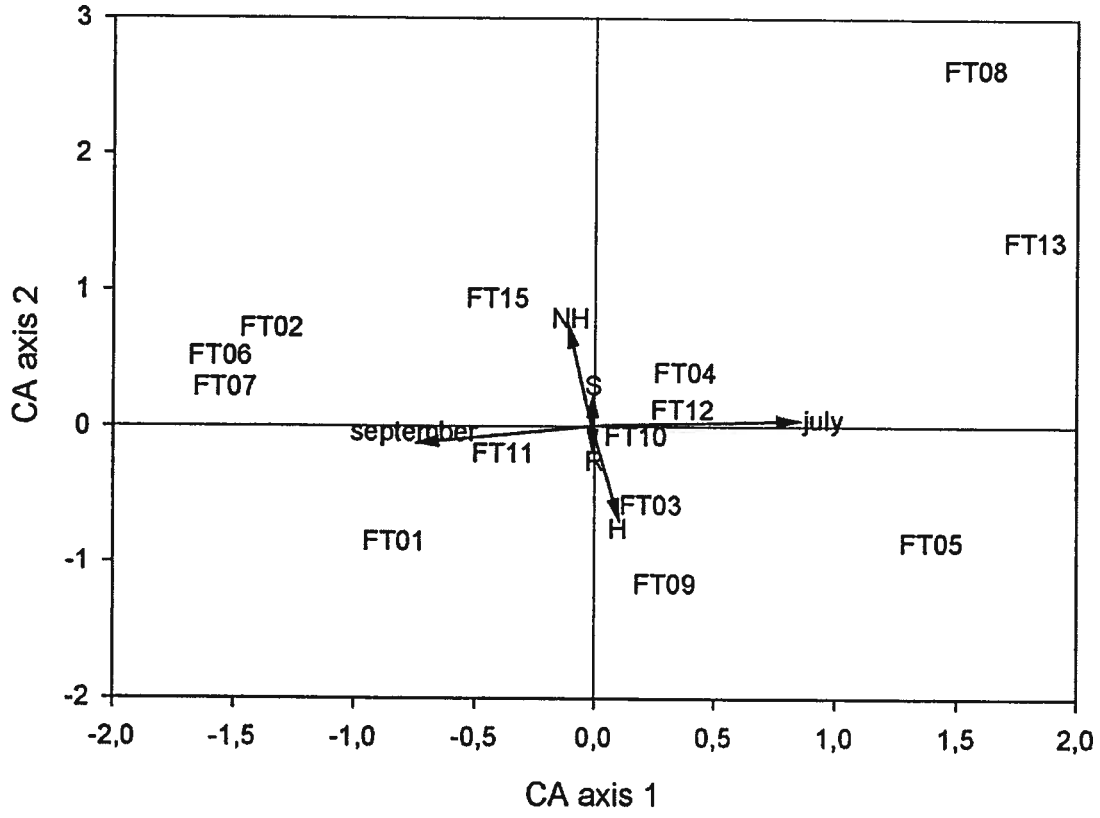

3
2
1
0
-1
-2
CA axis 2
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
2,0
CA axis 1
FT08
FT13
FT15
NH
FT02
FT06
FT07
FT04
S
FT12
july
september
FT11
FT10
R
FT03
H
FT01
FT05
FT09

FIGURE 3.

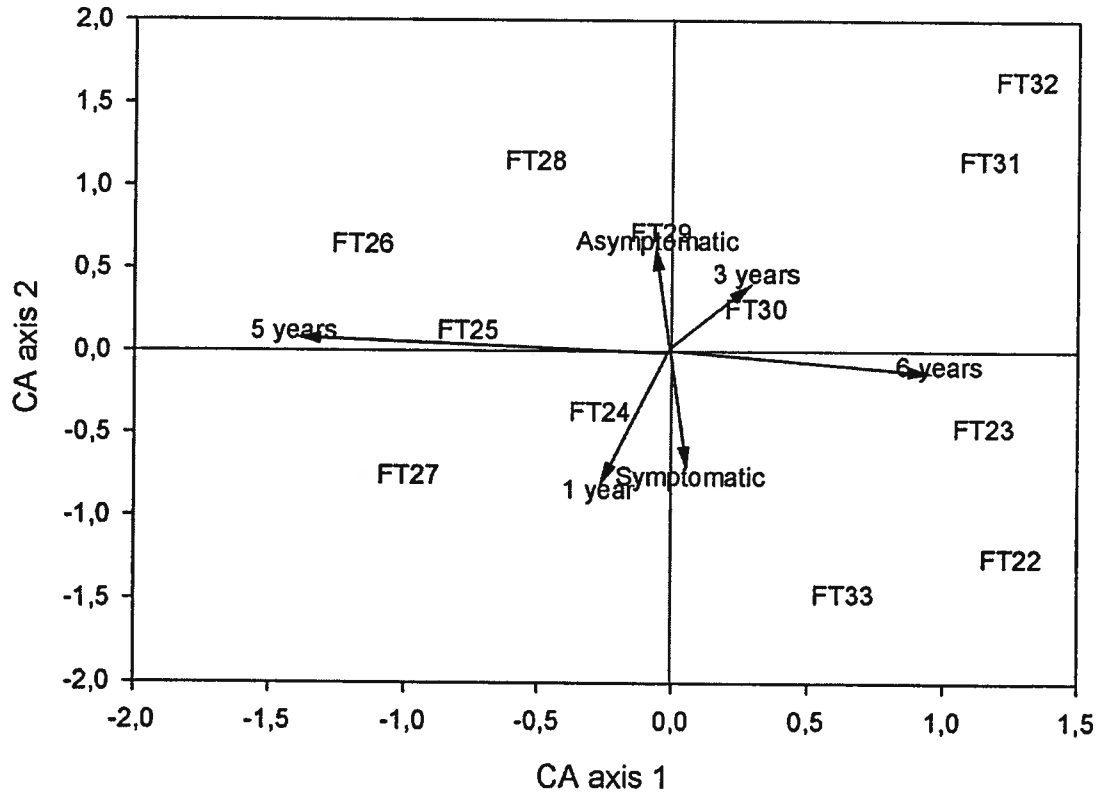

2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
CA axis 2
-2,0
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
CA axis 1
FT32
FT28
FT31
FT26
FT29
Asymptomatic
3 years
FT30
5 years
FT25
6 years
FT24
FT23
FT27
1 year
Symptomatic
FT22
FT33

FIGURE 4.

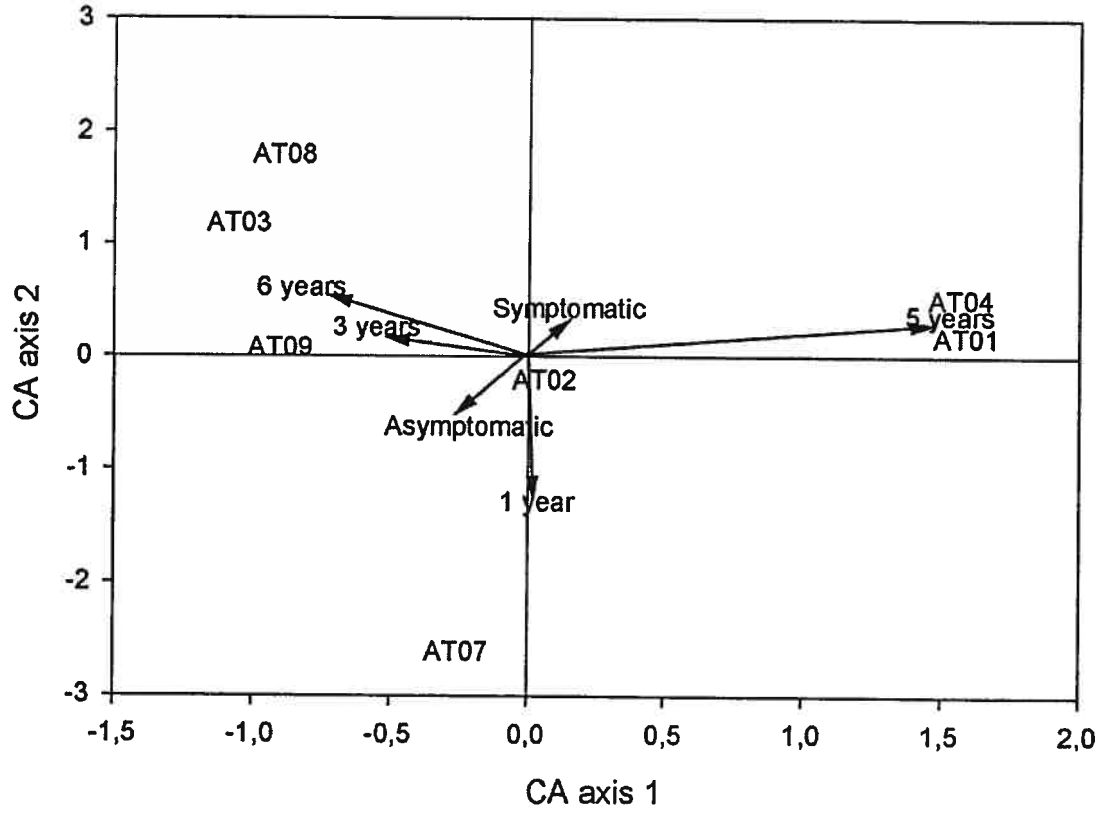

3
2
1
0
-1
-2
-3
CA axis 2
AT08
AT03
6 years
3 years
AT09
Symptomatic
AT04
5 years
AT01
AT02
Asymptomatic
1 year
AT07
-1,5
-1,0
-0,5
0,0
0,5
1,0
1,5
2,0
CA axis 1

FIGURE 5.

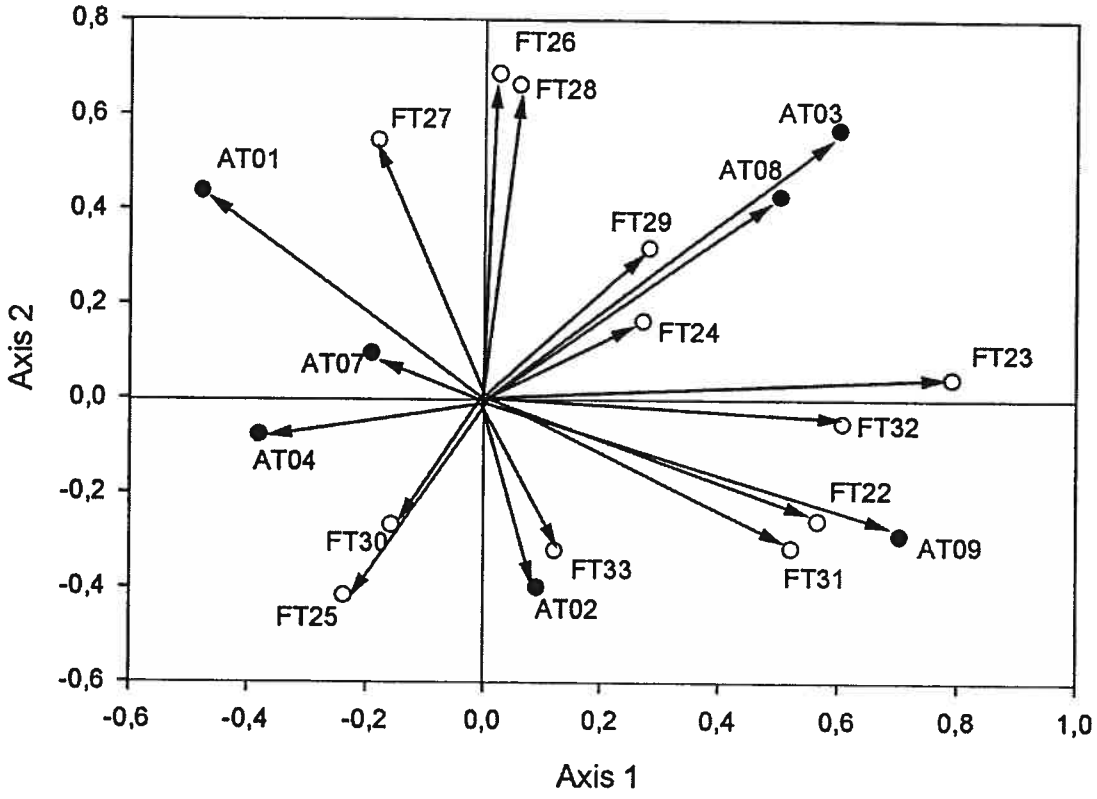
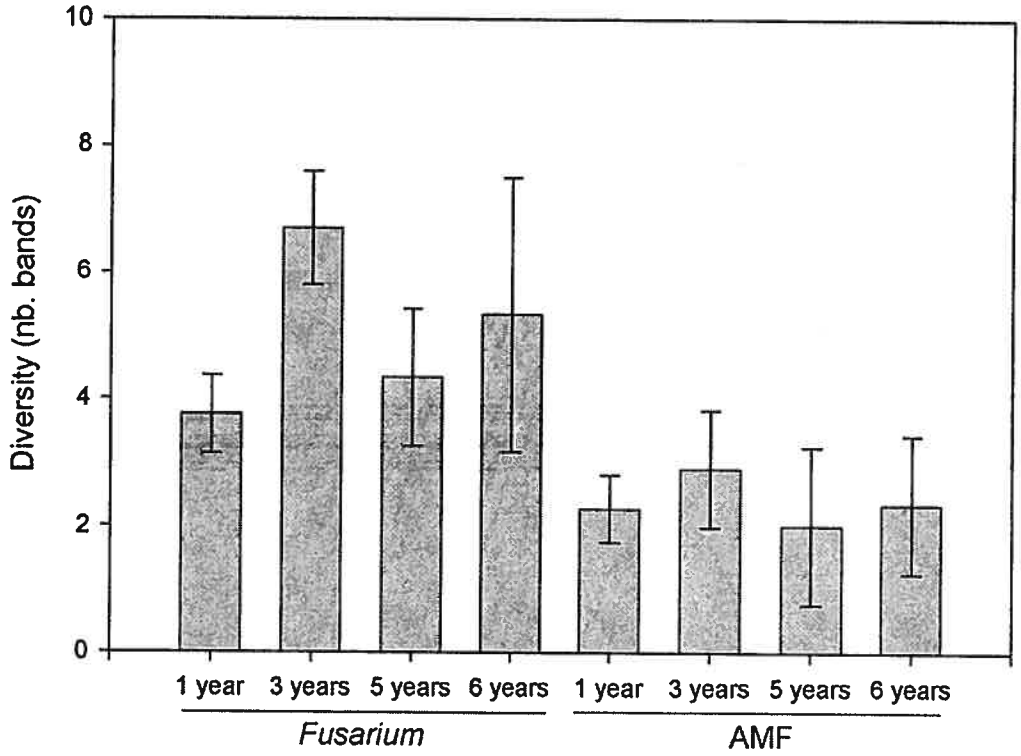

0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
-0,6
Axis 2
-0,6
-0,4
-0,2
0,0
0,2
0,4
0,6
0,8
1,0
Axis 1
FT26
FT28
FT27
AT01
AT03
AT08
FT29
FT24
AT07
FT23
FT32
AT04
FT22
AT09
FT30
FT33
FT31
FT25
AT02

FIGURE 6.


Diversity (nb. bands)
10
8
6
4
2
0
1 year
3 years
5 years
6 years
1 year
3 years
5 years
6 years
Fusarium
AMF

Chapitre

4 Discussion générale

4.1 Mise au point d'un outil d'étude de la biodiversité de *Fusarium* spp.

Le genre fongique *Fusarium* cause des ravages importants dans plusieurs cultures à travers le monde, mais son écologie demeure peu étudiée. Ceci réside dans le fait que les méthodes traditionnelles d'étude des micro-champignons sont souvent peu rapides et nécessitent une longue expérience. Le Chapitre 2 de ce mémoire tente de remédier à cela en présentant la mise au point d'un outil de détection moléculaire du genre *Fusarium* dans les plants d'asperges. Le principal avantage de cet outil est qu'il est rapide et qu'il ne dépend pas des étapes de culture et d'isolation qui donnent souvent un portrait biaisé de la biodiversité des micro-organismes (Bridge and Spooner 2001; Head et al. 1998). Cette méthode est, à ma connaissance, la première à s'appliquer à un grand nombre d'espèces du genre *Fusarium* et à être totalement indépendante de la culture. Il est à espérer que cet outil permettra éventuellement d'étudier plus facilement l'écologie de cet important genre dans les champs d'asperges de même que dans d'autres agro-écosystèmes.

La région du « translation elongation factor 1 alpha » (EF-1 alpha) a été choisie pour le PCR-DGGE à la suite d'un alignement de séquences déjà publiées et d'une recension de littérature qui mettaient en évidence le pouvoir discriminant de cette région (O'Donnell et al. 1998a; O'Donnell et al. 1998b). Des amorces spécifiques aux *Fusarium* ont donc été mises au point pour cette région et utilisées en DGGE. D'un autre côté, il serait intéressant et utile d'utiliser le DGGE pour suivre

les populations de *Fusarium* mais en ciblant des gènes possiblement impliqués dans la pathologie. De même, il serait possible de mettre au point une méthode utilisant le « reverse-transcriptase » (RT)-PCR afin d'avoir des patrons de diversité correspondant uniquement aux organismes vivants et actifs. En combinant ces deux approches, il serait possible de suivre spécifiquement par DGGE les espèces du genre *Fusarium* qui expriment activement un gène impliqué dans la pathologie.

Dans le Chapitre 2, le système de PCR-DGGE a été testé avec succès sur des échantillons d'asperges et a permis de discriminer la majorité des espèces testées sans difficulté. Afin d'utiliser cet outil avec d'autres types d'échantillons environnementaux, il faudrait tester le pouvoir discriminant de ce système sur les espèces que l'on suspecte être dominantes dans ces échantillons. Cela permettrait d'identifier les biais possibles ainsi que les espèces qui pourraient migrer à des positions identiques. À la suite de la mise au point de ce système pour les asperges, une étude écologique à plus grande échelle, présentée dans le Chapitre 3, a été menée sur des échantillons de plantes provenant de champs commerciaux.

4.2 Effet de la récolte des asperges sur les communautés fongiques

La récolte est un stress très important pour les asperges et on croit que ce stress contribue à l'augmentation de la fusariose, phénomène observé dans les champs plus âgés. Dans la deuxième partie de ce mémoire, il a été question que l'augmentation de la fusariose est probablement due à des changements dans les communautés fongiques associées aux racines des plantes et que de nombreux autres facteurs (paramètre du sol, communautés bactériennes, etc.) peuvent aussi avoir une influence directe ou indirecte sur l'incidence de fusariose et les communautés fongiques.

Dans un premier temps, des échantillons des parcelles expérimentales ont été récoltées et comparées avec ceux parcelles non-récoltées. Les parcelles ont été suivies durant toute la saison de croissance et échantillonnées à trois reprises (mai, juillet et septembre). Cette expérience nous a permis de suivre les changements à court terme qui surviennent dans les communautés de *Fusarium* à la suite de la récolte. Des différentes communautés de *Fusarium* et une augmentation significative de l'incidence de la fusariose ont été observées entre les parcelles récoltées et non-récoltées. Deux explications peuvent être avancées pour expliquer ce phénomène : 1) les asperges ont un métabolisme et une exsudation altérés à la suite de la récolte, ce qui provoquerait directement un changement dans les communautés de *Fusarium* qui leur sont associées et que cela entraînerait ensuite une augmentation de l'incidence de la fusariose; 2) les autres communautés fongiques et bactériennes pourraient aussi varier et influencer par la suite les communautés de *Fusarium*, entraînant une augmentation de la fusariose. Or, bien que le mécanisme exact soit inconnu, il est

évident que le changement observé dans les communautés de *Fusarium* à la suite de la récolte est fortement relié à l'augmentation de l'incidence de la fusariose.

D'un autre côté, les changements observés à court terme semblent aussi se produire à long terme. Ceci a été testé en échantillonnant des champs âgés de 1 an (établissement des plantes dans le champ), 3 ans (année avant le début de la récolte), 5 ans (première année de productivité complète) et 6 ans (champ en productivité complète). Dans ce cas, pour avoir un portrait plus complet des communautés fongiques, les communautés de champignons mycorhiziens à arbuscules (CMA) et celles de *Fusarium* ont été observées. Les champs d'âges différents avaient des communautés fongiques différentes autant pour les CMA que pour les *Fusarium*. Certaines espèces de CMA sont plus efficaces pour assurer une protection contre la fusariose chez les asperges, et certaines espèces et isolats de *Fusarium* sont plus virulents que d'autres. Il semble que les changements dans ces deux communautés soient dans une séquence qui n'est pas profitable aux asperges, car il y a une augmentation marquée de la fusariose avec l'âge (Hamel et al. 2004). Les changements sont peut-être dus aux composés autotoxiques que libèrent les plants d'asperge qui s'accumulent dans les champs avec l'âge (Yang 1982). Ces composés ont des effets sur les micro-organismes qui varient selon les espèces (Blok and Bollen 1996; Hartung and Stephens 1983). Il est aussi possible que cela soit relié avec certains facteurs physico-chimiques du sol, car il a été déterminé que l'âge du champ d'asperges était corrélé négativement avec le phosphore et positivement avec le pH du sol (Wacker et al. 1990c). Il est donc plausible que les communautés fongiques répondent directement aux composés libérés par les asperges ou encore aux

changements dans les sols, mais il est aussi probable que cette réponse soit indirecte. En effet, ces changements dans les paramètres abiotiques du sol influencent peut-être plusieurs micro-organismes autant bactériens que fongiques, qui à leur tour influencent les *Fusarium* et les CMA. La complexité des micro-organismes ainsi que leurs interactions avec le milieu abiotique et avec les plantes fait en sorte qu'aucune conclusion définitive ne peut être faite pour le moment. Malgré cela, il est fort probable que les changements à la suite de la récolte dans les communautés de champignons mycorhiziens ainsi que dans celles de *Fusarium* jouent des rôles non-négligeables dans le déclin des champs d'asperges.

4.3 Conclusions

La mise en place d'un protocole simple et rapide pour l'identification des espèces de *Fusarium* a permis d'effectuer une étude de grande envergure à l'aide d'un plan d'échantillonnage, ce qui permet de tirer des conclusions appuyées statistiquement. De telles études sont pratiquement impossibles à effectuer par des méthodes traditionnelles. De plus, l'application de méthodes statistiques multivariées sur les résultats est relativement innovatrice étant donné que peu d'études portant sur l'écologie moléculaire des micro-organismes tiennent compte de ces outils d'analyses et se concentrent plutôt sur les méthodes d'observation.

L'étude présentée ici s'inscrivait dans un projet multidisciplinaire comptant plusieurs chercheurs qui travaillent à trouver un moyen de contrôler la fusariose de l'asperge de façon biologique en utilisant une approche intégrée. Les nouvelles connaissances apportées dans le Chapitre 3 permettront vraisemblablement d'aider à atteindre cet objectif. Les résultats ont effectivement permis d'illustrer la complexité et la dynamique des populations de *Fusarium* dans les champs d'asperges du Québec. La connaissance des taxons impliqués et leur succession à la suite de la récolte permettra peut-être de mettre au point des stratégies de biocontrôle mieux adaptées ou mieux synchronisées avec l'apparition des différentes espèces. Les résultats établissent distinctement qu'il y a un changement dans les communautés pathogènes à la suite de la récolte et que ce changement a lieu à court terme (quelques mois) et à long terme (plusieurs années). Il serait donc intéressant d'étudier différents paramètres (façon de récolter, durée de la récolte, âge lors de la première récolte) par rapport à ces changements dans les communautés. D'autres résultats devraient aussi

être étudiés plus en profondeur comme l'opposition observée entre certains taxons de *Fusarium* et les CMA. De plus, des résultats en cours d'analyse qui ne cadraient pas dans les objectifs de ce mémoire, montrent des relations intéressantes entre la diversité des *Fusarium* et quelques variables environnementales, notamment la concentration en manganèse, en fer et en phosphore dans la plante.

Il sera intéressant de voir l'utilisation de l'outil présenté ici dans les prochaines années et son application dans d'autres cultures susceptibles aux attaques du genre *Fusarium* comme le maïs ou le blé. De plus, en continuant l'étude de *Fusarium* dans les champs d'asperges avec cet outil, tout en suivant simultanément d'autres communautés fongiques et bactériennes, il sera possible de découvrir quels sont les facteurs biotiques et abiotiques à contrôler pour réduire les dommages causés par la fusariose et, ultimement, de contrôler cette maladie biologiquement.

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