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IMPACT DES ARBRES GÉNÉTIQUEMENT MODIFIÉS SUR LES COMMUNAUTÉS FONGIQUES DU SOL

Thèse présentée
à la Faculté des études supérieures de l'Université Laval
dans le cadre du programme de doctorat en Sciences Forestières
pour l'obtention du grade de *Philosophiæ doctor* (Ph. D.)

DÉPARTEMENT DES SCIENCES DU BOIS ET DE LA FORÊT
FACULTÉ DE FORESTERIE, DE GÉOGRAPHIE ET DE GÉOMATIQUE
UNIVERSITÉ LAVAL
QUÉBEC

2010

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Résumé

La constante augmentation des surfaces cultivées avec des plantes génétiquement modifiées (PGM) pose la question du risque environnemental qu'elles représentent. Afin de documenter cette problématique, nous avons dans un premier temps fait la synthèse de 20 années de recherche sur l'impact potentiel des cultures et des arbres génétiquement modifiés sur les champignons. L'analyse des publications scientifiques sur ce sujet montre que les conséquences des plantes transgéniques tolérantes aux herbicides et aux insectes ravageurs sur les champignons sont sous-étudiées alors qu'elles représentent la majorité des PGM cultivées dans le monde. Des changements significatifs affectant les champignons ont été relevés dans 18 études, dans lesquelles les PGM étudiées n'exprimaient pas de caractères transgéniques laissant présager un effet sur les champignons. En dépit du fait qu'elles sont commercialisées depuis 1996, le risque que les PGM représentent à l'heure actuelle pour les communautés fongiques ne peut être clairement défini à partir des données disponibles.

L'impact potentiel de peupliers exprimant une résistance à la kanamycine a été évalué sur la communauté des ectomycorhizes (EM), après 8 années de déploiement au champ. Les mesures qualitatives et quantitatives de la diversité des EM n'ont pas mises en évidence de différence significative entre la structure de la communauté des EM provenant des peupliers témoins et celle provenant des peupliers transgéniques. Par contre, la communauté des EM identifée par l'analyse des extrémités racinaires était significativement différente de celle obtenue par le clonage de l'ADN fongique du sol. Ces deux stratégies d'échantillonnage, en les combinant, se sont révélées complémentaires pour une définition plus fine de la diversité des EM.

Des travaux effectués en serre sur les conséquences de la surexpression de l'endochitinase dans les racines et les exsudats racinaires d'épinettes blanches transformées avec le gène *ech*42 n'ont pas détecté d'effet délétère sur la symbiose ectendomycorhizienne et la biomasse fongique du sol. De plus, les effets potentiels de ces épinettes transgéniques sur deux communautés fongiques provenant de sols forestiers ont été suivis pendant 8 mois. Les analyses ont montré que l'insertion dans le génome des épinettes blanches du gène

*ech*42 et son expression n'affectaient pas de manière significative la biomasse, la diversité et la structure des communautés fongiques des deux sols analysés.

Cette thèse a permis de faire le point sur l'impact des PGM sur les champignons et d'évaluer le risque que peuvent représenter différents types d'arbres génétiquement modifiés sur les champignons du sol.

Abstract

The continuous increase of area cultivated with genetically modified plants (GMPs) addresses the issue of their potential impact on the environment. To document this issue, we first reviewed 20 years of research monitoring of the potential effects of GM crops and GM trees on fungi. The analysis of peer-reviewed publications showed that the consequences of GM plants expressing herbicide and insect tolerance on fungi are understudied while they dominate GM hectarage worldwide. Significant changes on non-target fungi were observed in 18 studies and all of them involved GM plants expressing traits that were not expected to affect fungi. Reviewing the literature currently available about the relationships between GM plants and fungi made it impossible to obviously determine if fungi are significantly at risk to GM plants. Then, the potential impact of poplars expressing kanamycin resistance was investigated on ectomycorrhizal fungi, eight years after field deployment. Based on qualitative and/or quantitative diversity measurements, we found no evidence of difference in the EM fungal community structure from control and transgenic poplars. On the other hand, we showed that EM fungal communities recorded through direct sequencing of root tips and soil fungal DNA cloning were significantly different. Coupling these two sampling strategies was a complementary approach to better document EM fungal diversity.

Next, the impact of transgenic white spruce overexpressing endochitinase was evaluated on soil fungal biomass and on the ectendomycorrhizal fungi *Wilcoxina* spp. using a greenhouse trial. Results indicated that the higher levels of chitinolytic activity in root exudates and root tissues from transformed lines did not alter the development of ectendomycorrhizal symbiosis involving *Wilcoxina* spp. or the soil fungal biomass. Furthermore, the potential impact of these endochitinase transformed white spruces was investigated on two soil fungal communities imported from natural forests and results showed they did not significantly affect biomass, diversity and structure of soil fungal communities.

This thesis made possible to establish the current state of the effects of GMPs on fungi and to evaluate under various experimental conditions if soil fungi were at risk to different types of GM trees.

Avant-propos

Mes études se sont enfuies très loin. Je les poursuis encore au moment d'écrire ces quelques lignes, depuis le Québec qui plus est. Douze années de course poursuite, qui m'ont d'abord emmené sur les rives du lac d'Annecy. Les objectifs étaient clairs. Devenir technicien forestier et faire des études courtes. Si le premier point fût atteint, le second est un échec cuisant... Le marché du travail à 20 ans, à quoi bon. Et puis l'université fuie deux ans plus tôt apparaissait soudainement plus séduisante qu'un poste d'agent forestier, dans un quelconque village de France à la moyenne d'âge d'outre-tombe. Ça sentait le sapin. Direction l'université donc, jusqu'au bout.

C'est ainsi l'occasion de remercier toutes les personnes qui ont permis la réalisation de cette thèse. Je tiens tout d'abord à remercier le Dr Richard Hamelin, co-directeur de la thèse, mais chef de fil du financement du projet et de l'étudiant. Merci Richard pour ta disponibilité, en dépit de ton détachement à l'université de Colombie-Britannique depuis... je ne m'en souviens plus. Malgré cette absence, tu as toujours répondu présent quand il le fallait. Merci aussi pour la déconcertante confiance que tu m'as accordée pour mener à bien ce projet. J'ai eu les mains et l'esprit libres pour poursuivre mes travaux, au risque parfois même de me disperser. Mais j'ai tellement appris. Merci enfin de m'avoir encouragé à participer au moins une fois par année à des congrès internationaux, peu de directeurs peuvent s'en vanter. Je remercie mon directeur de thèse le Dr Yves Piché. Merci Yves pour ces nombreuses réunions, souvent impromptues, qui ont permis de faire avancer mes réflexions sur mon sujet de thèse, merci aussi de ta complicité pour le financement de certains des congrès qui ont ponctué chacune de mes années au doctorat. Je te remercie également d'avoir accepté, financé et mis en œuvre les moyens nécessaires pour que je puisse apprendre des expériences moins moléculaires et plus physiologiques qui comptent beaucoup pour ma formation. J'ai pu trouver dans ton laboratoire et au contact de ton équipe une « culture mycorhizienne » que je considère comme indispensable à la maturation et l'achèvement de mon projet de doctorat.

Je remercie le Dr Jean Bérubé, dans l'ombre de ce doctorat. Été 2001 et premier stage en recherche, sous sa direction. Merci Jean de m'avoir offert la chance d'une telle expérience. D'endophytes en aiguilles d'épinettes, me voilà rendu au doctorat. Merci pour ta

participation aux financements de certains congrès, Hawaï restera dans les mémoires. Merci aussi pour tous ces à-côtés, 5 à 7, dîners, soupers, sorties terrains et autres, qui changent les idées et améliorent le quotidien d'un étudiant étranger. Je ne l'oublierai pas. Je remercie également le Dr Armand Séguin, ne serait-ce que parce que sans le travail de son équipe, je n'aurais pas eu de matériel d'étude. Merci aussi Armand pour les quelques avances de salaires perçues au début de mon doctorat, lorsque la paie était plutôt aléatoire.

Merci au Dr Philippe Tanguay de m'avoir transmis une petite partie de ses immenses connaissances expérimentales. Merci aux techniciennes de labo (Nicole, Françoise), aux biologistes (Marie-Josée, Josyanne, Gervais, Jean-Guy) et aux professionnels de recherche (Annie, André) qui ont répondu aux questions nombreuses que l'on peut avoir lorsqu'on débarque dans un laboratoire, et qui ne sont pas parmi les plus brillantes des interrogations. Merci à celles et ceux que j'ai pu solliciter pour satisfaire mes besoins en produits et matériels divers et variés, qui ont toujours réagi dans les plus brefs délais.

Je voudrais également remercier ma famille en général et mes parents en particulier pour leur soutien moral et financier. Je vous en suis profondément reconnaissant, tous les étudiants n'ont pas la chance d'un soutien sans frontière… Je tiens à remercier mon oncle, le Dr Jean-Baptiste Galeazzi, qui m'a souvent inspiré, par les récits de ses expériences personnelles et professionnelles glanés de-ci de-là, une certaine motivation quand au labeur qu'exigent des études doctorales.

Comment ne pas penser aux amis, ceux que l'on peut retrouver après des semaines voir des mois ou des années comme si l'on s'était laissé hier. Merci Margarita, Serge, JP, Guillaume, pour tout ce que vous avez pu m'apporter ces dernières années. Merci également à Agathe, Guillaume et David, doctorants et délirants, promoteurs de bonne humeur.

Enfin je tiens à remercier celle qui connaît le mieux la thèse qui suit, ainsi que son auteur, pour avoir été la première lectrice et souvent correctrice de mes manuscrits. Marie-Josée, tu as partagé mes espoirs et désespoirs, mes joies et mes rages, tu m'as laissé cette liberté et solitude, si précieuses pour rester soi-même, tu as toujours respecté et soutenu l'intensité du rythme de travail nécessaire à l'avancement et l'aboutissement de cette thèse. Pour tout ça et bien plus… merci.

À ma famille

Ce sont les hommes qui écrivent l'histoire,
mais ils ne savent pas l'histoire qu'ils écrivent.
(Raymond Aron)

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Liste des abréviations

ADN / DNA	acide désoxyribonucléique
ADNr / rDNA	acide désoxyribonucléique ribosomique
AGM / GMT	arbre(s) génétiquement modifié(s)
AM	*arbuscular mycorrhizas* (mycorhizes à arbuscules)
ANCOVA	*analysis of covariance* (analyse de covariance)
ANOVA	*analysis of variance* (analyse de variance)
ARN, RNA	acide ribonucléique
ARNr / rRNA	acide ribonucléique ribosomique
BLAST	*Basic Local Alignment Search Tool* (outil de recherche d'alignement local)
BSA	*bovine serum albumin* (albumine sérique bovine)
Bt	*Bacillus thuringiensis*
C	carbone
Ca	calcium
$CaCl_2$	chlorure de calcium
CEC	*cation exchange capacity* (capacité d'échange cationique)
CFU	*colony-forming unit* (unité formant colonie)
cm^3	centimètre cube
CsVMV	*cassava vein mosaic virus* (virus de la mosaïque des nervures du manioc)
Ct	*cycle threshold* (cycle seuil)
CTAB	bromure d'hexadécyltriméthylammonium
Ct-No	*Number of molecule at cycle threshold* (nombre de molécules au cycle seuil)
D	*Simpson index* (indice de Simpson)
db	*distance based* (basé sur la distance)
df	*degree of freedom* (degré de liberté)
DGGE	*denaturing gradient gel electrophoresis* (électrophorèse en gel à gradient denaturant)

dNTP	*deoxynucleotide triphosphate* (désoxyribonucléotide triphosphate)
DPCoA	*double principal coordinate analysis* (analyse en composantes principales doubles)
DES	*Dark septate endophyte*
E	*efficiency* (efficacité)
***ech*42**	*42 kDA endochitinase gene* (gène codant pour l'endochitinase 42 kDA)
ECM, EEM	*ectendomycorrhizas* (ectendomycorhize)
EDTA	*ethylenediaminetetraacetic acid* (acide éthylènediaminetétraacétique)
EM	*ectomycorrhizal or ectomycorrhizas* (ectomycorhizien ou ectomycorhize)
Et	*endochitinase transformed* (transformé endochitinase)
F	distribution de la valeur de Fisher
FAO	*Food and Agriculture Organization of the United Nations* (organisation des nations unies pour l'alimentation et l'agriculture)
Fg	*fungal* (fongique)
g	gramme
g	force gravitationnelle
gDNA	*genomic DNA* (ADN génomique)
GM	*genetically modified* (modifié génétiquement)
GR	*glyphosate-resistant* (résistant au glyphosate)
GUS	*β-glucuronidase reporter gene* (gène rapporteur codant pour la *β-glucuronidase*)
h	heure
H	*Shannon index diversity* (indice de diversité de Shannon)
Ha	hectare
HCl	acide chlorhydrique
HGT	*horizontal gene transfert* (transfert horizontal de gène)
HPLC	*high performance liquid chromatography* (chromatographie en phase liquide à haute performance)

H_0	*null hypothesis* (hypothèse nulle)
ISI	*Institute for Scientific Information* (Institut de l'Information Scientifique)
ITS	*Internal Transcribed Spacer* (Espaceur Interne Transcrit)
JSTOR	*Journal Storage* (conservation de journaux)
K	potassium
kDa	kilodalton
lme	*linear mixed-effects* (effets fixes linéaires)
M	molaire
min	minute
MG	*morphological group* (groupe morphologique)
Mg	magnésium
mg	milligramme
$MgCl_2$	chlorure de magnésium
MH	*mineral horizon* (horizon minéral)
ml	millilitre
mm	millimètre
mM	millimolaire
MRA	*mycelium radicis atrovirens*
MSS	*Murashige and Skoog basalt salt* (sel de Murashige et Skoog)
MU	méthylumbelliférone
N	*nitrogen* (azote)
N_2	*dinitrogen* (diazote)
Na	sodium
NAG	N-acetyl-glucosamine
NaOH	hydroxyde de sodium
NCBI	*National Center for Biotechnology Information* (centre national pour l'information sur les biotechnologies)
nLSU	*large subunit nuclear rDNA gene* (gène de la grande sous-unité de l'ADNr)

ng	nanogramme
nm	nanomètre
nM	nanomole
NOS	*nopaline synthase promoter* (promoteur de la nopaline synthase)
NPMLE	*non-parametric maximum likelihood estimator* (estimateur non-paramétrique du maximum de vraisemblance)
***npt*II**	*neomycin phosphotransferase gene* (gène de marquage conférant la résistance à la kanamycine)
ODS	octadécylsilane
OGM / GMO(s)	organisme(s) génétiquement modifié(s)
OH	*organic horizon* (horizon organique)
OM	*organic matter* (matière organique)
OTU(s) / UTO	*operational taxonomic unit(s)* (unité(s) taxonomique(s) opérationnelle(s))
P	phosphore
P*, *p	probabilité
pb/bp	paire de bases
PBO	Plant Biosafety Office (bureau de la biosécurité végétale)
PC	*principal component axis* (axe de composante principale)
PCA	*principal coordinate analysis* (analyse en composantes principales)
PCR	*polymerase chain réaction* (réaction de polymérisation en chaîne)
PDA	*potato dextrose agar*
PEG	polyéthylène glycol
PFTE	Polytetrafluoroéthylène
PGM / GMP(s)	plante(s) génétiquement modifiée(s)
pH	potentiel hydrogène
ppm	partie par million
PG, Pg	*Picea glauca*
PR	*pathogenesis-related protein* (protéine de pathogénicité)
r	coefficient de corrélation de Pearson

RDA	*redundancy analysis* (analyse canonique de redondance)
RFLP	*restriction fragment length polymorphism* (polymorphisme des fragments de restriction)
rpm	rotation par minute
RT	*root tip(s)* (extrémité(s) racinaire(s))
s	secondes
SES_{mpd}	*standardized effect size of the mean pairwise distance*
SDS	*sodium dodecyl sulfate* (dodécylsulfate de sodium)
Sp	*Chao index* (indice de Chao)
Ta	*annealing temperature* (température d'appariement)
Taq	*Thermus aquaticus*
TAE	TRIS-acide acetique-EDTA
TE	TRIS-EDTA
Ti	*Tumor inducing* (induction de tumeur)
TRIS	trishydroxyméthylaminométhane
T-DNA	*transfert DNA* (ADN de transfert)
T0, T8	temps 0 et temps 8 (mois)
U	unité
UAMH	*university of Alberta microfungus collection and herbarium* (herbier et collection des microchampignons de l'université d'Alberta)
v	volume
wt	*weight* (masse)
Zn	zinc
µg	microgramme
µl	microlitre
µm	micromètre
µM	micromolaire
ηM	nanomolaire
°C	degré Celsius
%	pour cent

PRÉFACE

L'histoire est riche en concordances des temps qui peuvent parfois nous éclairer sur notre présent. Il y a deux siècles, l'Angleterre débutait sa révolution industrielle et connaissait, par le fait même, de virulentes réactions ouvrières se traduisant par la destruction de machines textiles et agricoles. La fin du XXe siècle se caractérise par l'avènement de l'industrie agrobiotechnologique avec la commercialisation des premières semences transgéniques : les fauchages volontaires de plantes génétiquement modifiées (PGM) débutent en Angleterre et en France en 1997. Quel peut-être le point commun entre les révoltes ouvrières du début du XIXe siècle, s'attaquant aux premières machines des manufactures et de l'agriculture, et les faucheurs volontaires de la fin du XXe siècle, détruisant des champs de PGM ? Les fortes réactions sociales concernant ces deux évènements sont apparues à la suite de changements de paradigmes générés par le développement de la mécanisation dans les manufactures et l'agriculture et par la commercialisation d'organismes génétiquement modifiés. Dans ce dernier cas, un nouveau seuil a été franchi en développant des produits résultant non plus de l'assemblage de matériaux inertes mais de la modification du vivant et de ce qu'il a de plus fondamental, son ADN. Dans les deux situations, les technologies ne sont pas attaquées pour ce qu'elles sont en elles-mêmes mais pour les changements qu'elles annoncent et les détériorations potentielles qui pourraient en découler sur les conditions de travail, de vie et, plus récemment, sur la qualité de l'environnement. La destruction de champs de PGM est un cas de luddisme renouvelé (Chevassus-au-Louis[1]).

Depuis De Broglie (1892-1987) affirmant que « *le risque est la condition de tout succès* », à la mise en place du principe de précaution dans le droit international (conférences pour la protection de la Mer du Nord 1984, 1987, Rio 1992, traité de Maastricht, 1992), l'opinion publique au sujet des technologies et des produits, ainsi que la place de la société dans les processus de décisions, ont subi de profonds changements, impulsés par la succession de scandales ou de crises et par l'essor de la démocratie participative. Les drames sanitaires ou environnementaux de la fin du XXe siècle ont été les

[1] Nicolas Chevassus-au-Louis. 2006. Les briseurs de machines. 270 p. Éditions du Seuil.

déterminants de l'émergence de la notion de risque raisonné et de la prise de risque en connaissance de cause, qui constituent les fondements même du principe de précaution. Par leur ampleur, ces évènements ont touché et surtout sensibilisé un large public qui, grâce à la révolution des moyens de communication, est en mesure de prendre connaissance de façon indépendante du rapport bénéfice / risque associé à chaque technologie ou produit développé et de décider de son acceptabilité. Cela a pour conséquence de réduire l'influence des scientifiques comme celui des industriels qui ne peuvent plus proposer des avancées ou des produits sans que leur acceptabilité sociale ne soit prise en considération, définissant un nouveau principe de précaution. Ainsi, François Ewald[2] analyse la situation : « *la science propose son expertise, dans son ordre, et la société, souverainement, juge de l'usage qu'elle veut en faire. Pourquoi la société devrait se ranger à l'avis du savant ? Telle est la nouveauté du principe de précaution : il déconnecte la décision politique de l'expertise scientifique qui n'est plus qu'un élément de décision parmi d'autres, plus sociaux.* [...] *L'avis scientifique concernant l'usage d'une substance est une condition nécessaire de la décision politique mais désormais non suffisante* ».

Les produits issus des secteurs sensibles comme celui des biotechnologies, sont et seront fréquemment l'objet de réévaluation en fonction du progrès des connaissances. Le recours possible des États aux clauses de sauvegarde témoigne de l'importance qu'ils accordent aux réactions sociales et à la reconsidération des risques à la lumière des derniers progrès de la science. C'est ainsi, par exemple, que la culture du maïs MON810 a été suspendue en France en janvier 2008. Si, dans le monde judiciaire, en cas de doute on acquitte, il en va tout autrement avec les biotechnologies. En ce qui concerne les PGM, fruit des progrès de la science par excellence, le doute et la controverse caractérisent le débat du risque sanitaire et environnemental qu'elles représentent. Dans une société devenue précautionneuse, l'évaluation scientifique du risque, même si elle n'est plus l'unique élément de décision, demeure à la base du processus d'analyse de risque. Les autorités publiques, quant à elles, se retrouvent souvent entre le marteau et l'enclume, et doivent trouver le juste milieu entre sous-évaluation et excès de protection. À défaut de faucher, peut-on trancher le débat ?

[2] François Ewald. 2009. Aux risques d'innover. 218 p. Éditions Autrement.

Pour cela il faut des données, beaucoup de données, afin de faire reculer le plus possible l'incertitude qui caractérise les PGM.

Moins connues du grand public, les biotechnologies trouvent également des applications en arboriculture et sylviculture. La durée du cycle de vie des arbres étant ce qu'elle est, les exemples de commercialisation sont encore rares. Cependant, les arbres transgéniques suscitent un intérêt grandissant, le nombre de dispositifs expérimentaux au champ ne cessant de croître. Par leur durée de vie, la principale inquiétude associée aux arbres génétiquement modifiés (AGM) est d'ordre environnemental. Les arbres interagissent avec leur environnement principalement par l'intermédiaire de leur système racinaire. Les racines sont le lieu d'une intense activité biochimique et microbienne. L'activité microbienne implique principalement les champignons du sol, parmi lesquels les champignons mycorhiziens qui s'associent directement avec les extrémités racinaires des arbres pour développer des relations de type symbiotique. Les bénéfices de la mycorhization sont très importants pour les arbres. Les hyphes mycorhiziens prospectent un volume de sol beaucoup plus grand que ne le feraient les racines, ils assurent la nutrition minérale des arbres par la translocation des éléments minéraux ou la formation de réserve durant la saison morte. La colonisation des extrémités racinaires par les champignons mycorhiziens limite par ailleurs le développement de maladies fongiques. Les AGM se doivent de ne présenter aucun risque pour ces champignons pour deux raisons. Premièrement, en cas d'effets délétères sur la mycorhization (modifications physiologiques au niveau de l'association racinaire, changements de la diversité de la flore mycorhizienne), le bénéfice apporté par la transformation génétique ne compenserait certainement pas la perte partielle ou totale des bénéfices résultant de la mycorhization. Deuxièmement, de part les fonctions centrales qu'ils occupent dans le sol, une diminution de la diversité des champignons du sol aurait des conséquences écologiques désastreuses.

C'est pourquoi l'impact potentiel des AGM sur les champignons du sol est une question cruciale, dont les réponses permettront de mieux cerner les risques environnementaux qu'ils représentent. C'est dans ce cadre que s'inscrit l'objectif de cette thèse. Le chapitre 1 est une introduction générale qui fait le point sur 20 années de publications scientifiques au sujet de l'impact des plantes agricoles et des arbres transgéniques sur les champignons,

qu'ils soient ciblés ou non par les caractères transgéniques exprimés. Les résultats de l'impact d'une plantation de peupliers exprimant un gène marqueur de résistance à la kanamycine sur la communauté des champignons ectomycorhiziens, après 8 années d'interaction au champ, sont présentés dans le chapitre 2. Les effets potentiels de l'augmentation de l'activité chitinolytique dans les tissus et les exsudats racinaires d'épinettes blanches transformées avec le gène *ech*42 sont évalués sur la symbiose ectendomycorhizienne et la biomasse fongique du sol dans le chapitre 3. Enfin, dans le chapitre 4, les conséquences de la surexpression de l'endochitinase des épinettes blanches transformées avec le gène *ech*42 sont étudiées sur la diversité fongique de deux types de sol forestier après 8 mois de croissance en serre.

CHAPITRE I

The current state of genetically modified plant impact on target and non-target fungi

1.1 Avant-propos

Ce chapitre sera publié sous forme d'article de revue dans un journal qui reste à déterminer. Les auteurs sont FOP Stefani & RC Hamelin. FOP Stefani a rédigé l'ensemble du chapitre. RC Hamelin a permis le financement de cette étude.

1.2 Résumé

Depuis une vingtaine d'années, le génie génétique permet de développer des plantes agricoles et des arbres fruitiers ou forestiers plus ou moins « sur mesure » afin d'améliorer les rendements et de simplifier les conditions d'exploitation. Les cultures intensives en agriculture et foresterie se traduisent par le déploiement de monocultures sur de larges surfaces. Ces pratiques bénéficient du développement de plantes génétiquement modifiées exprimant des caractères nouveaux tels que la tolérance aux herbicides ou aux insectes ravageurs, une croissance rapide, des modifications de la composition en lignine/cellulose, une meilleure résistance contre les virus, les champignons et les bactéries pathogènes.

La commercialisation des plantes agricoles transgéniques et le récent développement d'arbres génétiquement modifiés ont suscité un certain nombre d'inquiétudes au sujet de leur impact potentiel sur l'environnement en général et sur la biodiversité (organismes non cibles) en particulier. Les champignons sont présents partout sur la planète, occupent des

fonctions clés dans tous les écosystèmes, et sont intimement liés aux végétaux depuis que ces derniers ont colonisé les terres émergées.

Cette revue de littérature fait le bilan de 20 années de recherche sur l'impact potentiel des cultures et des arbres transgéniques sur les champignons ciblés et non ciblés par l'expression des caractères d'origine transgénique. La recherche, dans les banques de données publiques, d'articles revus par les pairs traitants de l'impact des plantes génétiquement modifiées sur les champignons nous a permis de retrouver 149 études. L'analyse de ces publications montre que les conséquences sur les champignons des plantes transgéniques tolérantes aux herbicides et aux insectes ravageurs sont sous étudiées alors qu'elles représentent la majorité des PGM cultivées dans le monde.

Les expériences faisant le suivi sur les champignons pathogènes (cibles) de l'impact des cultures et des arbres transgéniques ayant une activité antifongique accrue montrent, en général, une diminution significative de la gravité des infections. Parmi 60 travaux étudiant les effets potentiels des plantes transgéniques sur les champignons non cibles, 18 présentaient des changements significatifs (augmentation ou diminution du développement des champignons, de leur abondance ou de leur diversité); les PGM impliquées n'exprimaient pas de caractères transgéniques laissant présager un effet sur les champignons. Bien que les plantes transgéniques soient commercialisées depuis 1996, le risque qu'elles représentent à l'heure actuelle pour les communautés fongiques ne peut être clairement établi à partir des données disponibles.

1.3 Abstract

For two decades, genetic engineering has allowed the development of crops and trees designed for yield improvement and simplified culture management. Intensive agricultural and forestry practices result in field deployment of monoculture on large areas. This, combined with field deployment of monoculture on large areas, can result in environmental stress and potentially unwanted side effects. The commercial production of genetically modified (GM) crops and the recent development of GM trees raise concerns about their potential impact on the environment in general and on biodiversity, i.e. non-target

organisms, in particular. Fungi are widespread worldwide and play key roles in ecosystems. They are intimately associated with plants since they have emerged from oceans. This review critically examines research monitoring the potential effects of GM crops and GM trees on target and non-target fungi. Parsing public databases for peer-reviewed publications about GM plants impact on fungi yielded 149 studies, a relatively modest number considering the diversity of crops and ecosystems studied. The analysis of these publications showed that the effect on fungi of GM plants expressing herbicide and insect tolerance are understudied while they dominate GM area worldwide. Experiments monitoring the impact on target fungi of GM crops and GM trees with enhanced antifungal activity showed, for the most part, significant decreases in disease severity caused by fungal pathogens. Significant changes (increase or decrease in fungal development, abundance, diversity) on non-target fungi were observed in 18 out 60 studies and all of them involved GM plants expressing traits that were unexpected to affect fungi. The remaining 42 studies did not find significant impact in the fungal populations. Therefore, in spite of the fact that GM plants are commercialized since 1996, no clear generalized trend can be identified and it appears that a case-by-case approach is the safest.

1.4 Introduction

Intensive agricultural practices have resulted in field-deployment of monocultures on large areas. The management of monocultures typically requires the use of chemical substances to control weeds and to limit insect ravages and microbial diseases. These practices stress the development of plant varieties with new traits such as herbicide tolerance or increased resistance against potential attacks from viruses, bacteria, fungi or insects. For two decades, genetic engineering (Box I) has allowed the development of some made-to-measure plants to facilitate the spreading of chemical pesticides and to improve yield. The surface planted with genetically modified (GM) crops has constantly increased since 1996 to reach 134 million hectares in 2009, distributed over 25 countries worldwide and representing 8.7% of the total area of arable lands (James 2009; FAO 2010). Soybean, cotton, maize and canola are the most subjected to genetic transformation as 70%, 48%, 24% and 20% of the surface

hosting these cultures have been planted with genetically engineered plants, respectively (James 2008). Herbicide tolerance and insecticidal toxin production are the most commonly traits used in GM crops (Brookes and Barfoot 2009).

In forestry, the development of trees displaying desirable characteristics based on conventional breeding is difficult because of the long-reproductive cycle of woody species. Genetic transformations (Box II) and *in vitro* micro-propagation circumvent these barriers and make it possible to easily and quickly develop trees with desirable traits from a large interspecific pool of genes. Since the 1990's, progress has been made in tree transgenesis and the number of transformed woody species and field-tests have increased during the period 1990-2000 (van Frankenhuyzen and Beardmore 2004). The most common woody species subjected to genetic transformation belong to the genera *Populus* (47%), *Pinus* (19%), *Eucalyptus* (7%), *Liquidambar* (5%) and *Picea* (5%) (Marchadier and Sigaud 2005). The desired new traits are mainly of commercial interest such as improved vigour, alteration of wood lignin content, abiotic-stress (frost, dryness, salt) and herbicide tolerance and increased resistance against insects or microbial pathogens (Tzfira *et al.* 1998; Marchadier and Sigaud 2005; Teulieres and Marque 2007). However, most GM trees are still under development and field-tested. There are only two instances of commercial cultivation of tree: GM papaya exhibiting resistance against ringspot virus field-deployed in USA and *Bacillus thuringiensis* (Bt)-transformed black poplars field- deployed in 2002 on 200-300 ha in China (www.gmo-safety.eu).

The commercial growing of genetically modified organisms (GMOs) in agriculture raises public ecological awareness. The effects due to transgene insertion and / or expression raise the issues of the potential risks for the environment. The application of this technology to woody species has also given rise to intense public debate about environmental risk issues (Mathews and Campbell 2000; Strauss *et al.* 2009). The hazards associated with growing GM crops and GM trees are nearly the same: vertical gene flow (contamination of wild plant genomes due to transgenic pollen, seeds and vegetative propagule dispersion), GM plants escape with potential weedy behaviour, horizontal gene transfer, and foreseeable / unforeseeable effects of the insertion / expression of the transgene on non-target organisms. Nevertheless, temporal and spatial scales of the

interactions of GM trees with their environment differ from transgenic crops (van Frankenhuyzen and Beardmore 2004). Trees are long-lived perennials and they develop several biotic interactions with soil microbial communities for a longer period than crops. Therefore one can consider that the hazard of GM trees on non-target organisms is potentially more important compared to GM crops due to the longer exposure of microbial communities to them.

GMO impact on non-target organisms typically involves plants genetically engineered to increase their resistance against insects and microbial pathogens. Plants mainly interact with their environment through their root system. Therefore, soil microorganisms are potentially exposed to the new traits expressed by GM plants, among which fungi are important. The main functions of soil fungi are decomposers and mutualists (Christensen 1989; Bride and Spooner 2001) and along with fungal pathogens and parasites, they are involved in shaping plant community structure and dynamics. Among soil fungi, mycorrhizae are the fungal group displaying the most intimate relationships with their hosts (Box III). They play key roles in the main biogeochemical processes (Read and Perez-Moreno 2003; Leake *et al.* 2004; Read *et al.* 2004) and are thereby deeply involved in soil fertility. Most terrestrial plants in temperate, boreal, tropical, and subtropical regions are colonized by mycorrhizal fungi.

The innocuousness of GMOs toward non-target organisms is one of the five points listed by the Plant Biosafety Office (PBO) of the Canadian Food Inspection Agency (CFIA) that must be demonstrated prior to the release of transformed plants (Finstad *et al.* 2007). Here we survey 20 years of research monitoring desirable and undesirable impacts of GM crops and GM trees on targeted fungi (fungal pathogens) and non-targeted fungi (mostly mycorrhizal fungi).

The ISI web of Science online database was parsed by targeting the following key words in publication titles: genetically engineered, genetically modified, transgenic and transformed. These key words were associated in publication titles with the following plant key words: alfalfa, barley, canola, carnation, cotton, maize, papaya, petunia, potato, rice, sweet pepper, soybean, squash, sugar beet, tobacco, tomato, wheat, apple, aspen, birch, eucalyptus, liquidambar, pine, poplar, spruce. Database parsing was repeated with the

previous plant key words in their plural form and finally with the corresponding genus name. Moreover, the database was parsed for articles which included in their title the words glyphosate, herbicide, and insect associated either with the words tolerant or resistant plus the words fungi, fungus or fungal included in all the field. Over 6700 publications were retrieved. Additional researches were performed by parsing Google Scholar search engine and PubMed and JStor databases with different combination of the following key words: GMOs, GM trees, GM crops, impact, target fungi, non-target fungi, fungus, fungal. Database parsing was concluded in April 2010. The software Papers v1.9.3 (http://mekentosj.com) was used to parse databases, store, organize and analyze publications.

1.5 Interaction of GM crops with fungi

1.5.1 Overview

From 1991 to 2010, we recorded 117 studies assessing the potential effect of transgenic crops on fungi (Table 1.1). Investigating the effect of GM plant on fungi was performed mainly under controlled conditions (in 96 studies out of 117). The analysis of these publications showed that the impact of transgenic plants on target fungi has been better monitored than the impact on non-target fungi. Thus, during this period, we recorded 84 studies examining GM crops impact on target fungi against 35 studies on non-target fungi. Figure 1.1A shows the progress in field planting GM crops with the number of impact studies on target and non-target fungi. While the average area planted with GM crops continually increased by 10 millions ha/year since 2000, the number of surveys of GM crop impact on fungi remained steady with 10 studies/year on average, including the year 2008 when 17 articles were published. From 1996 to 2009, studies on non-target fungi represented approximately 25 % of the annual articles published each year about GM crop relationships with fungi. The assessment of GM plant impacts on target and non-target fungi (Fig. 1.2A) mainly involved plants from genera *Nicotiana* (23%), *Oryza* (19%) and *Triticum* (16%). As might be expected, 69% of the new traits expressed in GM plants tested

against target and non-target fungi were related to fungal disease resistance (Fig. 1.2B). Taking into consideration only studies monitoring GM crops on non-target fungi, this trait represented 13% while insect resistance was the trait the most frequently monitored (40%, data not shown). Studies monitoring the impact of herbicide tolerant plants on fungi represented 4 % of the studies retrieved (Fig. 1.2B). Herbicide tolerance is the dominant trait in commercialized transgenic cultures, representing 63% of the surface cultivated with GM crops in 2008 (James 2008).

1.5.2 GM crops impact on target fungi

GM plants with enhanced antifungal activities have been developed and tested against fungal pathogens belonging mainly to the genera *Rhizoctonia* (22%), *Magnaporthe* (15%), *Fusarium* (11%), *Botrytis* (6%), *Sclerotinia* (6%) and *Erysiphe* (5%, Fig. 1.3A). One hundred and thirty nine assays showed the efficiency of GM plants to limit fungal pathogen establishment and development. Most of them were transformed with vectors carrying pathogenesis-related (PR) proteins like β-1,3-glucanase (PR-2), chitinase (PR-3), thaumatine like protein (PR-5) under the control of cauliflower mosaic virus 35S or ubiquitin promoters. The mean exposure time of target fungi to transgenic plants was 28 days (median = 13, min = 1, max = 473) and symptom severity based on counting and measuring necrotic lesions was the most common response variable recorded.

Among the few studies that tested GM crops resistance against target fungi in the field, six surveys out of seven involved transgenic wheat. GM plants with increased tolerance to fungal pathogen under controlled conditions did not necessarily display the same level of resistance when field-deployed. For example, Anand *et al*. (2003) showed that a wheat line expressing the thaumatin-like protein (tlp) transgene was significantly less susceptible to scab (*Fusarium graminearum*) when grown in greenhouse (reduced mean number of infected spikelets / head). However, disease severity observed on this transgenic line during field-test was either similar to or higher than the susceptible controls. They hypothesized that lesion-mimic phenotype could compromise the plant ability to undergo continuous pathogen pressure in the field. Shin *et al*. (2008) observed as well disparate results between greenhouse- and field-grown transgenic wheat carrying a barley chitinase transgene.

Chitinase-expressed levels were similar between controls and transgenic lines albeit these last ones had enhanced resistance to scab in greenhouse but similar susceptibility with controls in the field. Other studies that evaluated qualitatively or quantitatively GM plant performance in the field showed improved fungal resistance, with respect to controls (Cober *et al.* 2003; Schlaich *et al.* 2006; Zhao *et al.* 2006; Mackintosh *et al.* 2007; Luo *et al.* 2008).

1.5.3 GM crops impact on non-target fungi

No evidence of negative effects on non-target fungi

The issue of the potential effects of GMO cultures on non-target fungi has been addressed either by monitoring the level of colonisation and development of vesicular arbuscular mycorrhizal fungi (mainly represented by *Glomus mosseae* and *Glomus intraradices*) or by investigating soil fungal communities by the mean of fungal culturing, biomarkers or molecular tools (Fig. 1.3B). Forty-two assays out of 56 showed no effect on non-target fungi associated with GM plants or occurring in their vicinity. The mean exposure time of non-target fungi to transgenic plants was 63 days (median = 38, min = 12, max = 473). Thirteen studies out of 35 on non-target fungi were performed in the field. Only two studies evaluated the impact on both target and non-target fungi. Vierheilig *et al.* (1993) showed *Nicotiana sylvestris* genetically transformed with different chitinase genes to be more resistant to *Rhizoctonia solani* while the colonization by the endomycorrhizal fungus *G. mosseae* was not affected. It was proposed that proteins or alkalisoluble polysaccharides might cover the chitin layer of the fungal cell wall and prevent chitinase binding to endomycorrhizal hyphae. Turrini *et al.* (2004a) transformed aubergine plants to constitutively express the Dm-AMP1 antimicrobial defensin and monitored, under controlled conditions, the effects on the soilborne fungal pathogen *Verticillium albo-atrum*, phytopathogenic fungus infecting leave *Botrytis cinerea* and endomycorrhizal *G. mosseae*. They showed that the growth of *V. albo-atrum* colonies was reduced by 49 to 71% with respect to controls by the release of active antifungal protein from the roots of transformed aubergine plants. The necrotic areas of leaves infected with *B. cinerea* were also reduced by 36 to 100%. At the opposite, host recognition responses and establishment of the

endomycorrhizal symbiosis was not affected in these transgenic lines. To explain the differential effect of Dm-AMP1 transformed aubergines on the beneficial endomycorrhizal symbiosis, the authors hypothesized that *G. mosseae* hyphal membranes would not expose at their surface patches containing sphingolipids to which Dm-AMP1 binds then causes damages to membranes (Thevissen *et al.* 2000; 2003).

Three other studies assessed the potential effects of GM crops with enhanced fungal resistance on non-target fungi. Vierheilig *et al.* (1995) inoculated *G. mosseae* to tobacco constitutively expressing pathogenesis-related proteins like chitinase or glucanase and observed difference neither in the final level of root colonization nor during the time course of colonization between control and transformed tobacco. The roots of defensin-transformed aubergine were as susceptible as those of non-transformed aubergine to the colonization by *G. mosseae* (Turrini *et al.* 2004a; 2004b). Girlanda *et al.* (2008) investigated the potential impact on endomycorrhizal fungi and saprotrophic fungi associated with the rhizosphere and phyllosphere of tomatoes genetically engineered to express chitinase and glucanase proteins. They found no significant difference in the frequency and intensity of endomycorrhization and in the arbuscular quality between transgenic tomatoes and controls, after two and eight months of interaction. Furthermore, based on the isolation and identification of over 20500 fungal colonies retrieved in the rhyzosphere and phyllosphere of transgenic tomatoes and controls, no evidence of change within the two fungal communities was observed, after the same time of interaction with transgenic tomatoes.

GM plants galvanize fungi: unexpected effects

Up until now, techniques used to transform plants (Box II) do not allow to control neither the number nor the position of the transgene(s) incorporated within the recipient plant genome. This leads sometimes to the expression of unexpected traits (pleiotropic effects) that are not directly related to the transgene expression. Major changes can be observed when pleiotropic effects result in modifying plant root exudation. Indeed, root exudates play a key role in structuring soil microbial communities (Kowalchuk *et al.* 2003; Bais *et al.* 2006; Broeckling *et al.* 2008). The most acute consequence recorded in the literature due to pleiotropic effect was about transgenic insect-resistant lines of cotton. In China, 64

varieties of pest-resistant cotton are grown on 3.7 million hectares representing 70% of total cotton culture area (Stone 2008). Since their field-deployment, it has been observed that certain GM cotton lines were as susceptible to Fusarium and Verticillium wilts as cultivars lacking resistance. Li *et al*. (2009b) showed that root exudates of Cry1Ac/CpTI- and Cry1Ac-transformed lines contained more sugar (fructose, maltose and an unknown sugar) than parental control lines. This augmentation of sugar concentration in root exudates favoured the development of the fungal pathogen *Fusarium oxysporum* within the rhizosphere, increasing cotton plant mortality. Despite the fact that these GM plants circumvent mortality due to Lepidoptera attacks and avert the use of 650000 tons of insecticides, fungicide use is now required to control Fusarium and Verticillium (Stone 2008; Li *et al*. 2009b). Another instance of unexpected increase fungal activity is transgenic wheat expressing both chitinase and glucanase. Bieri *et al*. (2003) showed that some transgenic wheat lines were more susceptible to *Blumeria graminis* f.sp. *tritici* while other transgenic lines displayed a resistant phenotype in a leaf bioessay. They also observed that plants displaying the highest level of these two anti-fungal enzymes within their tissues were the most susceptible to the powdery mildew. The authors suspected the high level of β-1,3-glucanase to be responsible of the decrease in plant resistance against *B. graminis*. They hypothesized that glucanase could interfere with papillae formation during the infection stage as they are made of callose (Aist 1976) which is a substrate of β-1,3-glucanase. Kremer et al. (2005) observed that the interaction between glyphosate treatment and glyphosate-resistant (GR) soybean significantly increased the biomass of three out of four *Fusarium* strains in root exudates of GR soybean compared to non-GR cultivars treated with glyphosate. The authors showed that the root exudates of GR soybean released higher carbohydrate and amino acid contents than non-GR cultivars. Moreover, glyphosate treatment also enhanced these compounds in root exudates in both GR and non-GR cultivars.

Five other studies demonstrated that endomycorrhizal fungi and other soil fungi were stimulated when associated with GM plants. Donegan *et al*. (1995) observed a transient increase in the colony-forming units (CFU) of cultivable fungi from soil samples associated with two lines of insect-tolerant cotton. Staehelin *et al*. (2001) showed that the gene

enod40, involved in formation of root nodules, upregulates mycorrhizal formation and development. The overexpression of enod40 in alfalfa significantly increased the frequency of arbuscules and vesicles in roots. Hénault *et al.* (2006) investigated the potential impact of lignin-modified tobacco residues on soil fungal community. They observed by measuring the proportion of double unsaturated chain fatty acids, which are indicative of fungi, that the soil fungal community incubated for 14 days with residues from one tobacco line significantly increased compared to controls. O'Callaghan *et al.* (2008) monitored fungal communities associated with three lines of potato genetically modified to express the antimicrobial peptide magainin, by counting the number of fungal CFU on the surface of leaves, roots and tubers, after two and four months of field-growth. Results showed contrasting effects at time of harvest. The number of fungal CFU was significantly higher in roots and lower in tubers of the transgenic line D9, with respect to unmodified parental line and unrelated cultivars. The two other transformed lines did not show difference with the controls. Contrasting results were also observed in the abundance of saprophytic fungi isolated from the rhizosphere of maize expressing the Cry1Ab protein (Oliveira *et al.* 2008). Thirty days after sowing, the fungal CFU were significantly more abundant in soil samples from one transformed line while significantly less abundant in soil samples from another transformed line. Finally, Weinert *et al.* (2009) analyzed the soil fungal diversity during three developmental stages of two GM potato lines genetically modified to accumulate the carotenoid zeaxanthin in their tubers. They observed a significant shift in fungal denaturing gradient gel electrophoresis (DGGE) fingerprints based on the internal transcribed spacer (ITS) sequences between GM potato lines and their parental cultivar. Nevertheless, the differences observed between the soil fungal communities associated with four commercial potato cultivars were higher than those observed between the two transgenic potato lines and their parental counterparts.

GM plants with negative effects on non-target fungi

Five assessments of GM impact on non-target fungi observed deleterious effects (Staehelin *et al.* 2001; Turrini *et al.* 2004b; Castaldini *et al.* 2005; O'Callaghan *et al.* 2008; Oliveira *et al.* 2008). None of these transgenic plants were transformed to express anti-fungal proteins. Staehelin *et al.* (2001) observed that the downregulation of the enod40 transcription within

transgenic lines of *Medicago truncatula* resulted in a significant lower colonization of roots by *G. intraradices* compared to controls. In three cases out of five, these assessments involved plants genetically engineered to increase their insect-tolerance through the expression of the insecticidal toxin encoded by the Cry1Ab gene from *Bacillus thuringiensis*. Turrini *et al.* (2004b) compared the effect of root exudates of the lines Bt corn 176 and Bt corn 11 on *G. mosseae* pre-symbiotic growth and hyphal differential morphogenesis. The line Bt corn 176 significantly reduced the length of *G. mosseae* mycelium and the number of appressoria developing infection units as 35.7 % of appressoria were not able to colonize roots, 35 days after inoculation. A higher level of Cry1Ab toxin was measured in the line Bt corn 176 (80.63 Cry1Ab / g protein) that negatively affected *G. mosseae* compared to the line Bt corn 11 (< 0.55 Cry1Ab / g protein) that was shown to be not deleterious on the endomycorrhizal symbiosis. Castaldini *et al.* (2005) monitored the development of endomycorrhizal infection units and the endomycorrhizal colonization of the lines Bt corn 176 and Bt corn 11. In both transformed lines, the intraradical colonization by *G. mosseae* was significantly lower (about 50 %) compared to wild type, after eight and ten weeks of interaction under controlled conditions. The number of entry points developing arbuscules at eight days was significantly reduced in the roots of the lines Bt corn 11 and Bt corn 176 by 72% and 67% respectively. The percentage of root length colonized by arbuscular mycorrhizal fungi was significantly lower in *Medicago sativa* grown for four months in soil containing Bt corn 11 line residues. The reasons for which Bt corn lines are less susceptible to endomycorrhizal colonization remain unknown.

1.6 Interaction of GM trees with fungi

1.6.1 Overview

The potential impact of transgenic trees on fungi has been assessed in 32 studies published between 1996 and 2010 (Table 1.2 and Fig. 1.1B). Since 2000, three studies on average monitoring the potential impact of GM trees on fungi are published each year, which is

three times less than studies related to GM crop impact on fungi during the same interval. The potential impact of GM plants on non-target fungi has prompted more attention in forestry as they accounted for 50 % of the studies recorded compared to 25 % in agriculture (Figs. 1.1A and 1.1B). Valenzuela *et al.* (2006) showed that the number of experiments field-testing GM trees worldwide increased from seven to 36 in the period 1996-2001. The number of outdoor plots recorded in USA and Europe was 46 in 2003, 354 in 2005 (Robischon 2006) and it reached 528 in 2010 (www.gmo-safety.eu). Poplar, pine, apple and eucalyptus were the dominant species transformed and field-deployed. The three principal traits expressed in these outdoor experiments were disease resistance (20%), selectable marker gene (16%) and herbicide tolerance (14%) (Fig. 1.4A). Fungal disease resistance was the most frequent trait (66%) expressed by GM trees that were studied about their potential impact on fungi, far followed by altered lignin composition (12%) (Fig. 1.4B). These transgenic trees belong for the most part to the genera *Populus* (35%), *Malus* (29%), *Betula* (21%) and *Picea* (8%) (Fig. 1.5). No data about the potential impact of GM eucalyptus and GM pine on fungi was retrieved.

Fungal disease resistance was mainly increased within GM trees by the insertion and expression of genes encoding for chitinolytic enzymes. Chitinases have been isolated from bacteria, fungi and plants and can be classified in two groups according to the way they cleave chitin. Endochitinase degrades chitin wall (a major component of the fungal cell wall) by randomly cleaving internal sites of the chitin molecule whereas exochitinase attacks the chitin molecule from it nonreducing end (Cohen-Kupiec and Chet 1998). The insertion of gene(s) encoding chitinase(s) within a plant genome promotes the fungal disease resistance of this plant via a chitinolytic activity increased.

1.6.2 GM trees impact on target fungi

Thirty-seven assays out of 59 investigated the impact of GM trees on target fungi, mainly under controlled conditions. Trees genetically modified to enhance their fungal disease resistance were tested against fungal pathogens from the genera *Venturia* and *Septoria* in over 50 % of the experiments recorded between 1996 and 2010. Disease severity between transgenic lines and control trees was evaluated by counting the number of sporulating

lesions and conidia on inoculated foliar disks or detached leaves and by estimating the percentage of leaf area infected. The exposure time with the pathogen was 32.4 days on average (median = 14, min = 4, max = 157). Table 1.2 lists 24 assays where GM tree resistance was increased against fungal pathogens. Among these successful assays, Bolar *et al.* (2001) took advantage of the synergistic effect of endochitinase and exochitinase to enhance apple resistance against *Venturia inaequalis*. They developed apple transgenic lines by the means of *Agrobacterium tumefasciens* containing plasmids carrying both genes encoding endochitinase (ech42) and exochitinase (Nag70). Results showed that the percent of leaf area infected was 30% in non-transformed apple trees while it was reduced to 1.3-12% in transgenic lines expressing both chitinases. Resistance against *V. inaequalis* in transgenic lines expressing both enzymes was higher than in transgenic lines transformed with either ech42 or Nag70 gene alone. Transgenic lines displaying the highest resistance against apple scab were as vigorous as control trees. Bolar *et al.* (2000) observed that there was a significant negative correlation between the level of expressed endochitinase and growth of ech42-transformed apple trees.

Only one study investigated the potential impact of GM tree with enhanced fungal disease resistance on both target and non-target fungi. Newhouse *et al.* (2007) evaluated the effects of *Ulmus americana* trees genetically modified with the gene ESF39A, encoding for a synthetic antimicrobial peptide, on *Ophiostoma novo-ulmi* (Dutch-elm disease) and on mycorrhizal fungi after three months of field-planting. They demonstrated that sapwood staining spread on a significantly shorter distance in one transformed line after *O. novo-ulmi* inoculation. The percentage of root length colonized by mycorrhizal fungi was similar between transgenic (75%) and control trees (76%).

Four studies investigating the potential impact of GM trees transformed with anti-fungal or anti-microbial genes or even with genes involved in plant defence mechanism showed failure in increasing tree fungal disease resistance whereas two studies showed GM trees with opposite desired traits. Mohamed *et al.* (2001) transformed two hybrid poplars with the bacterio-opsin (*bO*) gene from *Halobacterium halobium* to stimulate their hypersensitive response. GM poplars were then challenged with *Melampsora occidentalis, Venturia populina, Septoria musiva and Septoria populicola* under greenhouse conditions

and in the field. Results showed that the overexpression of *bO* gene did not decrease poplar susceptibility to fungal pathogens tested while the growth rate of one transgenic line was severely affected. Liang *et al.* (2002) enhanced fungal disease resistance of the hybrid poplar clone Ogy (*Populus* × *euramericana*) when transformed with the antimicrobial peptide gene ESF12. On the other hand, the hybrid poplar clone NM6 (*Populus nigra* × *P. maximowizii*) similarly transformed was as susceptible as controls to *S. musiva* infection. Faize *et al.* (2004) transformed two varieties of apple tree (one carrying the natural resistance *Vf* gene and the other not) with gene encoding wheat puroindoline B (pinB). Under controlled conditions, they inoculated leaves of GM trees and controls with two different races of *V. inaequalis*. Only few transgenic lines exhibited a significant increased resistance against one of the two races of *V. inaequalis*. This result emphasized the limited resistance potential of these GM apples under field conditions where they would face up to several different races of fungal pathogens. Pasonen *et al.* (2004) monitored in the field, during three growing seasons, the response of silver birches (*Betula pendula*) transformed with chitinase IV gene from sugar beet to natural infection by *Pyrenopeziza betulicola* (leaf spot disease) and *Melampsoridium betulinum* (birch rust). They showed that the number of rust pustules on leaves from four transgenic lines in 2002 and three transgenic lines in 2003 was significantly reduced. However, only one single transgenic line showed a significant decrease of the number of rust pustules on its leaves during these two consecutive growing seasons. Two other disease parameters were measured, the general disease score and percent area covered by birch rust. Similar results were recorded between all transgenic lines and control trees in 2002 and 2003. Monitoring the resistance of these chitinase-transformed silver birches against *P. betulicola* showed either a similar or significant increased susceptibility of some transgenic lines to the leaf spot disease, compared to controls. This result was inconsistent with the analyses performed by Pappinen *et al.* (2002) on the same transgenic lines in greenhouse. They showed that the transgenic lines displaying the highest accumulation of chitinase IV transcripts had a significant increased resistance against *P. betulicola*. The exposure in the field of transgenic silver birches to different genotypes of *P. betulicola* may explain the differential susceptibility observed between greenhouse and field trials. The authors also hypothesized that the overexpression

of chitinase may have a fitness cost with potential backlash on cell functions and plant defence. Seppänen *et al.* (2004) described another instance of unexpected increased susceptibility of transgenic trees to fungal pathogen. They performed a wood decay assay involving the fungal pathogen *Phellinus tremulae* causing white trunk rot of aspens and hybrid aspens transformed with the pinosylvin synthase gene to increase their antifungal activity. Results showed that two transgenic hybrid aspen lines decayed significantly faster than the controls while one transgenic aspen line was more resistant to *P. tremulae*. It was hypothesized that the low concentration of stilbenes measured into the two transgenic hybrid aspen lines might stimulate the *P. tremulae* activity.

1.6.3 GM trees impact on non-target fungi

Half of the studies monitoring the potential impact of GM trees on non-target fungi was performed in the field and mainly focused on fungal communities occurring in soil or leaf litter. The exposure time of non-target fungi to transgenic trees was 1.9 year on average (median = 259 days, min = 28 days, max = 9 years). Among the different types of mycorrhizal fungi, those forming ectomycorrhizal (EM) association with GM trees were the most studied. Under controlled conditions, potential impact of GM trees was principally assessed on the EM fungus *Paxillus involutus* (Pasonen *et al.* 2005; Seppänen *et al.* 2007; Tiimonen *et al.* 2008; Sutela *et al.* 2009). Five studies observed direct or indirect impact on non-target fungi. Kaldorf *et al.* (2002) modified the phytohormone balance of hybrid aspen by the introduction of the *rolC* gene from *Agrobacterium rhizogene*. Transgenic aspens were field-deployed in 1996 and root samples were collected in 1998, 1999 and 2000. Ninety-eight percent of the EM root tips identified belonged to eight morphotypes. No significant difference between transgenic aspens and controls was found in root mycorrhizal colonization and diversity but one of the four dominating EM morphotypes was significantly less abundant and poorly developed on roots of one transgenic line. This effect was clone-specific as the roots from the two other transgenic aspen lines showed no difference in the abundance and development of this morphotype, with respect to controls. Wei *et al.* (2006) observed changes in soil microbial rhizosphere after nine months of growth of papaya tree genetically modified to enhance its resistance against the ringspot

virus. The number of fungal CFU was significantly higher in soil samples from transgenic papaya trees than soil samples from non-transformed papaya trees. Moreover, the number of kanamycin-resistant fungal colonies increased in soil planted with transgenic papaya trees carrying the kanamycin marker gene. Bradley *et al*. (2007) monitored soil microbial responses in three different soils planted with three lines of transgenic poplar genetically modified to alter its lignin biosynthesis. They observed that the abundance of fungal biomarkers (neutral lipid fatty acids) was significantly reduced (30 to 50% compared to controls) in soil samples associated with one transgenic line grown in silt loam. In this case, the negative effect of GM trees on non-target fungi was not only clone-specific but also soil-specific. Seppänen *et al*. (2007) showed that transgenic silver birches with modified lignin biosynthesis developed normal EM structures. The level of root tip colonization was similar between transgenic birches and controls, 35-39 days after inoculation with *P. involutus*. However, the fungal biomass retrieved in bags containing transgenic leaves let to decay in the field during seven and eleven months was either significantly lower (for two transgenic lines) or higher (for one transgenic line) than the fungal biomass retrieved in bags filled with leaves from control trees. Tiimonen *et al*. (2008) observed that the number of lateral roots covered with *P. involutus* hyphae and the in-depth development of the Hartig net was reduced in some lignin-modified transgenic lines of silver birch with respect to controls. The decrease in Hartig net development seemed not to have consequence on the beneficial effect resulting from the symbiosis establishment, as the percentage of viable plant increased due to *P. involutus* inoculation. In all the previous studies that observed an impact on non-target fungi, the effects were transgenic line-specific.

One study investigated the potential impact of insect-tolerant transformed white spruce on non-target fungi. Stefani and Bérubé (2006) isolated and identified foliar fungal endophytes inhabiting needles of Cry1Ab-transformed and non-transformed trees. They observed no significant effect of the transformation on the incidence of the most common fungal endophytes retrieved. Numbers of endophytic isolates and endophytic taxa per clonal line were similar to those of controls.

As regards the horizontal gene transfer, Zhang *et al*. (2005) inoculated *Amanita muscaria* to hybrid poplars genetically engineered to enhance their herbicide tolerance

through the insertion and expression of the *bar* gene. Four to six weeks after inoculation, 35000 EM root tips of these transgenic hybrid poplars were plated on a selective media containing 200 μg ml^{-1} Basta herbicide. One hundred and two EM fungal colonies developed. However, the subculture of these colonies on fresh media containing Basta stopped fungal growth. Moreover, the *bar* gene was not recovered from the amplification of the genomic DNA extracted from these colonies. They were considered as false positives and their occurrence was explained by a too weak herbicide concentration in the growth medium.

1.7 Conclusion

Fungi are found everywhere in the environment and develop complex relationships with plants. Soil fungi and mycorrhizal fungi in particular are major players in ecological systems on which we depend. They are involved in soil formation, stabilization and fertility. Therefore, gauging the environmental risks of GM plants cannot be properly done if their effect on non-target fungi is not well defined. The spreading of GM plants on 8.7 % of the arable land in 2009 urges to accurately monitor GM plants impact on fungi, to increase the number of field-scale studies and to focus on widely used commercial GM plants. This review clearly shows that there is a knowledge gap about the potential impact on non-target fungi of GM plants that are today planted globally. Indeed, herbicide and insect tolerance traits dominate areas grown with GM crops while the consequences of GM plants expressing these traits on fungi remain understudied. Liu and Du (2008) highlighted that interactions between GM plants expressing the insecticidal crystal proteins from *Bacillus thuringiensis* and vesicular arbuscular mycorrhizal fungi are a new urgent soil ecology issue in agro-ecosystems. Our review shows that the impact of herbicide tolerant plants on fungi is also a pressing question that needs to be addressed. Moreover, the success of GM plants like glyphosate resistant plants in agriculture leads to an increase in land surface spread with glyphosate while there are evidence in the literature that glyphosate can have adverse effect on fungal pathogen (Anderson *et al*. 2005; Feng *et al*. 2005; 2008).

Transgenic-line specific effects on fungi have been recorded, emphasizing that the unpredictable side effect from transgene insertion requires evaluating each transformed line. Moreover some experiments showed that results observed under controlled conditions cannot be extrapolated to what would be observed under field conditions. This is somewhat troubling while most of the impact studies reviewed were performed under controlled conditions, although the frequency of field-scale studies monitoring GM plants impact on fungi has increased since 2006 in forestry and 2008 in agriculture. It stresses the need to systematically assess GM plants impact in field trial prior stating about their innocuousness.

Studies showing significant perturbation on fungi due to the occurrence of GM plants comprise a small proportion of the 149 studies reviewed. These effects were from GM plants expressing traits that were unexpected to affect fungi. This highlights that inadvertent trait expression of GM plants is the main source of risk to fungi. Among the unexpected trait observed in GM plants that lead to significant change in non-target fungi, modification in root compound exudates have been recorded. Therefore GM plant root exudates should be systematically characterized and contrasted to their non-transformed counterparts to assess GM plant effect on the environment.

Among studies monitoring the potential impact of GM plants on fungal diversity in outdoor experiments, they are some instance where soil type, plant genotype (i.e. different cultivars), growing stage, season, and environmental conditions bring stronger effects on fungal diversity and fungal community structure than the occurrence of GM plants (Naef *et al.* 2006; O'Callaghan *et al.* 2008; Weinert *et al.* 2009; Yi *et al.* 2009; Tan *et al.* 2010; Hart *et al.* 2009). Therefore, interpreting the variability observed between transgenic and non-transformed plants under field conditions should systematically be adjusted to the natural variability.

1.8 Acknowledgements

We are grateful to Marie-Josée Bergeron, Dr. Philippe Tanguay, Dr. Yves Piché and Dr. Jean Bérubé for insightful comments in the course of this study and on an earlier draft of

the manuscript. This work was supported by a grant from the Canadian Regulatory System for Biotechnology Fund.

Box I. Glossary

Biotechnology: represents the whole methods and techniques that use microorganisms or some of their elements like proteins, enzymes or genes to create products profitable to pharmaceutical, food-processing industries and agronomy.

Genetic engineering: represents the whole techniques used in molecular biology to isolate, characterize, modify, clone, transfer and express genes within the genome of a host organism. The origin of the genetic engineering is difficult to date as it includes breeding prior gene manipulation. Nevertheless, the modern meaning of genetic engineering can be rooted in 70's. The first publication showing in vitro the assemblage of two DNA molecules of different origin (a monkey virus and the bacteriophage lambda) is attributed to Paul Berg research team in 1972 (Jackson et al. 1972). Also, Werner Arber, Hamilton O. Smith and Daniel Nathans shared the Nobel price in 1978 for their discovery of restriction enzymes giving birth to recombinant genetic engineering.

GMOs (genetically modified organisms): organism displaying a new phenotype by the mean of genetic engineering. New phenotypes are the result of endogenous gene knockdown or overexpression, or they can be acquired by the insertion and expression of exogenous genes into the nuclear genome of the recipient organism.

Risk: the probability that an undesirable event will happen.

Target and non-target fungi: target fungi are fungal pathogen, involved in major crop and tree diseases and that were inoculated to plants genetically engineered to increase their antifungal activity. Rhizosphere, epiphyte and endophyte fungi, ranging from pathogenic to mutualistic, and that can be affected by the occurrence of GM plants, whatever the new trait expressed, are defined as non-target fungi.

Box II. Genetic transformation

Since the advent of the modern genetic engineering, physical, chemical and biological methods to transfer foreign DNA molecules within a host genome have been used with variable efficiencies. Common methods used to incorporate foreign DNA into plant genomes are electroporation (Wong and Neumann 1982), polyethylene glycol treatment, microinjection (Yamamoto *et al.* 1982), *Agrobacterium*-mediated transformation and particle bombardment (Sanford *et al.* 1987).

Electroporation (or electrically mediated gene transfer) allows transferring linear or circular DNA into cells by increasing membrane permeability through short electric impulses above a certain field strength. Transformation by the mean of electroporation can be performed on protoplasts or intact cells, and transformation efficiency can be increased when coupled with polyethylene glycol.

Polyethylene glycol (PEG) increases the osmotic pressure that involves protoplast contraction and foreign DNA molecules are subsequently incorporated through the plasma membrane by endocytosis (Barcelo and Lazzeri 1998).

Microinjection makes possible to introduce foreign DNA into the nucleus or cytoplasm of a host cell under optical control and without damaging cells using a fine cannula. The targeted cells can be immature embryos, meristems, pollen, ovules, calli and protoplasts.

***Agrobacterium*-mediated transformation** requires to co-cultivate explants, cells or protoplasts with the soil-born *Agrobacterium tumefasciens* or *A. rhizogenes* involved in crown gall or hairy root formation. *Agrobacteria* contain tumor-inducing (Ti) plasmids. Ti plasmids are genetically modified to replace the original transferred DNA (T-DNA) by a gene of interest that will be inserted into the chromosomal DNA of the plant cell. Originally, *Agrobacteria* only infect dicotyledonous plants, but modifications of plasmid vectors and fine tuning transformation conditions allow now to transform monocotyledonous plants, i.e. most of the cereal plants.

Particle bombardment (also called biolistic particle delivery) is a process that can transform plant embryogenic cell suspensions, meristems, embryos, pollen and leaf tissues (Sanford 1990). Cell walls and cell membranes are crossed by high velocity microparticles (tungsten or gold) coated with DNA molecules of interest. Surviving cells incorporate the foreign DNA in their genome. This method requires a minimum of biological material preparation and it allows introducing DNA in many cells simultaneously. *Agrobacterium*-mediated transformation and particle bombardment are the two methods that predominate to genetically modify plant genome.

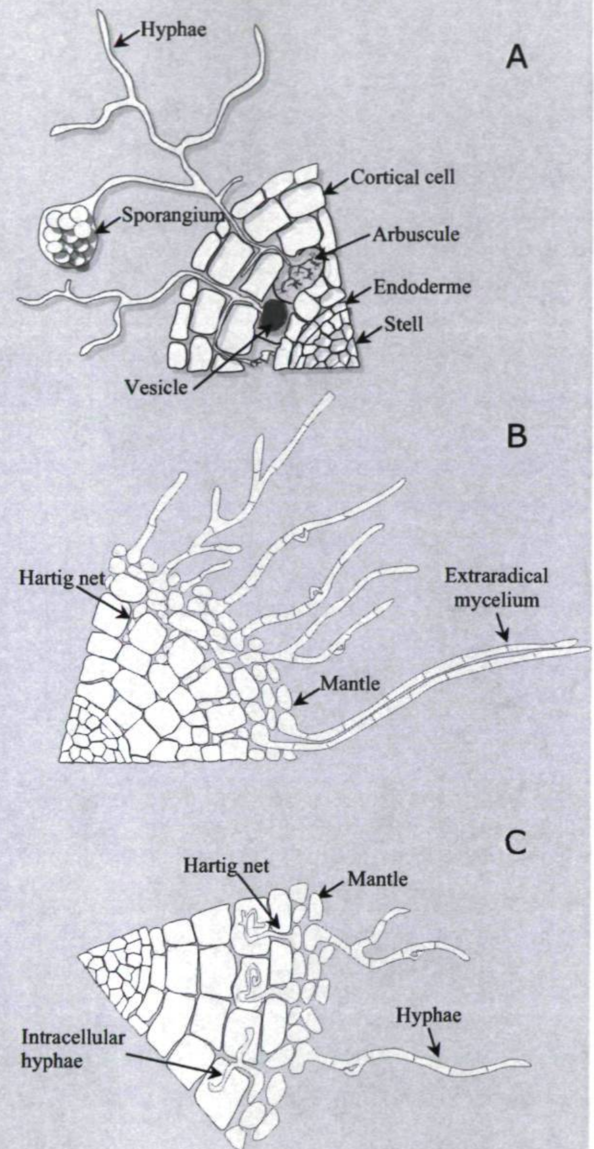
Box III. Mycorrhizal symbioses

Mycorrhizal fungi can be distinguished according seven types of structures that they develop with roots of their host plant (see Peterson and Massicotte 2004 for a detailed review). Below are summarized features of the three main mycorrhizal symbioses that can be observed on crops and forest species.

Arbuscular mycorrhizas (AM, Fig. A.) are ubiquitous and play a keystone role in soil ecology. They originated more then 550 million years ago (Berbee and Taylor 1993) and probably helped the first plant colonizing land. All phyla of terrestrial plant are susceptible to be colonized by AM and about 150 species have been described (Smith and Read 2008). Arbuscular mycorrhizas are obligate biotrophic organisms and colonize roots to develop vesicles (storage structure located within or between cortical cells) and arbuscules (within cortical cells) where nutrient exchange takes place (Bonfante 1994).

Ectomycorrhizas (EM, Fig. B) differ from AM by the occurence of a layer of hyphae (fungal mantle) enclosing root tips, hyphae colonizing intercellular spaces between cortical cells (Hartig net) and hyphae scavenging soil for nutrients (extraradical mycelium) and making the link with sporocarps. Hartig net and the inner part of the mantle represent the two main interfaces for bidirectional nutrient exchange (Massicotte *et al.* 1987; Dexheimer and Gérard 1994). Most of the woody species in temperate, boreal, tropical, and subtropical forests are colonized by EM (Read 1991) while it is less common to observe the roots of herbaceous species associated with EM. No intracellular structure is present within root tip colonized by EM. Ectomycorrhizas are a polyphyletic group comprising over 5000 species (Molina *et al.* 1992), mainly composed of Agaricomycetes (Hibbett and Matheny 2009).

Ectendomycorrhizas (ECM, Fig. C) share two structural characteristics with ectomycorrhizal fungi as they form a thin mantle enclosing root tips and a Hartig net. Contrary to EM, root tips associated with ECM exhibit intracellular penetration of hyphae. The interface involved in nutrient exchange between the ECM and their host remains unknown (Yu *et al.* 2001). All the ectendomycorrhizal fungi identified until now are ascomycetes, mainly member of the Pezizales and Helotiales. ECM are very common under greenhouse conditions and nurseries on roots of *Pinus, Larix, Picea, Abies, Tsuga, Pseudotsuga, Betula, Populus* (Scales and Peterson 1991; Smith and Read 2008). They are also very common on roots of plants in disturbed sites like post-fire forest (Vrålstad *et al.* 2002; Fujimura *et al.* 2005).



Modified from Le Tacon (1985)

The four other mycorrhizal symbioses recognized are ericoid, arbutoid, monotropoid, and orchid mycorrhizas. **Ericoid mycorrhizas** form intracellular hyphal coils in epidermal cells with roots of plants species belonging to the Ericaceae and Epacridaceae, and are mainly ascomycetes of *Oidiodendron* and *Hymenoscyphus* groups (Monreal *et al.* 1999). **Arbutoid** and **monotropoid mycorrhizas** colonized the Ericales and the nonphotosynthetic subfamily of the Monotropoideae, respectively, and involved basidiomycetes that also form EM. They display the same structures as ECM except the inter- and intracellular structures are limited to the epidermis (Peterson and Massicotte 2004). Monotropoid mycorrhizas develop fungal pegs within epidermal cells. **Orchid mycorrhizas** involved basidiomycetes of Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae families that form complex hyphal coils (pelotons) in cortical cells of the parenchyma (Smith and Read 2008).

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Table 1.1 Studies investigating the impact of genetically modified crops on target and non-target fungi from 1991 to 2010.

First author	Year	Transformed plant	Event [a]	Desired phenotypes	Impact on target fungi [b]	Impact on-non target fungi	Study type	Exposure time [c]	Variable response
Broglie	1991	*Nicotiana tabacum*	*CaMV35S*-CH5B	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	11-18-23 d	Seedling mortality and root fresh weight
Logemann	1992	*Nicotiana tabacum*	*wun1*-RIP	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	4-12 d	Plant heights
Vierheilig	1993	*Nicotiana sylvestris*	*CaMV35S*-ChitA	Fungal disease resistance	↘*Rhizoctonia solani*	→*Glomus mosseae*	Controlled conditions	5 w	Degree of infected/colonized root length
		Nicotiana sylvestris	*CaMV35S*-ChitAΔH		↘*Rhizoctonia solani*	→*Glomus mosseae*			
		Nicotiana sylvestris	*CaMV35S*-ChitAΔT		↘*Rhizoctonia solani*	→*Glomus mosseae*			
Donegan	1995	*Gossypium* sp.	*CaMV35S*-Cry1Ab	Insect resistance	—	↗Soil fungi	Controlled conditions	4-8 w	*Fungal CFU/g soil*
		Gossypium sp.	*CaMV35S*-Cry1Ac			↗Soil fungi			
Jach	1995	*Nicotiana tabacum*	*CaMV35S*-CHI	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	15-20 d	A five class disease severity scale
		Nicotiana tabacum	*CaMV35S*-GLU		↘*Rhizoctonia solani*				
		Nicotiana tabacum	*CaMV35S*-RIP		↘*Rhizoctonia solani*				
		Nicotiana tabacum	*CaMV35S*-CHI/*CaMV35S*-GLU		↘*Rhizoctonia solani*				
		Nicotiana tabacum	*CaMV35S*-CHI/*CaMV35S*-RIP		↘*Rhizoctonia solani*				
Jongedijk	1995	*Lycopersicon esculentum*	*CaMV35S-35S*-Chi-I	Fungal disease resistance	→*Fusarium oxysporum*	—	Controlled conditions	25-30 d	Disease severity
		Lycopersicon esculentum	*CaMV35S-35S*-Glu-I		→*Fusarium oxysporum*				
		Lycopersicon esculentum	*CaMV35S-35S*-Chi-I/*CaMV35S-35S*-Chi-II/*CaMV35S-35S*-Glu-I/*CaMV35S-35S*-Glu-II		↘*Fusarium oxysporum*				
Vierheilig	1995	*Nicotiana tabacum*	*CaMV35S*-PRs	Fungal disease resistance	—	→*Glomus mosseae*	Controlled conditions	2-8 w	Degree of colonized root length
Donegan	1996	*Solanum tuberosum*	*CaMV35S-35S*-CryIIIA	Insect resistance	—	→Phylloplane fungi	Field trial	14 w	Fungal CFU/g dry wt. leaves
Grison	1996	*Brassica napus*	*CaMV35S*-Endochitinase gene	Fungal disease resistance	↘*Phoma lingam*	—	Field trial	3 w after flowering	Scoring severity of stem canker
					↘*Cylindrosporium concentricum*			End of flowering	Percentage of diseased plants, diseased leaves and contaminated leaf surface
					↘*Sclerotinia sclerotium*			24-52 d	Mean of necrosis length on rape stems
Masoud	1996	*Medicago sativa*	*CaMV35S*-Aglu1	Fungal disease resistance	→*Stemphylium alfalfae* →*Phoma medicaginis*	—	Controlled conditions	10-20 d	Symptom severity
		Medicago sativa	*CaMV35S*-RCH10		→Stemphylium alfalfae →Phoma medicaginis				
		Medicago sativa	*CaMV35S*-RCH10/CaMV35S-Aglu1		→*Stemphylium alfalfae* →*Phoma medicaginis*				
Maddaloni	1997	*Nicotiana tabacum*	*wun1*-RIP	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	30 d	Symptom severity
Bliffeld	1999	*Triticum aestivum*	*Act*-Glu/*Ubi*-Chi	Fungal disease resistance	↘*Erysiphe graminis*	—	Controlled conditions	7 d	Counting the number of *E. graminis* colonies developed
		Triticum aestivum	*Act*-RIP/*Ubi*-Chi		↘*Erysiphe graminis*				
Chen	1999	*Triticum aestivum*	*Ubi*-tlp	Fungal disease resistance	↘*Fusarium graminearum*	—	Controlled conditions	7 -10 d	Infected spikelets per inoculated spike
Datta	1999	*Oryza sativa*	*CaMV35S*-TLP-D34	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	6 w	Percent of sheath infection
Donegan	1999	*Medicago sativa*	Mac-MnP	Lignin degradation	—	→Soil fungi	Field trial	1 to 473 d	Fungal CFU/g dry wt. soil
			Mac-Amyl	Starch degradation		→Soil fungi			

Nishizawa	1999	*Oryza sativa*	*CaMV35S*-Cht-2	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	7 d	Disease severity index
		Oryza sativa	*CaMV35S*-Cht-3		↘*Magnaporthe grisea*				
Tabaeizadeh	1999	*Lycopersicon esculentum*	*CaMV35S-pcht28*	Fungal disease resistance	↘*Verticillium dahliae*	—	Controlled conditions	4-5 w	Foliar disease symptoms and vascular discoloration
Wang	1999	*Brassica napus*	*CaMV35S*-PR10.1	Fungal disease resistance	→*Leptosphaeria maculans*	—	Controlled conditions	5 w	Disease scores
		Brassica napus	*CaMV35S*-DRR206		↘*Leptosphaeria maculans*				
		Brassica napus	*CaMV35S-chi1*		→*Leptosphaeria maculans*				
		Brassica napus	*CaMV35S*-DRR230		→*Leptosphaeria maculans*				
Bieri	2000	*Triticum aestivum*	*CaMV35S*-RIP	Fungal disease resistance	→↘*Erysiphe graminis*	—	Controlled conditions	Boot stage	Detached leaf infection assay
Clausen	2000	*Triticum aestivum*	*Ubi1*-KP4	Fungal disease resistance	↘*Ustilago maydis*	—	Controlled conditions	1 d	Scoring spore formation
Hipskind	2000	*Medicago sativa*	*CaMV35S*-RS	Fungal disease resistance	↘*Phoma medicaginis*	—	Controlled conditions	8-10 d	Measuring the extent of necrosis around the wound sites
Kesarwani	2000	*Nicotiana tabacum*	*CaMV35S*-OXDC	Fungal disease resistance	↘*Sclerotinia sclerotiorum*	—	Controlled conditions	1 w	Disease symptoms
		Nicotiana tabacum	*CaMV35S*-SOVA		↘*Sclerotinia sclerotiorum*				
		Lycopersicon esculentum	*CaMV35S*-SOVA		↘*Sclerotinia sclerotiorum*				
Liang	2000	*Triticum aestivum*	*Ubi1*-Vstl	Fungal disease resistance	↘*Erysiphe graminis*	—	Controlled conditions	8-10 d	Five grades method
Ming	2000	*Oryza sativa*	*CaMV35S*-TCS	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	2 w	Observation of infected symptom
Mitsuhara	2000	*Nicotiana tabacum*	*PR1*-mSarco	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	4 w	Disease severity
Powell	2000	*Lycopersicon esculentum*	*CaMV35S-TMV U1 Ω*-pPGIP	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	7 d	Scoring disease progress, lesion diameter on leaves; measuring on fruit the diameter of the macerated zone around each site of inoculation
Rajasekaran	2000	*Nicotiana tabacum*	*CaMV35S*-cpo	Fungal disease resistance	↘*Colletotrichum destructivum*	—	Controlled conditions	7 d	Disease severity
Schaffrath	2000	*Oryza sativa*	*CaMV35S*-Rir1b	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	4 d	Counting blasts per leaf
Donaldson	2001	*Glycine max*	*CaMV35S*-gf-2.8	OxO activity	↘*Sclerotinia sclerotiorum*	—	Controlled conditions	5 d	Lesion length on cotyledon and stem
Gandikota	2001	*Oryza japonica*	*Ubi*-C2	Increase flavonoid levels	↘*Magnaporthe grisea*	—	Controlled conditions	10 d	Symptom severity
Krishnamurthy	2001	*Oryza* sp.	*Ubi1*-PinA	Fungal disease resistance	↘*Magnaporthe grisea* ↘*Rhizoctonia solani*	—	Controlled conditions	7 d	Leaf blast symptoms; dividing total lesion length by plant height for sheath blast symptoms
		Oryza sp.	*Ubi1*-PinB		↘*Magnaporthe grisea* ↘*Rhizoctonia solani*				
		Oryza sp.	*Ubi1*-PinA / -PinB		↘*Magnaporthe grisea* ↘*Rhizoctonia solani*				
Lewis	2001	*Zea mays*	MON810	Insect resistance	—	→Beauveria bassiana	Controlled conditions	5 w	Occurrence of endophytic B. bassiana
		Zea mays	Bt176			→Beauveria bassiana			
		Zea mays	MON802			→Beauveria bassiana	Field trial	30-45-60 d	
		Zea mays	MON810			→Beauveria bassiana			
		Zea mays	Bt176			→Beauveria bassiana			
Oldach	2001	*Triticum aestivum*	*Ubi1*-afp	Fungal disease resistance	↘*Erysiphe graminis* ↘*Puccinia recondita*	—	Controlled conditions	9-13 d / 12 d	Number and size of formed colonies
		Triticum aestivum	*Ubi1*-chi		↘*Erysiphe graminis* ↘*Puccinia recondita*	—			
		Triticum aestivum	*Ubi1*-rip		→*Erysiphe graminis* →*Puccinia recondita*	—			
Saxena	2001	*Zea mays*	Cry1Ab	Insect resistance	—	→Soil fungi	Field trial	40 d	Fungal CFU/g dry wt. soil

Staehelin	2001	*Medicago truncatula*	*CaMV35S*-Mtenod40	Increased root nodules	—	↗*Glomus mosseae*	Controlled conditions	12 to 80 d	Percentage of root length containing mycorrhizal structures
						↗*Glomus intraradices*			
		Medicago truncatula	Suppression of enod40	Decreased root nodules		↘*Glomus intraradices*		12 to 48 d	
Wang	2001	*Brassica napus*	*CaMV35S*-DRR206	Fungal disease resistance	↘*Leptosphaeria maculans*	—	Controlled conditions	9-13 d	Disease scores
					↘*Rhizoctonia solani*			8 d	Percentage of mortality of seedlings
					↘*Sclerotinia sclerotiorum*			2 d	Diameter of necrotic lesions
Banzet	2002	*Nicotiana tabacum*	*CaMV35S-35S*-PR1a-helio	Fungal disease resistance	↘*Cercospora nicotianae*	—	Controlled conditions	14 d	Percentage of the necrotic area versus total leaf area
		Nicotiana tabacum	*CaMV35S*-PG-droso		↘*Cercospora nicotianae*				
Chen	2002	*Daucus carota*	*Ubi*-tlp	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	8 d	Length of the lesion developing along the petiole and onto leaf- lets
					↘*Sclerotinia sclerotiorum*			5 d	
Kanzaki	2002	*Oryza sativa*	*Ubi1*-EKHSubWT	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	10-14 d	Lesion size on leaves
Yuan	2002	*Oryza sativa*	*CaMV35S*-TCS	Fungal disease resistance	↘*Pyricularia oryzae*	—	Controlled conditions	5 d; 3 w	Lesions on leaves; seedling survival rate
Way	2002	*Nicotiana tabacum*	*CaMV35S*-ShPAL	Fungal disease resistance	↘*Cercospora nicotianae*	—	Controlled conditions	10 d	Measuring leaf area affected and symptom development
Anand	2003	*Triticum aestivum*	*Ubi*-PR2	Fungal disease resistance	→*Fusarium graminearum*	—	Controlled conditions Field trial	Greenhouse 7-10-14 d Field 14 d	Number and percentage of spikelets infected per head
		Triticum aestivum	*Ubi*-PR3		→*Fusarium graminearum*				
		Triticum aestivum	*Ubi*-PR5		→*Fusarium graminearum*				
		Triticum aestivum	*Ubi*-PR3 / *Ubi*-PR2		→*Fusarium graminearum*				
Bieri	2003	*Triticum aestivum*	*actin*-Glu / Ubi-Chi	Fungal disease resistance	→↗↘*Blumeria graminis*	—	Controlled conditions	5-7 d	Colony number on leaf sections
		Triticum aestivum	*CaMV35S*-RIP		↘*Blumeria graminis*				
		Triticum aestivum	*CaMV35S*-Bn-B*		↘*Blumeria graminis*				
		Triticum aestivum	*actin*-Glu / Ubi-Chi × *CaMV35S*-RIP		↘*Blumeria graminis*				
Carstens	2003	*Nicotiana tabacum*	*CaMV35S*-CTS1-2	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	3 - 7 d	Disease lesions were scored
Chakrabarti	2003	*Nicotiana tabacum*	*UBQ3*-MSI-99	Fungal disease resistance	↘*Sclerotinia sclerotiorum* ↘*Alternaria alternata* ↘*Botrytis cinerea*	—	Controlled conditions	3 d	Lesion diameter
		Musa sp.	*UBQ3*-MSI-99	Fungal disease resistance	↘*Fusarium oxysporum* ↘*Mycosphaerella musicola*		Controlled conditions	6 w	Tolerance to disease infection Area of lesion
Cober	2003	*Glycine max*	*CaMV35S*-gf-2.8	Fungal disease resistance	↘*Sclerotinia sclerotiorum*	—	3 field trials	Across 3 y	Disease severity index
Emani	2003	*Gossypium hirsutum*	*CaMV35S*-ech1	Fungal disease resistance	↘*Rhizoctonia solani* ↘*Alternaria alternata*	—	Controlled conditions	2 w	Disease index Percentage of leaf area with necrosis
		Nicotiana tabacum	*CaMV35S*-ech1		↘*Alternaria alternata*				
Herrera Medina	2003	*Nicotiana tabacum*	*CaMV35S*-NahG	Reduced levels of SA	—	→*Glomus mosseae* →*Glomus intraradices*	Controlled conditions	11-84 d	Percentage of root length containing mycorrhizal structures
		Nicotiana tabacum	*CaMV35S*-CSA	Increased levels of SA		→*Glomus mosseae* →*Glomus intraradices*			
Kachroo	2003	*Oryza sativa*	*PAL*-GOX	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	14 d	Lesion types were scored
Kim	2003	*Oryza sativa*	*rbcS*-MOD1-*Act1*-RCH10	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	10 d	Length of the diseased area on the leaf sheath
					→*Bipolaris oryzae*		NA	NA	NA
					→*Magnaporthe grisea*		NA	NA	NA
Velazhahan	2003	*Nicotiana tabacum*	*CaMV35S*-tlp-D34	Fungal disease resistance	↘*Alternaria alternata*	—	Controlled conditions	15 d	Chlorosis, necrosis
Blackwood	2004	*Zea mays*	*CaMV35S*-Cry1Ab	Insect resistance	—	→Soil fungi	Controlled conditions	12 d	Fungal community-level physiological profiles

Coca	2004	*Oryza sativa*	*Ubi*-afp	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	9 d	Degree of disease symptoms caused by *M. grisea*
Gazendam	2004	*Solanum tuberosum*	*CaMV35S*-pgip1	Fungal disease resistance	↘*Verticillium dahliae* [⌘]	—	Controlled conditions	16 w	Disease index
Milling	2004	*Solanum tuberosum*	RNA-antisensing	Altered starch composition	—	→Soil fungi	Field trial	3 growing seasons	18S rDNA DGGE profiles
O'Callaghan	2004	*Solanum tuberosum*	*CaMV35S*-magainin II	Bacterial resistance	—	→Endophytes from leaf, root and tuber	Field trial	11 w	Fungal CFU/g leave - roots - tubers (fresh weight)
Sawada	2004	*Oryza sativa*	*CaMV35S*-OsSBP	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	7 d - 14 d	Lesion size on leaves
Turrini	2004a	*Solanum melongena*	*CaMV35S*-Dm-AMP1	Fungal disease resistance	↘*Verticillium albo-atrum*	—	Controlled conditions	18 d	Fungal colony diameters
					↘*Botrytis cinerea*		Controlled conditions	3 d	Measuring the necrotic lesions sizes
					—	→*Glomus mosseae*	Controlled conditions	4 w	Percentage of root length containing mycorrhizal structures
Turrini	2004b	*Zea mays*	*CaMV35S*-Cry1Ab	Insect resistance	—	↘*Glomus mosseae*	Controlled conditions	35 d	Counting appressoria and percentage of root length containing mycorrhizal structures
		Solanum melongena	*CaMV35S*-Dm-AMP1	Fungal disease resistance	—	→*Glomus mosseae*			
Villarroel	2004	*Glycine max*	CP4-EPSPS	Herbicide tolerance	—	→Pod and seed mycoflora	Controlled conditions	NA	Fungal isolation and culturing in Petri dishes
Wei-xiang	2004	*Oryza japonica*	*Ubi*-Cry1Ab	Insect resistance	—	→Soil fungi	Controlled conditions	7-84 d	Fungal CFU/g dry wt. soil
Castaldini	2005	*Zea mays*	*CaMV35S*-Cry1Ab	Insect resistance	—	↘*Glomus mosseae*	Controlled conditions	1 to 16 w	Counting fungal appressoria and percentage of root length containing mycorrhizal structures
Chye	2005	*Solanum tuberosum*	*CaMV35S*-BjCHI1/HbGLU	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	2 w	Root health observation
		Solanum tuberosum	*CaMV35S*-BjCHI1		↘*Rhizoctonia solani*				
		Solanum tuberosum	*CaMV35S*-HbGLU		↘*Rhizoctonia solani*				
Janoušková	2005	*Nicotiana tabacum*	*CaMV35S*-HisCUP	Increased cadmium accumulation	—	→*Glomus intraradices*	Controlled conditions	12 w	Percentage of root length containing mycorrhizal structures and counting the extraradical mycelium of the AMF
Kremer	2005	*Glycine max*	CP4-EPSPS (Pioneer 94B01)	Herbicide tolerance	—	↗Fusarium spp.	Controlled conditions	28 d	Fungal biomass
Moreno	2005	*Oryza sativa*	*ZmPR4*-afp	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	6 d	Quantification of the infection using a Burker counting chamber; Visual observation of symptom development
Takahashi	2005	*Lolium multiflorum*	AcC-RCC2	Fungal disease resistance	↘*Puccinia coronata*	—	Controlled conditions	10 d	Counting chlorotic regions and the uredospore colonies
Tesfaye	2005	*Medicago sativa*	*CsVMV*-ech42-APSP	Fungal disease resistance and increased root exudation	↘*Phoma medicaginis*	—	Controlled conditions	1 d	Counting the number of germinated spores
		Medicago sativa			↘*Colletotrichum trifolii*				
Yevtushenko	2005	*Nicotiana tabacum*	win3.12T-CEMA	Fungal disease resistance	↘→*Fusarium solani*	—	Controlled conditions	Over 3 w	Disease symptoms
Coca	2006	*Oryza sativa*	*Ubi*-Ap-CecA	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	6 d	Counting the number of spores and estimating the leaf area covered with lesion
		Oryza sativa	*Ubi*-ER-CecA		↘*Magnaporthe grisea*				
de las Mercedes Dana	2006	*Nicotiana tabacum*	*CaMV35S*-CHIT33	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	7 d	Survival rates of transgenic and control plants were estimated
		Nicotiana tabacum	CaMV35S-CHIT42		↘*Rhizoctonia solani*				
		Nicotiana tabacum	CaMV35S-CHIT33-CHIT42		↘*Rhizoctonia solani*				
Hénault	2006	*Nicotiana tabacum*	antisens COMT	Modification of lignin biosynthesis	—	→Soil fungi	Controlled conditions	14 d	Proportion of double unsaturated chain fatty acids
		Nicotiana tabacum	antisens CAD			↗Soil fungi			

Ikeda	2006	*Lycopersicon esculentum*	*CaMV35S*-HMGR	Modification of secondary metabolic pathway	—	→Soil fungi	Controlled conditions	4 w	Fungal RISA profiles
Joubert	2006	*Nicotiana tabacum*	*CaMV35S-Vvpgip1*	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	3 - 15 d	Average lesion diameter
Makandar	2006	*Triticum aestivum*	*Ubi1*-AtNPR1	Fungal disease resistance	↘*Fusarium graminearum*	—	Controlled conditions	3- 21 d	Percentage of spikelets to which the infection had spread
Naef	2006	*Zea mays*	*CaMV35S*-Cry1Ab	Insect resistance	—	→*Fusarium graminearum* →*Trichoderma atroviride*	Controlled conditions	7-14 d	Fungal biomass quantification via microsatellites
Roy-Barman	2006	*Triticum aestivum*	*Ubi*-Ace-AMP1	Fungal disease resistance	↘*Blumeria graminis*	—	Controlled conditions	NA	Counting the number of fungal colonies developing on leaf pieces
					↘*Neovossia indica*			Booting stage, ears maturity	Observation of infection development on mature ears
Schlaich	2006	*Triticum aestivum*	*Ubi1*-KP4	Fungal disease resistance	↘*Tilletia* caries	—	Controlled conditions	NA	Stinking smut symptoms
					↘*Tilletia* caries		Field trial	16 w	
Zhao	2006	*Triticum aestivum*	*CaMV35S*-Glu	Fungal disease resistance	↘*Blumeria graminis*	—	Controlled conditions	NA	Number of the lesion spots
					↘*Blumeria graminis*		Field trial	NA	
Balconi	2007	*Triticum aestivum*	*CaMV35S*-RIP	Fungal disease resistance	↘*Fusarium culmorum*	—	Controlled conditions	7-14 d	Percentage of infected spikelets
de Vaufleury	2007	*Zea mays*	*CaMV35S*-Cry1Ab	Insect resistance	—	→Mycorrhizal fungi	Controlled conditions	21 d	Frequency of mycorrhization (F); Intensity of mycorrhization (M); Frequency of arbuscules (A); Mycorrhizal infectivity (MSI_{50})
Gomez-Ariza	2007	*Oryza sativa*	*CaMV35S*-PRms	Fungal disease resistance	↘*Magnaporthe oryzae* ↘*Fusarium verticillioides* ↘*Helminthosporium oryzae*	—	Controlled conditions	7 d	Disease severity was inferred from the lesion size developed at the inoculated spots on the leaves
Jayaraj	2007	*Daucus carota*	*CaMV35S-35S*-Ltp	Fungal disease resistance	↘*Alternaria radicicola* ↘*Botrytis cinerea*	—	Controlled conditions	15 d	Percent of leaf area disease
		Daucus carota	*CaMV35S-35S*-Chi2		↘*Alternaria radicicola* ↘*Botrytis cinerea*				
		Daucus carota	*CaMV35S-35S*-Ltp / *CaMV35S-35S*-Chi2		↘*Alternaria radicicola* ↘*Botrytis cinerea*				
Mackintosh	2007	*Triticum aestivum*	*Ubi*-KM1	Fungal disease resistance	↘*Fusarium graminearum*	—	Controlled conditions Field trial	21 d	Percentage of spikes wit symptomatic spikelets
		Triticum aestivum	*Ubi*-PR5		↘*Fusarium graminearum*				
		Triticum aestivum	*Ubi*-Gluc-3		↘*Fusarium graminearum*				
Marchive	2007	*Nicotiana tabacum*	*CaMV35S*-VvWRKY1	Fungal disease resistance	↘*Peronospora tabacina*	—	Controlled conditions	10 d	Disease intensity
					↘*Erysiphe cichoracearum*			10 - 13 d	Percentage of leaf surface covered by powdery mildew mycelium
Maruthasalam	2007	*Oryza sativa*	*Ubi*-chi11	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	3-7 d	Functional resistance/susceptibility index (FRI)
		Oryza sativa	*Ubi*-tlp						
Powell	2007	*Glycine max*	CP4-epsps	Herbicide tolerance	—	→AM fungi	Controlled conditions	Pod formation - maturity	Percentage of root length containing mycorrhizal structures
Rajam	2007	*Nicotiana tabacum*	*CaMV35S*-35S-SN1	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	6 d	Disease scoring
Sohn	2007	*Nicotiana tabacum*	*OsCc1*-hrpNEP	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	5 d	Evaluation of diseased incidence

Almasia	2008	*Solanum tuberosum*	*CaMV35S-35S*-SN1	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	2-4-6-8 d	Disease symptoms
Chen	2008	*Oryza sativa*	*Ubi*-OPBP1	Fungal disease resistance	↘*Magnaporthe oryzae* ↘*Rhizoctonia solani*	—	Controlled conditions	13 d	Counting the number of lesions per leaf and measuring the size of the lesions
Deo Prasad	2008	*Oryza sativa*	*Ubi*-Mj-AMP2	Fungal disease resistance	↘*Magnaporthe oryzae*	—	Controlled conditions	10 d	Scoring lesions
Girlanda	2008	*Solanum lycopersicum*	*CaMV35S*-chiI-GluI	Fungal disease resistance	—	→AM fungi →Rhizosphere saprotrophic microfungi →Phyllosphere saprotrophic microfungi	Controlled conditions	2-8 m	Frequency of mycorrhization (F); Intensity of mycorrhization (M); Frequency of arbuscules (A); Fungal species richness and abundance
Huang	2008	*Nicotiana tabacum*	*CaMV35S*-curcin 2	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	30 d	Disease index scale
Janni	2008	*Triticum aestivum*	*Ubi1*-Pvpgip2	Fungal disease resistance	↘*Bipolaris sorokiniana*	—	Controlled conditions	3 d	Measuring infected area
Jiang	2008	*Gossypium* sp.	Cry1Ac	Insect resistance	—	→*Pleurotus ostreatus*	Controlled conditions	0-24 d	Fungal growth
Knox	2008	*Gossypium hirsitum* *Gossypium hirsitum*	Cry1Ac - Cry2Ab CP4-epsps	Insect resistance Herbicide tolerance	—	→AM fungi	Field trial	14-100 d	Percentage of root length containing mycorrhizal structures
Liu	2008	*Oryza japonica rice*	*Ubi*-Cry1Ab	Insect resistance	—	→Soil fungi	Field trial	2 growing seasons	Fungal diversity based on ITS T-RFLP
Luo	2008	*Triticum aestivum*	*Ubi*-pinA	Fungal disease resistance	↘*Puccinia triticina*	—	Field trial	28 d	Observation of uridenia size and the occurrence of chlorosis and necrosis; Measurements of different plant characteristics
O'Callaghan	2008	*Solanum tuberosum*	*CaMV35S*-magainin II	Bacterial resistance	—	→↗↘Soil fungi	Field trial	3-5 m	Fungal CFU/g fresh wt. leaves - roots- tubers
Oliveira	2008	*Zea mays* Compa Cb *Zea mays* Elgina	Cry1Ab Cry1Ab	Insect resistance Insect resistance	—	↘Saprophytic fungi ↗Saprophytic fungi	Field trial Field trial	1-4 m during 2 y	Colony-forming units (CFU) of cultivable fungi
Shin	2008	*Triticum aestivum*	*Ubi*-Chit	Fungal disease resistance	↘*Fusarium graminearum*	—	Controlled conditions Field trial	20 d 21 d	Percentage of symptomatic spikelets per spike
Sridevi	2008	*Oryza sativa*	*CaMV35S*-gluc-*Ubi1*-Chi11	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	7 d	Disease Index
Swathi Anuradha	2008	*Nicotiana tabacum* *Arachis hypogaea*	*CaMV35S*-BjD	Fungal disease resistance	↘*Fusarium moniliforme* ↘*Pheaoisariopsis personata* ↘*Cercospora arachidicola*	—	Controlled conditions	10 d 21-28 d	Disease symptoms Number and average diameter of the lesions
Takakura	2008	*Oryza sativa*	*CaMV35S*-N1141	Fungal disease resistance	↘*Magnaporthe oryzae*	—	Controlled conditions	7 d	Disease lesions

Zhang	2008	*Oryza sativa*	*CaMV35S*-OsWRKY31	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	7 d	The lengths and numbers of lesions were averaged from at least five leaves from each line
Campos-Soriano	2009	*Oryza sativa*	CaMV35S-PRms	Fungal disease resistance	↘*Magnaporthe orizae*	—	Controlled conditions	NA	Confocal laser scanning microscopy (CLSM) analysis of root and leave tissues
Guo	2009	*Nicotiana tabacum*	*CaMV35S*-GhZFP1	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	20 d	Disease symptoms
Hart	2009	*Zea mays*	C4-EPSPS	Herbicide tolerance	—	→Soil fungi	Field trial	19-97-167 d	Determination of soil fungi abundance and soil fungal community structure by qPCR and T-RFLP, respectively
Kumar	2009	*Gossypium hirsutum*	*CaMV35S*-ech42	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	2-5 d	Disease index
Li	2009a	*Oryza sativa*	*Ubi*-McCHIT1	Fungal disease resistance	↘*Magnaporthe grisea* ↘*Rhizoctonia solani*	—	Controlled conditions	14 d	Five class disease severity scale (*R. solani*); lesion size scale of 0–9 grades (*M. grisea*)
Li	2009b	*Gossypium* sp.	Cry1Ac-CpTI	Insect resistance	—	↗*Fusarium oxysporum*	Controlled conditions	2-38 d	Disease incidence, fungal spore germination and mycelial growth
		Gossypium sp.	*Cry1Ab/Cry1Ac*	Insect resistance		↗*Fusarium oxysporum*	Controlled conditions		
Qiu	2009	*Oryza sativa*	*Ubi*-pemG1	Fungal disease resistance	↘*Magnaporthe grisea*	—	Controlled conditions	2-4-7 d; 8 d	Fungal colonization of leaves, number of lesions in leaves
Shah	2009	*Oryza sativa*	*CaMV35S*-cht42	Fungal disease resistance	↘*Rhizoctonia solani*	—	Controlled conditions	7 d	Sheath blight symptom
Weinert	2009	*Solanum tuberosum*	*GBSS*-zeaxanthin epoxidase (sens and anti-sens)	Altered zeaxanthin level	—	≠Soil fungi	Field trial	EC30-60-90	Fungal diversity based on DGGE profiles
Yi	2009	*Citrinus lanatus*	*CaMV35S*-CGMMV-CP	Virus resistance	—	→Soil fungi	Field trial	3 m	Fungal diversity based on T-RFLP profiles
Tan	2010	*Zea mays*	Cry1Ab 34B24 (MON810)	Insect resistance	—	→Soil fungi	Controlled conditions	15 to 82 d	Fungal diversity based on DGGE profiles and cloning-sequencing the 18S rRNA gene
		Zea mays	Cry1A (Nongda 1246*1482)			→Soil fungi			

a: abbreviations are detailed in table 1.3.

b: ↘ indicates a significant decrease in the variable response measured compared to control, ↗ indicates a significant increase in the variable response measured compared to control, → indicates an absence of significant difference with respect to control, ⌘ indicates a pleiotropic effect.

Table 1.2 Studies investigating the impact of genetically modified trees on target and non-target fungi from 1996 to 2010.

First author	Year	Transformed plant	Event	Desired phenotypes	Impact on target fungi	Impact on non-target fungi	Study type	Exposure time	Variable response
Hampp	1996	*Populus tremula* × *P. tremuloides*	*mas*-iaaM-iaaH	IAA over production	—	→*Amanita muscaria*	Controlled conditions	4 w	EM fungi establishment and development
Nakamura	1999	*Actinidia chinensis*	*CaMV35S*-EG	Fungal disease resistance	↘*Botrytis cinerea*	—	Controlled conditions	3 d - 4 d	Diameter of the necrotic lesion on leaves
Bolar	2000	*Malus domestica* cv. Marshall McIntosh	*CaMV35S*-ech42	Fungal disease resistance	↘*Venturia inaequalis*	—	Controlled conditions	2 w	Number of sporulating lesions, percentage of leaf area infected, conidia from leaves
		Malus domestica cv. Marshall McIntosh	*CaMV35S-35S*-ech42		↘*Venturia inaequalis*				
Bolar	2001	*Malus domestica* cv. Marshall McIntosh	*CaMV35S-35S*-ech42	Fungal disease resistance	↘*Venturia inaequalis*	—	Controlled conditions	2 w	Number of sporulating lesions, percentage of leaf area infected, conidia from leaves
		Malus domestica cv. Marshall McIntosh	*CaMV35S-35S*-nag70		↘*Venturia inaequalis*				
		Malus domestica cv. Marshall McIntosh	*CaMV35S-35S*-ech42 /*CaMV35S-35S*-nag70		↘*Venturia inaequalis*				
Liang	2001	*Populus × euramericana*	*CaMV35S*-OxO	Fungal disease resistance	↘*Septoria musiva*	—	Controlled conditions	1 w	Necrotic areas on leaf disks
Mohamed	2001	*Populus trichocarpa x P. deltoïdes*	*CaMV35S*-Ω-Cab-t-bO	Fungal disease resistance	→*Melampsora occidentalis* →*Venturia populina* →*Septoria musiva* →*Septoria populicola*	—	Controlled conditions Field trial	2-12 w	Proportion of rusted leaves / tree; Percentage of blighted tissues / tree; Percentage of green leaf tissue; Canker size
		P. trichocarpa × P. nigra	*CaMV35S*-Ω-Cab-t-bO		→*Melampsora occidentalis* →*Venturia populina* →*Septoria musiva* →*Septoria populicola*				
Liang	2002	Populus × euramericana	*CaMV35S*-Ac-AMP1.2	Fungal disease resistance	↘*Septoria musiva*	—	Controlled conditions	1 w	Necrotic areas of the leaf disks
		Populus × euramericana	*CaMV35S-win3.12*-Ac-AMP1.2/ESF12		↘*Septoria musiva*				
		Populus nigra × P. maximowizii	*CaMV35S*-Ac-AMP1.2/ESF12		→*Septoria musiva*	—			
		Populus nigra × P. maximowizii	*CaMV35S-win3.12*-Ac-AMP1.2/ESF12		→*Septoria musiva*				
Kaldorf	2002	*Populus tremula x P. tremuloides*	*CaMV35S*-RolC *rbcS*-RolC	Modification of the phytohormone balance	—	↘One EM morphotype →EM fungal community	Field trial	Over 15 m	Percentage of root length containing mycorrhizal structures; EM diversity based on ITS-RFLP and morphotyping
Pappinen	2002	*Betula pendula*	*CaMV35S*-Kit4	Fungal disease resistance	↘*Pyrenopeziza betulicola*	—	Controlled conditions	2 w	Percentage of leaf area displaying spots
Belfanti	2004	*Malus × domestica* cultivar Gala	*CaMV35S*-HcrVf2	Fungal disease resistance	↘*Venturia inaequalis*	—	Controlled conditions	10-15-21 d	Macroscopic symptoms and infection severity
Faize	2004	*Malus × domestica*	*CaMV35S*-pinB	Fungal disease resistance	↘*Venturia inaequalis* race 6 →*Venturia inaequalis* race 1	—	Controlled conditions	12-14 d	Percentage of scabbed leaves; percentage of leaf area with sporulating lesions
Pasonen	2004	*Betula pendula*	*CaMV35S*-Kit4	Fungal disease resistance	→↗*Pyrenopeziza betulicola*	—	Field trial	3 growing seasons (157 d/year)	Number of leaf spots or rust pustules on the surface of the leaves; percentage of the leaf area covered by leaf spots or rust pustules
					→↘*Melampsoridium betulinum*				
Seppänen	2004	*Populus tremula*	*4X35S*-pinosylvin synthase	Fungal disease resistance	↘*Phellinus tremulae*	—	Controlled conditions	7-8 w	Weight loss of wood samples during the incubation with *Phellinus tremulae*
		Populus tremula X tremuloides	*4X35S*-pinosylvin synthase		↗*Phellinus tremulae*				

Noël	2005	*Piceae mariana*	*CaMV35S-35S*-ech42	Fungal disease resistance	↘*Cylindrocladium floridanum*	—	Controlled conditions	10 d	Percentage of the foliar area affected by needle discoloration
		Populus nigra x P. maximowiczii	*CaMV35S-35S*-ech42		↘*Melampsora medusae*	—			Counting the number of uredial pustules and necrotic spots per leave disk
Pasonen	2005	*Betula pendula*	*CaMV35S*-Kit4	Fungal disease resistance	—	→*Paxillus involutus*	Controlled conditions	28-30 d	Total number of root tips, percentage of mycorrhizal root tips, root and shoot fresh weight, total fresh weight and root/shoot ratio
Zhang	2005	*Populus tremula × P. tremuloides*	*GPD*-Bar	Herbicide tolerance	—	→*Amanita muscaria*	Controlled conditions	6-8 w	Growth on a selective media to screen for horizontal gene transfer
Hsieh	2006	*Papaya*	*CaMV35S*-CP	Virus resistance	—	→Soil fungi	Field trial	9 years	Fungal CFU/g soil
Stefani	2006	*Picea glauca*	Cry1Ab	Insect resistance	—	→*Lophodermium picea*	Field trial	4 y	Diversity of foliar endophyte measured by plating sterilized needles on Petri dishes, ITS PCR-RFLP-sequencing
Vauramo	2006	*Betula pendula*	*CaMV35S*-Kit4	Fungal disease resistance	—	→Leaf litter fungal biomass	Field trial	8-11 m	Leaf litter content in ergosterol
Wei	2006	red-fleshed papaya	*CaMV35S*-RP	Virus resistance	—	↗Soil fungi	Field trial	1 y	Fungal CFU
Bradley	2007	*Populus tremuloides*	*Pt4CL1P*-Pt4CL (line 23)	Altered lignin composition	—	→Soil fungi	Controlled conditions	6 m	Measures of phospholipid and neutral lipid fatty acids in soil
		Populus tremuloides	*Pt4CL1P*-Pt4CL/*Pt4CL1P*-LsCald5H (Lines 72 - 141)			↘→Soil fungi			
Malnoy	2007	*Malus × domestica cultivar Galaxy*	*pin2*-MpNPR1-1	Fungal disease resistance	↘*Venturia inaequalis*	—	Controlled conditions	21 d	Macroscopic symptoms and infection severity
		Malus × domestica cultivar Galaxy	*CaMV35S*-MpNPR1-1		↘*Venturia inaequalis*				
		Malus × domestica cultivar Galaxy	*pin2*-MpNPR1-1		↘*Gymnosporangium juniperi-virginianae*	—			
Newhouse	2007	*Ulmus americana*	*ACS2*-ESF39A	Fungal disease resistance	↘*Ophiostoma novo-ulmi*	→EM fungal community	Field trial	11-14 w	Measures of the stained distance above and below the inoculation site; Level of mycorrhizal colonization
Seppänen	2007	*Betula pendula*	*CaMV35S*-bp4CL1 (antisens orientation)	Altered lignin composition	—	→*Paxillus involutus*	Controlled conditions	35-39 d	Total number of mycorrhizal and non mycorrhizal root tips, Microscope observation of EM structure
					—	↗↘leaf litter fungal biomass	Field trial	7 - 11	Ergosterol content
Oliver	2008	*Populus tremula X Populus alba*	*CaMV35S-35S*-PPO1	Fungal and bacterial resistance	—	→Soil fungi	Controlled conditions	5 m	Soil fungal diversity by cloning and sequencing fungal 18S rRNA
Tiimonen	2008	*Betula pendula*	*UbB1*-PtCOMT	Altered lignin composition	—	→↘*Paxillus involutus*	Controlled conditions	8 w	Evaluation of the number of lateral roots covered with fungal hyphae, examination by light microscopy
		Betula pendula	*CaMV35S*-PtCOMT		—	→↘*Paxillus involutus*			
Pasonen	2009	*Betula pendula*	*CaMV35S*-Kit4	Fungal disease resistance	—	→Soil fungi and EM fungi	Field trial	3 growing seasons	ECM colonization intensity and soil fungal diversity estimated by PCR DGGE and ITS sequencing

Stefani	2009	*Populus alba X P. grandidentata*	*nos*-nptII-*pin2*-GUS	Kanamycin resistance	—	➔EM fungal community	Field trial	8 years	ECM fungal diversity measured by cloning and sequencing soil fungal ITS and sequencing fungal ITS of root tips
Sutela	2009	*Betula pendula*	*CaMV35*-PtCOMT	Altered lignin composition	—	➔*Paxillus involutus*	Controlled conditions	8 w	Evaluation of the number of ECM root tips per root system
		Betula pendula	*UbB1*-PtCOMT			➔*Paxillus involutus*			
Szankowski	2009	*Malus × domestica* cvs 'Elstar' and 'Gala'	5`UTR fragments of the lengths of 115 bp-HcrVf2	Fungal disease resistance	↘*Venturia inaequalis*	—	Controlled conditions	21 d	Symptom scale
		Malus × domestica cvs 'Elstar' and 'Gala'	5`UTR fragments of the lengths of 288 bp-HcrVf2		↘*Venturia inaequalis*				
		Malus × domestica cvs 'Elstar' and 'Gala'	5`UTR fragments of the lengths of 779 bp-HcrVf2		↘*Venturia inaequalis*				
Flachowsky	2010	*Malus x domestica cv.* 'Holsteiner Cox'	*CaMV35*-Lc	Fungal and bacterial resistance	↘*Venturia inaequalis*	—	Controlled conditions	6 w	Scab incidence according a six value scale and number of infected leaves
Stefani	2010	*Picea glauca*	*CaMV35S-35S*-ech42	Fungal disease resistance	—	➔*Wilcoxina* spp. ➔Soil fungi	Controlled conditions	5 years	Quantitative PCR of root tips colonized by *Wilcoxina* spp; Soil content in ergosterol

Table 1.3 Abbreviation of promoter and gene used to transform crops and trees.

Abbreviation	Definition	Origin
AcC	Actin 1 gene promoter	Rice
Ace-AMP1	Antimicrobial protein gene	*Allium cepa*
ACS2	Vascular specific promoter	American chestnut
Act, actin, act1	Actin 1 promoter	Rice
afp	Anti-fungal protein	*Aspergillus giganteus*
Aglu1	Acidic glucanase gene	Alfalfa
Ac-AMP1.2	Chitin-binding protein	*Amaranthus caudatus*
Ap-CecA; ER-CecA	Cecropins antimicrobial peptides	*Hyalophora cecropia*
APSP	Signal peptide of the APase gene	White lupin
AtNPR1	Activation of systemic acquired resistance	*Arabidopsis thaliana*
bar	Phosphinotricine (PPT) resistance	*Streptomyces hygroscopicus*
BjCHI1	Chitinase with two chitin-binding domains	*Brassica juncea*
BjD	Defensin gene	Mustard
Bn	Apoplastic version of barnase (unspecific Rnase)	*Bacillus amyloliquefaciens*
B*	Barstar	*Bacillus amyloliquefaciens*
bp4CL1	4-coumarate:coenzyme A ligase	Birch
CaMV35S	35S - promoter	Cauliflower mosaic virus
C2	Chalcone synthase CHS	Mayze
CEMA	Novel cecropin A-melittin hybrid peptide	
CGMMV-CP	Coat protein gene	Cucumber green mottle mosaic virus
CH5B	Endochitinase gene	Bean
chi, chit	Antifungal barley-seed class II chitinase.	Barley
Chi-I	32 kD class I endochitinase	*Nicotiana tabacum*
Chi-II	28 kD class II endochitinase	*Nicotiana tabacum*
Chi1	Class I basic endochitinase gene	Pea
chi11	Chitinase gene	Rice
Chi2	Chitinase gene	Barley
chiI	32 kDa class I endochitinase	Tobacco
CHIT33; CHIT42	Endochitinase genes	*Trichoderma harzianum*
Cht-2, Cht-3	Class-I chitinase gene	Rice
Cht42	42-kDa endochitinase gene	Trichoderma virens
CP	Coat protein	Tobacco mosaic virus
CP4 EPSPS	5-enol-pyruvylshikimate 3-phosphate synthase	*Agrobacterium* sp. CP4
cpo	Chloroperoxidase gene	*Pseudomonas pyrrocinia*
CpTI	Trypsin inhibitor	Cowpea
cry1Ab, cry1Ac	Crystal protein genes	*Bacillus thuringiensis*
cryIIIA	Parasporal crystal protein	*Bacillus thuringiensis* var. *tenebrionis*
CSA	Constitutive SA biosynthesis	
CsVMV	Promoter	Cassava vein mosaic virus
CTS1-2	Chitinase enzyme	*Saccharomyces cerevisiae*
curcin 2	Ribosome-inactivating protein	*Jatropha curcas*
Dm-AMP1	Antimicrobial defensin	*Dahlia merckii*
DRR206	Defence gene	Pea
DRR230	Defensin gene	Pea
ech1	Endochitinase cDNA	*Trichoderma virens*
ech42	42 kDa endochitinase gene	*Trichoderma harzianum*
EG	β-1,3-endoglucanase cDNA	Soybean
EKHSubWT	Defensin gene	Wasabi
ESF12	Synthetic peptide that mimics the amphipathic α-helix found in magainins	
ESF39A	Synthetic antimicrobial peptide	
gf-2.8	Gene for germine	Wheat
GhZFP1	Zinc finger protein 1	*Gossypium hirsutum*
Glu-I	33 kD class I β-1,3-endoglucanase	*Nicotiana tabacum*
Glu-II	40 kD class II β-1,3-endoglucanase	*Nicotiana tabacum*
Glu	β-1,3-glucanase	Barley
Glu	β-1,3-glucanase	Tobacco
Gluc	β-1,3-glucanase	Tobacco
Gluc-3	β-1,3-glucanase	Barley
gluI	33 kDa class I β-1,3 glucanase	Tobacco
GOX	Glucose oxidase gene	*Aspergillus niger*

GPD	Glyceraldehyde-3-phosphate dehydrogenase - promoter	*Cochliobolus heterostrophus*
Gus	β-glucuronidase reporter gene	
HbGLU	cDNA encoding β-1,3-glucanase	*Hevea brasiliensis*
HcrVf2	Receptor-like protein	*Malus floribunda* 821
HisCUP	Polyhistidine cluster combined with yeast metallothionein CUP1	
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase gene	Melon
$HrpN_{EP}$	Harpin group of proteins	*Erwinia pyrifoliae*
iaaH	Indole- 3-acetamide hydrolase gene	*Agrobacterium tumefaciens*
iaaM	Trp-2-mono-oxygenase gene	*Agrobacterium tumefaciens*
Kit 4	Chitinase 4 gene	Sugarbeet
KM1	A-1-purothionin	Wheat
KP4	Antifungal protein	Ustilago maydis-infecting virus
Lc	Leaf colour gene	*Zea mays*
LsCAld5H	Coniferaldehyde 5-hydroxylase	*Liquidambar styraciflua*
Ltp	Lipid transfer protein	Wheat
mas	Mannopine synthase promoter	*Agrobacterium tumefaciens*
McCHIT1	Class I secretory endochitinase	*Momordica charantia*
Mj-AMP2	Knottin-type antimicrobial peptide	*Mirabilis jalapa*
MOD1	Ribosome inactivating protein gene	Mayze
MpNPR1-1	Activation of systemic acquired resistance	*Malus × domestica*
mSarco	Sarcotoxin IA gene	*Sarcophaga peregrina (Diptera)*
MSI-99	Mangainin antimicrobial peptides	*Xenopus laevis*
Mtenod40	Over expression of a nodulin gene	
N1141	Flagellin gene	*Acidovorax avenae*
nag70	Exochitinase, N-acetyl-β-D-hexosaminidase	*Trichoderma atroviride*
nos	Nopaline synthase promoter	
nptII	Neomycin phosphotransferase II	
OPBP1	Osmotin promoter binding protein 1	Tobacco
OsCc1	Cytochrome c gene - promoter	Rice
OsSBP	Selenium-binding protein	Rice
OsWRKY31	Transcriptional activator in defense signaling cascades	Rice
OXDC	Cytosolic expression of oxalate decarboxylase	*Collybia velutipes*
OxO	Germin-like oxalate oxidase gene	Wheat
PAL	Wound inducible phenylalanine ammonialyase promoter	Rice
pcht28	Acidic endochitinase gene	*Lycopersicon chilense*
pemG1	Elicitor-encoding gene	*Magnaporthe grisea*
PG-droso	Signal peptide of maize polygalacturonase 1 and mature drosomycin	*Drosophila melanogaster*
pin2	Wound inducible promoter	
pin2	Proteinase inhibitor II gene	Potato
pinA, pinB	Puroindoline protein	Wheat
pPGIP	Pear fruit poly-galacturonase inhibitor protein	Pear
PPO1	Polyphenol oxidase	Hybrid poplar
PR10.1	Pathogenesis-related (PR) protein family (PR10)	Pea
PR1a	Pathogenesis-related 1a promoter	Tobacco
PR1a-helio	Signal peptide of tobacco PR1a protein and mature heliomicin	*Heliothis virescens*
PR2	β-1,3-glucanase	
PR3	Chitinase	
PR5	Thaumatin-like protein 1	Barley
PRms	Pathogenesis-related gene	Mayze
PRs	Pathogenesis-related proteins (chitinases and β-1,3-glucanases)	
Pt4CL	4-coumarate:CoA ligase	*Populus tremuloide*
Pt4CL1P	Xylem specific promoter	*Populus tremuloide*
PtCOMT	Caffeate/5- hydroxyferulate O-methyltransferase	*Populus tremuloides*
Pvpgip2	Polygalacturonase-inhibiting proteins	Bean
rbcS	Light-inducible promoter	Potato or rice
RCC2	Chitinase Cht-2	Rice
RCH10	Chitinase gene	Rice
RIP	Ribosome-inactivating proteins	Barley or Mayze
Rir1b	11 kDa protein rich in glycine and proline	Rice
rolC	Root locus C	*Agrobacterium rhizogenes*
RPTW	Replicase (RP) mutant gene	Papaya ringspot virus
RS	Resveratrol synthase	*Arachis hypogaea*
ShPAL	Phenylalanine ammonia-lyase cDNA	*Stylosanthes humilis*
SN1	Snakin-1 antimicrobial peptide	*Solanum tuberosum*
SOVA	Oxalate Decarboxylase	*Collybia velutipes*
TCS	Trichosanthin: type I ribosome-inactivating protein	*Trichosanthes kirilowii*
TLP-D34	Thaumatin-like protein	Rice
TMV U1 Ω	Tobacco mosaic virus strain U1 enhancer	Virus
TobA	Vacuolar chitinase A	Tobacco

TobAΔT	Vacuolar chitinase A without its C-terminal vacuolar targeting peptide	Tobacco
UbB1	Polyubiquitin gene	Sunflower
Ubi	Ubiquitin 1 promoter	Mayze
UBQ3	Ubiquitin promoter	Arabidopsis
UTR	Untranslated region	
Vstl	Stilbene synthase	*Vitis vinifera*
Vvpgip1	Polygalacturonase-inhibiting proteins	*Vitis vinifera*
VvWRKY1	Transregulatory proteins	*Vitis vinifera*
win3.12T	Wound inducible promoter	Poplar
wun1	Wound inducible gene (promoter region)	Potato
ZmPR4	Fungal inducible promoter	Mayze
Ω-Cab-t-bO	Synthetic bacterio opsin gene, chlorophyll a/b transit peptide and an Ω translation-enhancing sequence	*Halobacterium halobium*

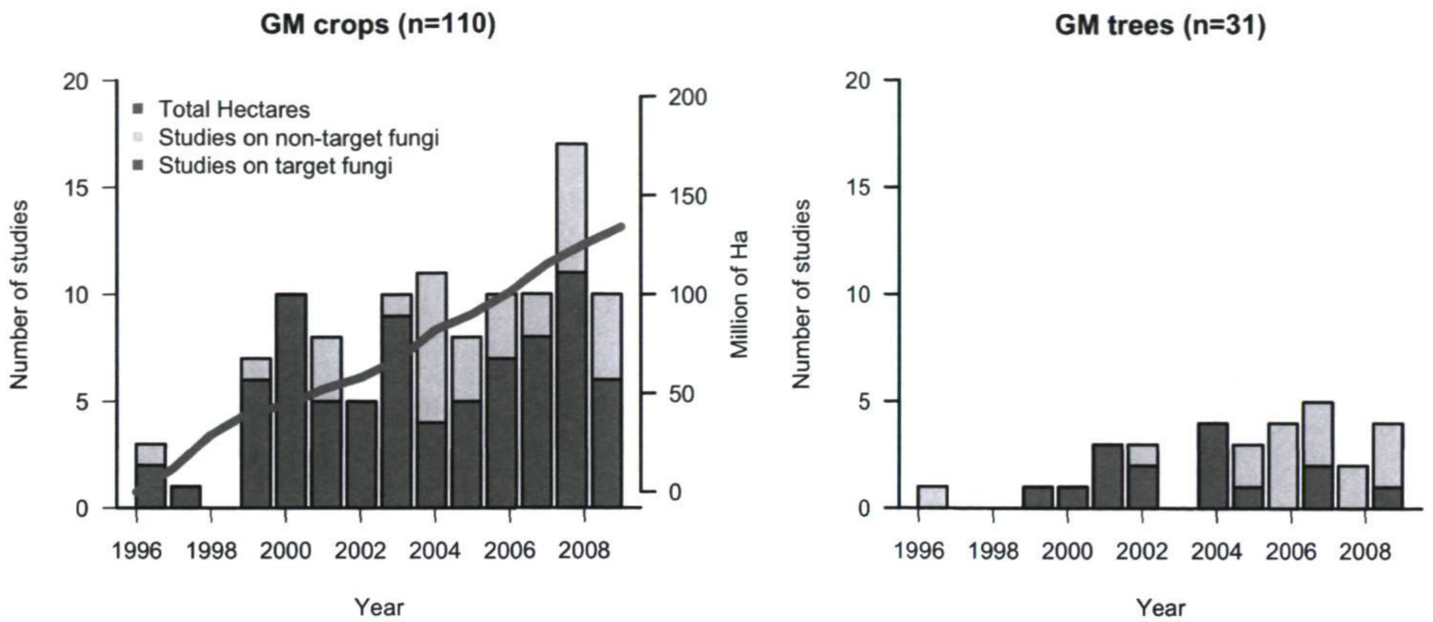
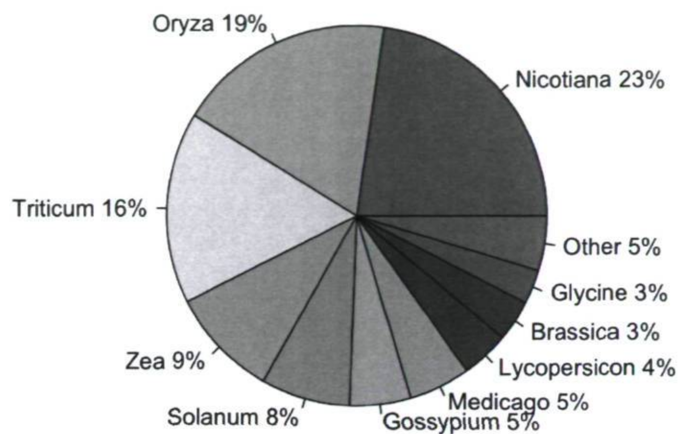


Fig. 1.1 Increase of the global hectarage of biotech crops in the period 1996-2009 (red line). Stack histograms show the number of studies investigating the potential impact of GM crops (A) and GM trees (B) on target fungi (dark grey) and on non-target fungi (bright grey) in the period 1996-2009.

A. Genera of GM plant tested against fungi (n=164)



B. Traits expressed in GM plants tested against fungi (n=125)

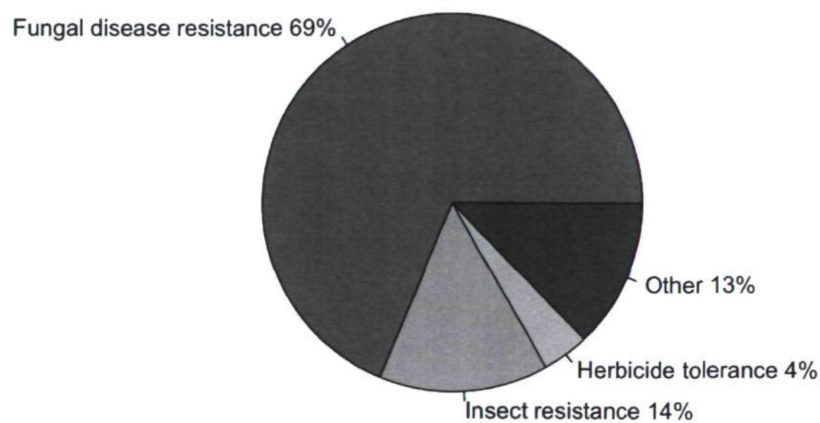
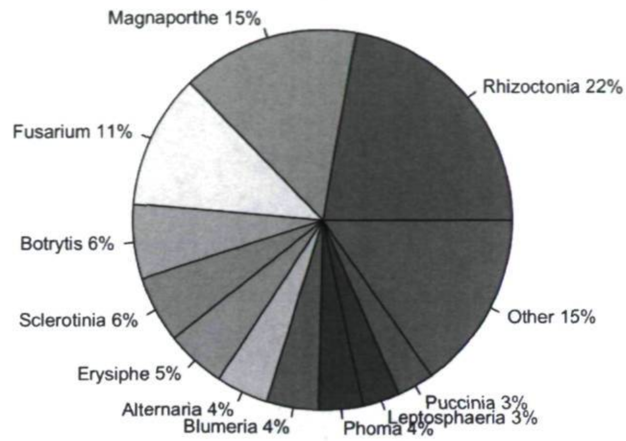


Fig. 1.2 Pie charts of genera to which belong GM crops that were investigated for their potential impact against fungi (A) and new traits expressed in the GM crops monitored (B).



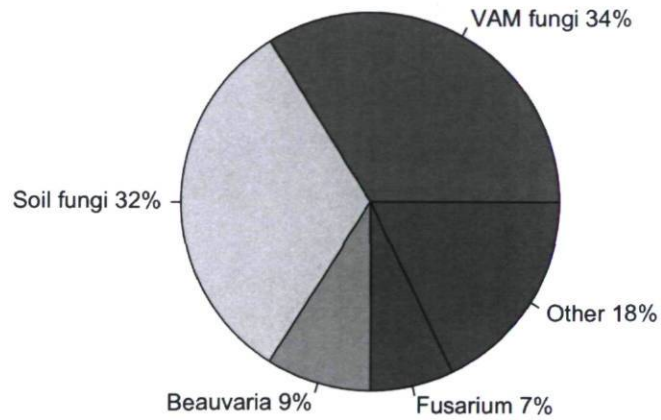
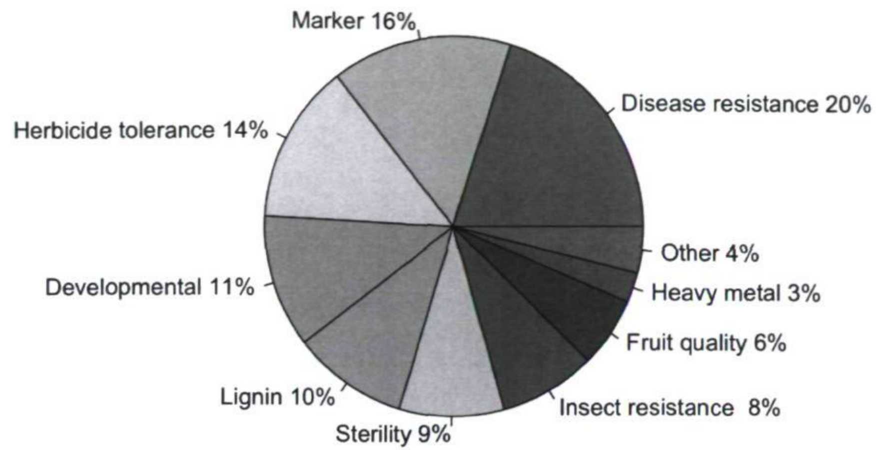


Fig. 1.3 Pie charts of the fungal genera targeted by GM crops (A) and types and genera of the non-target fungi monitored (B).



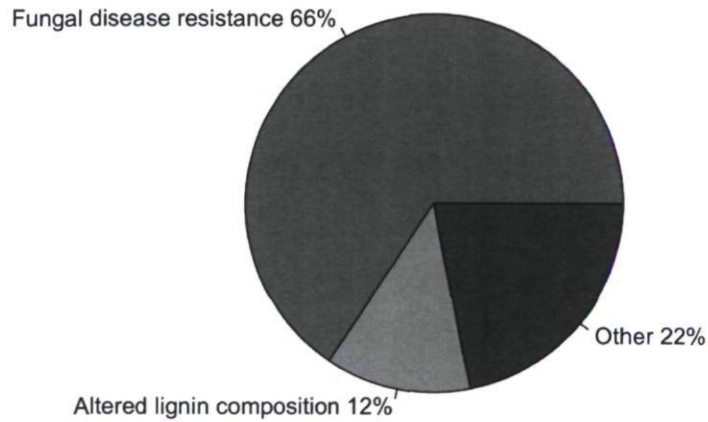


Fig. 1.4 Pie charts of the traits expressed in GM trees field deployed worldwide in 2005 (A) and new traits expressed in GM trees monitored for their potential impact on fungi (B).

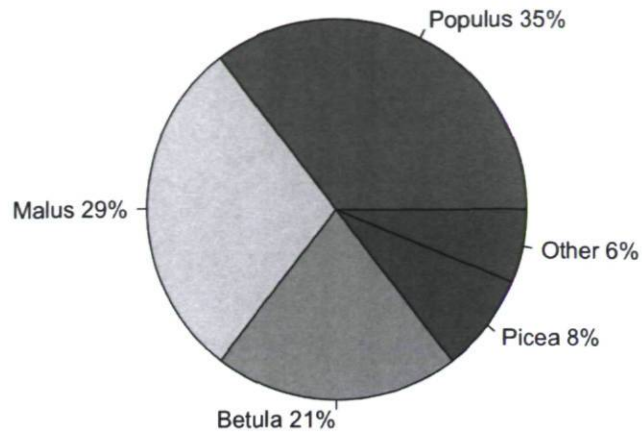


Fig. 1.5 Pie chart of genera to which belong the GM trees investigated for their potential impact on fungi.

CHAPITRE II

Impact study of an 8-year-old transgenic poplar plantation on the ectomycorrhizal fungal community

2.1 Avant-propos

Ce chapitre a été publié en 2009 dans APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 73(23) : 7527-7536 (FOP Stefani, J-M Moncalvo, A Séguin, JA Bérubé & RC Hamelin). FOP Stefani a défini l'approche méthodologique et a réalisé l'ensemble des manipulations, des analyses statistiques et de la rédaction du manuscrit. Les peupliers génétiquement modifiés et le dispositif au champ ont été développé et réalisé par l'équipe d'A Séguin. J-M Moncalvo a accueilli le premier auteur au sein de son laboratoire et a contribué à sa formation aux techniques moléculaires appliquées à l'étude des champignons du sol. Les quatre co-auteurs ont participé aux corrections du manuscrit. RC Hamelin a permis le financement des travaux effectués dans son laboratoire du Service canadien des forêts.

2.2 Résumé

Les conséquences à long terme du déploiement d'arbres génétiquement modifiés sur les organismes mutualistes du sol sont peu connues. Cette étude a pour objectif d'évaluer l'impact de peupliers transformés avec le gène marqueur *npt*II et le gène rapporteur *GUS* sur la communauté des ectomycorhizes (EM), après huit années de croissance au champ. Nous avons produit 2229 séquences ITS à partir de l'analyse de 1150 extrémités racinaires

colonisées par les EM et 1079 clones fongiques du sol issue de l'échantillonnage des horizons organique et minéral de la rhizosphère de trois peupliers témoins et de trois peupliers transgéniques. Cinquante unités taxonomiques opérationnelles (UTO) ont été identifiées à partir de l'analyse de 1706 séquences ITS de champignons ectomycorhiziens. Les courbes de raréfaction représentant les extrémités racinaires colonisées par les EM et les banques de clones fongiques étaient proches de la saturation, indiquant que la plupart des espèces ectomycorhiziennes présentes sur le site ont été identifiées. Les analyses statistiques des mesures qualitatives et / ou quantitatives des diversités α et β montrent qu'il n'y a pas de différence significative entre la communauté des EM associées aux peupliers témoins et celle des EM associées aux peupliers transgéniques. Cependant, la communauté des EM identifiées par l'analyse des extrémités racinaires était significativement différente de celle identifiée par le clonage du sol. Nous n'avons pas trouvé de différence dans la structure des communautés d'EM associées à long terme avec des peupliers transgéniques, et nous avons montré que l'analyse des extrémités racinaires colonisées par les EM est complémentaire de l'analyse des clones fongiques du sol. L'utilisation des deux approches permet d'avoir une image plus fine de la diversité des EM.

2.3 Abstract

The long-term impact of field-deployed genetically modified trees on soil mutualistic organisms is not well known. This study aimed at evaluating the impact of poplars transformed with a binary vector containing the selectable *npt*II marker and *GUS* reporter genes on ectomycorrhizal (EM) fungi, 8 years after field deployment. We generated 2229 fungal ITS PCR products from 1150 EM root tips and 1079 fungal soil clones obtained from the organic and mineral soil horizons within the rhizosphere of three control and three transformed poplars. Fifty EM fungal OTUs were identified from the 1706 EM fungal ITS amplicons retrieved. Rarefaction curves from both the root tips and soil clones were close to saturation, indicating that most of the EM species present were recovered. Based on qualitative and/or quantitative α- and β-diversity measurements, statistical analyses did not reveal significant differences between EM fungal communities associated with transformed

poplars and the untransformed controls. However, EM communities recovered from the root tips and soil cloning analyses differed significantly from each other. We found no evidence of difference in the EM fungal community structure linked to the long-term presence of the transgenic poplars studied, and we showed that coupling root tips analysis with a soil DNA cloning strategy is a complementary approach to better document EM fungal diversity.

2.4 Introduction

Poplar has become a model tree species in genetic engineering as it can easily be transformed, clonally propagated, and has a small genome size (7, 78, 81). Tree growth, agronomic traits and timber quality can be improved through genetic engineering (62), thereby avoiding the long reproductive cycles of conventional breeding (47, 60, 84). However, concerns have arisen about the potential impact of genetically modified (GM) trees on the environment (10). The potential environmental hazards linked to GM trees differ from those associated with transgenic crop plants at both spatial and temporal scales (85) because trees are long-lived perennials, unlike annual crop plants. They display several biotic interactions with soil microbial communities such as bacteria and fungi. Interactions between GM trees and these communities could result in exposure to the expression of new traits over several decades, a period longer than with GM crop plants.

Impact studies of GM plants on non-target organisms usually focus on the potential risk linked to the transgene expression (expected effects) that confers a genetic advantage to the transformed plant rather than on unforeseen (pleiotropic) effects from transgene insertion or the expression of other transgene components such as selection markers or reporter genes. The *npt*II gene, encoding neomycin phosphotransferase II (NPTII, EC 2.7.1.95), and the *GUS* gene, encoding β-glucuronidase (GUS, EC 3.2.1.31), are frequently used for genetic selection of transformed cells and for monitoring transgene presence and expression during transgenic plant lifetime (77). The products of the *npt*II and *GUS* genes have been subjected to safety assessment studies and were shown to be non-deleterious to human and animal health (21, 23, 27, 51). Nevertheless, pleiotropic effects in crop plants transformed

with the *npt*II and *GUS* genes have been observed (2, 15, 17, 43). Pleiotropic effects have also been recorded from GM trees co-expressing such selectable markers. For example, Pasonen *et al*. (57) showed a significant decrease in the number of root tips colonized by *Paxillus involutus* associated with a line of chitinase-transformed silver birch *in vitro*. Similar results have been observed *in vivo* with *P. involutus* associated with a line of lignin-modified silver birches (73).

Many trees in temperate, boreal, tropical and sub-tropical forests establish mutualistic interactions with EM fungi (42, 67, 68, 69). EM fungi are a polyphyletic group comprising over 5,000 species (49) that play key roles in biogeochemical soil processes and plant health. They represent one third of the total microbial biomass in the soil of boreal forests (32). Fine roots colonized by EM fungi, also called EM root tips or ectomycorrhizae, display a fungal mantle from which extends the extraradical mycelium to prospect the soil for nutrient uptake. These two anatomical parts can be sampled for EM fungi molecular identification, but some studies have highlighted dissimilarities between the EM fungal diversity recorded in root tip sampling and that recorded in extraradical mycelium sampling (26, 37, 39).

Given the potential cumulative effects caused by the presence and stable constitutive expression of transgenes over years on GM trees fitness and on the environment, impact studies of GM trees require long-term field trials (5, 73, 85). In this study, we investigated the potential long-term impact on the EM fungal community of hybrid poplars transformed with the binary vector containing the selectable *npt*II marker and *GUS* reporter genes, field-deployed for 8 years. This plantation was part of the first confined field trial of transgenic trees in Canada. Hybrid poplars constitutively expressed the *npt*II gene for kanamycin resistance driven by the *NOS* promoter (30). The activity of *NOS* promoter has been shown to increase in the lower part of transgenic tobacco plants (4). Such a vertical gradient has also been observed in transgenic hybrid poplars where the *NOS* promoter activity was 2.4 fold-higher in roots than in leaves (88).

As no direct negative impact of the *npt*II or *GUS* genes expression on fungal organisms has been reported in the literature, we first tested the null hypothesis H_0 that the EM fungal community recorded from transgenic poplars was similar to that from untransformed

poplars. Second, since the EM fungal diversity picture can be influenced by the sampling method, we contrasted the EM fungal community recovered from root tips with that recorded in soil cloning analyses. Intenal transcribed spacer (ITS) sequences from the nuclear ribosomal RNA (rRNA) were produced from both EM root tips and extraradical mycelia to compare the EM fungal communities associated with three control and three transgenic poplars. EM fungal communities were characterized by measuring the usual qualitative and quantitative EM species diversity within each community (α-diversity) and then estimating the nucleotide diversity between EM communities in relation to EM phylotype relative abundances (quantitative β-diversity).

2.5 Materials and Methods

2.5.1 Field site and sampling strategy

The study site was located at the Valcartier Research Station (Natural Resources Canada, Canadian Forest Service) and the plantation area was 540 m^2 (0.05 ha). Geographic, climatic and pedologic features of the plot are summarized in supplemental Table S2.1. The site belongs to the balsam fir–white birch bioclimatic domain. The plot is surrounded mainly by mature white pine (*Pinus strobus* L.) and red pine (*Pinus resinosa* Ait.) stands. It was the first trial with outplanted transgenic trees in Canada and many precautions were taken to avoid transgenic root escape and contact with the neighbouring forest. The transformed poplars and controls were deprived of physical contact with their surroundings by a ditch and a geotextile membrane at the bottom of the plot. The underground part of the plot was composed of superposed horizons of gravel, sand (40 cm), and organic soil (20 cm). Poplar clones 5339 (*Populus alba* L. X *P. grandidentata* Michx) were transformed using the binary vector pRT210, containing the wound-inducible promoter from proteinase inhibitor II (*pin2*) that drives the expression of the *GUS* reporter gene and the *npt*II gene driven by the NOS promoter for plant kanamycin selection (30). Transgenic and control poplars were planted in August 1997. Two guard rows of non-transgenic *Populus nigra* L. (clone 3051) surrounded the plantation. The experimental plantation system consisted of 10

blocks; two repetitions of each cloning line were randomly distributed within each block with a 1 x 1 m tree spacing. In early October 2005, a total of 24 soil cores (5 cm in diameter, 30 cm in length) and 24 root samples were collected from the three control and three transgenic poplars belonging to the same transformed line from three separate blocks. To limit biases in recovering EM fungal diversity due to a patchy EM distribution, the four cardinal points around each tree were sampled at 30 cm from the trunk. Each soil core presented a 20-cm-thick organic horizon (OH), very dark and compact, and a 10-cm-thick mineral horizon (MH), sandy and gravelly, at the bottom. From each core, around 20 cm^3 of organic and mineral soil were sub-sampled and sieved. Root samples were collected at the same point cored from the OH and tracked to ensure that they were linked to the targeted tree. Soil cores and root samples were kept at -80°C until processing.

2.5.2 Soil analyses

Moisture, organic matter, total C, primary nutrients (N, P, K), secondary nutrients (Mg, Ca), micronutrients (Zn), $H_2 0$ pH, cation exchange capacity (CEC), and texture were measured from the OH of each of the 24 cores collected in the plantation on a LECO CNS-2000 analyzer (Leco Corp., St. Joseph, MI). Table S2.1 presents the average values of these parameters computed from the 24 soil sub-samples.

2.5.3 DNA extraction and amplification

Soil core sub-samples were wet sieved (mesh size: 1 mm) to remove rocks and root fragments. Two hundred and fifty milligrams (wet weight) were used for total genomic DNA extraction using the Mo Bio UltraClean ™ soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA) according to the manufacturer's instructions, except that the final elution volume was 50 µl rather than 100 µl. From each root sample, a minimum of three thin roots, 5-10 cm in length, were analyzed for root tip isolation. All the root tips observed (one per aggregate) were sampled along the thin root to obtain a collection of 96 root tips per cardinal point. Root tips were individually frozen in liquid nitrogen, and ground in a 1.5 ml eppendorf tube with a polypropylene micropestle. Ground tissues were resuspended in 30 µl of ultra pure ™ DNAse/RNAse-free distilled water (Gibco, New

York, NY), then added at 150 µl of 15% Chelex® 100 (Bio-Rad) suspension with proteinase K (1.6 µg μl^{-1}) (Invitrogen, Carlsbad, CA). Samples were incubated for 2 h at 65°C, then for 20 min at 95°C to inactivate proteinase K, and centrifuged at 1000 rpm for 5 min. One microlitre of the supernatant was used as genomic DNA (gDNA) template for PCR. Internal transcribed spacer (ITS) regions were amplified using the ITS1-F / ITS4 primer set (89) to sequence the root tips data set and to build soil clone libraries from the organic horizon (OH) and mineral horizon (MH). PCR mixture was made up of 1X PCR buffer, 1.6 mM $MgCl_2$, 1.25 mM of each deoxynucleotide triphosphate, 25 µg of bovine serum albumin (BSA) (SIGMA, St. Louis, MO), 0.5 µM of each primer, and 1 unit of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), in a total volume of 25 µl. Thermal cycling conditions to amplify root tips and white colonies from each soil clone library were as follows: initial denaturation at 95°C for 2 min, 37 cycles at 94°C for 45 s, 58°C for 1 min, 72°C for 1 min 30 s, and a final elongation at 72°C for 10 min. Annealing temperature and the number of cycles were respectively decreased to 50°C and 30 for the pre-cloning PCR step to reach low stringent conditions. PCR reactions were done on a MJ research PTC-200 (MJ Research Inc., Waltham, MA).

2.5.4 Library construction and sequencing

PCR products were purified with QIAquick PCR Purification Kit and cloned with QIAGEN PCR Cloning plus Kit (QIAGEN, Rockville, MD) according to the manufacturer's instructions. Five-fold molar excesses of PCR products were incubated for 2 h at 14°C with the pdrive-cloning vector. After an overnight incubation at 37°C, 24 white bacterial colonies from each core sub-sample were spiked and transferred into the 25 µl PCR mixture for amplification as described above. Preliminary data indicated a low EM species richness in the soil, thus we screened soil clone amplicons by restriction fragment length polymorphism (RFLP) analysis prior to sequencing. Enzymatic digestion was performed with HhaI and AluI at 37°C for 2 h. RFLP mixture was made up of 1X PCR buffer, 2.5 units of each endonuclease, 1 µg of BSA, and 1 µl of PCR product, in a total volume of 10 µl. Enzymes were inactivated at 80°C for 20 min. We systematically sequenced 10% of amplicons belonging to the same restriction profile observed on each

agarose gel. Sequencing was performed on a 96-capillary 3730xl DNA analyzer at the Genomic Sequencing and Genotyping Platform of the Centre hospitalier de l'Université Laval Research Centre (RCCHUL, Québec, Canada).

2.5.5 Bioinformatic analyses and clustering

ITS sequences were edited and assembled with Sequencher v4.6 (GeneCodes, Ann Arbor, MI). The similarity threshold for sequences belonging to the same operational taxonomic unit (OTU) was set to 98%, which corresponds to values used in O'Brien *et al.* (54) (97%) and Arnold *et al.* (6) (95 and 99%), to serve as a proxy for 'species'. Each OTU consensus sequence was identified with the closest sequences found in the NCBI GenBank database using BLAST (3). PCR-generated chimeric sequences were determined from BLAST hits displaying conspicuous incongruence between the ITS1 and ITS2 regions and were excluded from the data sets. Sequences were aligned with MUSCLE software v3.5 (20) with two iterations. Sequences were clustered with those retrieved from the GenBank database by running a Neighbor-Joining analysis in PAUP v4.0b10 (79). Significant support in clusters displayed by the Neighbor-Joining trees was assessed with 1000 bootstrap re-samplings. Ascomycetes and basidiomycetes were analyzed separately. The number of OTUs, rarefaction curves, richness estimators, Shannon and Simpson species diversity indices (74, 75) were computed with DOTUR (71). Similarities between the EM fungal communities recorded from control and transgenic poplars and the different sampling methods were compared with the non-parametric maximum likelihood estimator θ (NPMLE), based on shared and non-shared species proportions between samples (90) computed with SONS program (72).

2.5.6 Statistical analyses

Comparisons between control and transformed poplars were based on a matrix made up of the EM fungal OTU relative abundance recorded from the root tip, the OH and the MH data sets, and observed at each of the 24 points sampled. Normal distribution and homogeneity of variance of the data used for comparisons were assessed with a Shapiro-Wilk normality test and an *F* test. Synthetic descriptors computed between control and transformed poplars,

such as the observed EM fungal species richness, the Chao richness estimator index (11) and the Shannon species index, were compared with a two-sample permutation test (10,000 permutations).

A canonical redundancy analysis (RDA; 64) was performed to investigate the variation in EM fungal composition recorded from the two data sets among the 24 points sampled as a function of transformed poplars and soil chemical variables. The raw relative abundance matrix of EM fungal OTUs was transformed with the Hellinger asymmetric association coefficient (65). The explanatory variables selected for the RDA were tested to be uncorrelated using the Pearson coefficient. The explanatory variables considered were the transformation event (defined as a binary variable 0/1 for objects associated with control and transformed poplars, respectively) and the soil chemical variables Ca, K, N_2, Zn, and $H_2 0$ pH. The remaining soil chemical variables were not considered as they were correlated. Finally, we tested the significance of the explanatory variables by running an ANOVA-like permutation test (1000 permutations, $\alpha = 0.05$, $\beta = 0.01$).

EM fungal communities from i) control and transformed poplars, and ii) root tips sampling and soil cloning were also investigated with a double principal coordinate analysis (DPCoA; 59). DPCoA displays the first two orthogonal principal axes, based on the relation between an OTU dissimilarity matrix (one consensus sequence per OTU was used as input) and the corresponding abundance matrix. The Rao diversity index is computed from the dissimilarity matrix and is decomposed into between-community and within-community diversities (59). It takes into consideration the genetic distance between each OTU, the diversity decreases when OTUs composing a sample are similar, and increases when they are more distant.

To assess the significance of the differences between EM fungal communities associated with control/transformed poplars or recovered according to the different sampling methods, point coordinates given by the DPCoA in the S-dimensional space (44) were analyzed with a RDA and the corresponding ANOVA test statistic *F* (1000 permutations). Two explanatory variables were considered: the sampling method and the transformation event, all defined as binary variables. All the statistical analyses were performed using the R

statistical language (63). Clustering, RDA and DPCoA analyses were computed with the vegan 1.8-6 (55) and ade4 (12) packages.

2.5.7 Nucleotide sequence accession numbers

Nucleotide sequences were deposited in the NCBI GenBank database and are registered under the accession numbers EU554677 to EU555003 and FJ626911 to FJ626949.

2.6 Results

2.6.1 Fungal baseline description

Fungal ITS data obtained with the ITS1-F / ITS4 primer set were produced from 1150 EM root tips identified by sequencing (≈48 root tips sequenced per cardinal point) and 1079 extraradical soil clones (≈45 clones sampled per cardinal point) from the organic (OH) and mineral (MH) horizons, identified either by sequencing (53.1% of the soil clone amplicons) or by PCR-RFLP. Eighty-four OTUs were recovered from these two ITS data sets. Clustering the 84 fungal OTUs with the closest sequences available in the NCBI GenBank database showed that 50 OTUs were related to ectomycorrhizal fungal groups (Table 2.1 and Fig. S2.1A, B, published as supplemental material). The rarefaction curves for each sampling method (Fig. 2.1A) and each sampled tree (Fig. 2.1B) appeared to level off. The Chao and bootstrap estimators confirmed that we achieved ectomycorrhizal OTU richness saturation for each data set with values close to the observed EM species richness (Table 2.2).

2.6.2 Comparisons of EM communities between transgenic poplars and controls

Rarefaction curves plotted by control and transformed poplars (Fig. 2.1B) displayed a similar level of EM fungal species richness. Using a permutation test (10,000 permutations), no statistical difference between control and transformed poplars was found

for the species richness observed ($P = 0.4$), the species richness estimated with the Chao index ($P = 0.3$) and with the Shannon species index ($P = 1$).

The heatmap in Figure 2.2 shows the distribution among the 24 cardinal points sampled around the three control and three transgenic poplars of the 50 EM fungal OTUs recovered by pooling the two ITS data sets. A single OTU identified as an uncultured EM *Cortinarius* was recovered at each sampled point with a mean frequency of 59% (min=17.6, max=86). Other OTUs had a patchy distribution with a mean frequency of 0.80% (min=0.04, max=4.77). The NPMLE clustering (top of Fig. 2.2), based on the Euclidean distance of the non-parametric maximum likelihood estimator θ computed at each cardinal point, showed an absence of partitioning in relation to control and transgenic poplars.

The redundancy analysis (RDA, Fig. 2.3A) showed no apparent cluster of the 24 points sampled according to the treatment or the soil chemical variables considered (Ca, K, N_2, Zn and pH). The combination of these six explanatory variables explained 8.2% of the variance and their effect was not significant (pseudo-*F* statistic under the null hypothesis $H_0 = 0.11$). Analysis using the treatment as the unique explanatory variable showed that the treatment explained 1.6% of the total variance (pseudo-*F* statistic under the null hypothesis $H_0 = 0.2$).

Divergence between EM fungal communities from transformed poplars and controls based on phylotype dissimilarities in relation to their relative abundance was also investigated using a double principal coordinate analysis (DPCoA, Fig. 2.3B). The DPCoA showed no more difference, in regard to the relation between the dissimilarity of the 50 EM fungal phylotypes and their relative abundance in the 24 soil samples analyzed, between and within control and transformed poplars. Decomposition of the Rao diversity showed that the differences between EM communities recorded from the 24 points sampled represented 7.8% of the total diversity. A RDA performed on the DPCoA point coordinates showed that the difference between the sampled points from control and transgenic poplars was not significant (pseudo-*F* statistic under the null hypothesis $H_0 = 0.89$), indicating that EM fungal species occurring in soil samples associated with transformed poplars were as genetically-diversified as those recorded in soil samples from control poplars.

2.6.3 Root tip sampling vs. soil cloning

The sequencing of 1150 root tips yielded 42 OTUs, of which 39 were identified to be EM fungi. Shannon and Simpson species diversity indices were the highest for the root tips data set. The identification by sequencing or PCR-RFLP of 1079 soil clones produced 58 OTUs, of which 26 were determined to be EM fungi (Tables 2.1 and 2.2). Root tips and soil cloning data sets shared only 15 EM fungal OTUs (Table 2.1).

Members of the Cortinariaceae family were most abundant in the EM community. They were sampled from 632 root tips out of 1146 (55.3%), representing five OTUs of this family. Among them, a single OTU matching an "uncultured *Cortinarius*" sequence in GenBank represented 478 out of the 1146 (41.7%) root tips sampled. Most OTUs from the Cortinariaceae, Hymenogastraeceae, Hydnangiaceae and Inocybaceae families were recovered from both root tips and soil clone libraries (Fig. S2.1B in supplemental material). This contrasts with the Thelephorales and Pezizales-clade I for which 6 out of 7 and 5 out of 6 OTUs, respectively, were found only on root tips. In all, 24 EM fungal OTUs were exclusive to the root tips data set and had a relative abundance of <1%, except *Cortinarius atrocoeulaeus* (8.9%, the second most abundant OTU within the root tips data set), *Russula emetica* (7.9%, the third most abundant OTU), *Cortinarius favrei* (2.6%), and an uncultured EM Thelephoraceae (2.4%).

The soil clone data set was made by pooling libraries from the OH and MH. The MH clone library had a much lower number of EM ITS amplicons than the OH library (Table 2.2). A large majority of the ITS amplicons from the MH clone library (77.8%) were identified as *Acremonium* spp., a genus of mitosporic Hypocreales (contig 02, Table 2.1).

Decomposition of the Rao diversity from the double principal coordinate analysis showed that there was a 20.8% difference between EM communities recorded from the 24 root tips data sets and the 24 soil clone libraries. There was a significant relationship between phylotypes distribution and the sampling method used (pseudo-F statistic = 0.001) while the difference between points from control and transformed poplars was still not significant (pseudo-F statistic under the null hypothesis H_0 = 0.96). This showed that the sampling method had an influence on the EM diversity recovered while the treatment did not.

2.7 Discussion

2.7.1 Transgenic poplar impact on EM fungal community

This study of a long-established transgenic poplar plantation is the most exhaustive assessment of transgenic tree impact on the diversity of the EM fungal community to date. After 8 years of mycorrhizae-GM host interaction, qualitative and/or quantitative α- and β-diversity measurements show no difference in EM fungal community structure between poplars transformed with the binary vector containing the selectable *npt*II marker and *GUS* reporter genes and the controls.

Studies monitoring the impact of field-deployed transgenic trees on mycorrhizal fungi are scarce and not extensively documented. Kaldorf *et al.* (36) found no difference in mycorrhizal colonization and diversity (15 EM fungi identified) between the changed phytohormone balance in *rolC*-transformed aspens and controls after being field-deployed for 3 years, but one of the four most common EM species recorded was significantly less abundant when associated with one transformed line. Vauramo *et al.* (86) showed that fungal biomass was not different between the leaf litter associated with chitinase-transformed silver birches and controls in a field trial that lasted 11 months. Newhouse *et al.* (53) field deployed American elms transformed to express the synthetic antimicrobial peptide ESF39A in an attempt to improve their resistance to Dutch elm disease caused by the fungus *Ophiostoma novo-ulmi*. Whereas staining from *O. novo-ulmi* was significantly reduced on transgenic sapwood, they noted that transgenic and wild-type elms had similar mycorrhizal colonization rates after three months of field deployment. Similar results have also been observed *in vitro*. Hampp *et al.* (29) did not detect any difference in the formation and morphology of the ectomycorrhizae *Amanita muscaria* associated with transgenic aspens expressing an hygromycin marker gene and IAA-biosynthetic genes. Pasonen *et al.* (57) investigated the ability of silver birches constitutively expressing the *npt*II marker gene and the sugar beet chitinase IV gene to form normal ectomycorrhizae with *Paxillus involutus*. Although one transformed line showed a significant decrease in the number of root tips due to a reduced root system, they concluded that the morphology of mycorrhizae

and mycorrhization efficiency was not altered. Similarly, Seppänen *et al.* (73) found that genetically-modified lignin biosynthesis pathway silver birches containing the *npt*II gene for kanamycin selection exhibited a significant pleiotropic effect decreasing the root biomass; however, the association between the EM fungus *Paxillus involutus* and transgenic silver birches was not affected.

Selection of transformed tissues based on selectable marker expressing antibiotic resistance in genetically engineered plants is widely used. The *npt*II gene is the most frequently used antibiotic-resistant marker in GM plants (24, 48), and among the above-mentioned studies, all the transgenic trees developed were kanamycin- or hygromycin-resistant. The hazard of using antibiotic resistant markers for the environment lies particularly in the potential horizontal gene transfer (HGT) from GM plants to soil bacteria. The likelihood of shifts in natural soil microorganism communities due to the emergence of new resistant bacterial strains from HGT between GM plants and soil bacteria is considered almost null (16) or offset by antibiotic prescription in clinical practice (24). Although mycorrhiza helper bacteria modulate mycorrhizal symbiosis (see Frey-Klett *et al.* (22) for a review), the impact of antibiotic-resistant markers on the mycorrhizal community as a consequence is hardly to be expected, and the absence of effect on an EM fungal community exposed for over 8 years to the expression of the *npt*II gene supports the absence of impact from the *npt*II gene when used as a selectable marker system in plants.

2.7.2 Ectomycorrhizal fungal species richness

After extensive root tips and soil analyses, we recovered 84 fungal OTUs ("species"), of which 50 were EM fungi. Based on the rarefaction curves depicted in Fig. 2.1A, this is an accurate estimate of fungal diversity at the study site. The original plantation site was devoid of established roots in the soil which play a major role in seedling mycorrhizal colonization (18). Therefore, the transgenic and control trees could only be colonized by wind- or animal-carried spores, or vegetative tissues from the neighbouring forest or from the soil used at the time of planting. In this highly artificial community, the combination of two sampling approaches with a high sequencing effort led to the recovery of a higher level of EM fungal diversity than most of the previous studies focusing on EM fungal

communities associated with forest trees, in plantations or natural stands. This agrees with the point raised by Horton and Bruns (33) who suggested the use of different methods and increased direct sequencing to better represent EM fungal diversity. They reviewed 14 studies investigating belowground EM fungal diversity that did not manage to saturate EM fungal species accumulation curves. The average number of soil samples collected was 35 and species richness averaged 33, except for the study with the highest sampling effort (198 soil samples), which recorded 200 morphologically distinct EM fungi. On the other hand, EM fungal species richness averaged 44 (min = 21, max = 79) in other studies using molecular tools mainly based on the identification of root tips with ITS-RFLP and denaturating gradient gel electrophoresis (DGGE) or soil cloning (9, 14, 18, 19, 25, 28, 34, 37, 38, 41, 50, 52, 61, 80, 83, 87). Some studies investigating EM fungal species richness observed values at least twice greater than those observed in this study. However, those studies were conducted on sites with higher ecological complexity and/or for a longer time period with more intense sampling (35, 45, 46, 54, 56, 70, 76, 82).

2.7.3 Methodological considerations

We showed that the differences observed among EM fungal communities were more influenced by the sampling method (root tips versus sieved soil) than by the occurrence of transformed poplars over an 8-year period. Increasing the sampling effort by cloning and sequencing is faster and easier than root tips sequencing, and despite their non-demonstrated function as EM fungi, it could recover a level of species richness not previously reported by sporocarp or root tip samplings. In our site, root tips provided 24 EM fungal OTUs not observed in the soil clone data set. Despite our attempts to minimize PCR artifacts by performing amplification in low stringency conditions, a PCR bias toward the dominant *Cortinarius* OTU could be expected, as it would outcompete rare EM fungal species during primer annealing. The consequence would be a statistical saturation of EM fungal diversity but not a biological saturation, as shown by the EM fungal species richness recovered from the root tips data set. Nevertheless, the soil clone data set brought 11 new OTUs compared with root tips, which represents an EM fungal species richness increase of 22%, while root tips rarefaction curves tend to be saturated.

Our results also revealed that investigating EM extraradical mycelia failed to detect the four common species recorded on root tips. This suggests that these four species – two *Cortinarius*, one Russulales and one Thelephoraceae – may produce limited extraradical mycelium in the soil, thus affecting their detection by soil cloning. It contrasts with Agerer (1) who classified species from the *Cortinarius* genus into the Medium-distance exploration type, featuring an extended contact with the soil. Parrent and Vilgalys (56) also noticed disparities between EM fungal communities recovered from root tips and from extraradical mycelium, and observed that some species occurring with an important frequency as extraradical mycelium or fruiting bodies were rarely or not detectable as root tips. This highlights the different foraging types and ecological roles of EM fungi (1), and explains why the detection of some EM fungal species would depend on the sampling method, an issue already raised by Koide *et al.* (37).

In conclusion, changes in the EM fungal community associated for 8 years with transformed and untransformed poplars were not detectable, while the analysis of root tips and extraradical mycelium cloning provided contrasting views of the EM fungal community colonizing the experimental poplar stand. The present study is in line with the trend according to which no major effect from transgenic trees on mycorrhizal, soil fungal or soil bacterial communities has been observed from the GM trees studied so far. Up until now, most of the studies investigating the effect of transgenic trees or transgenic crops on soil microbial communities have observed changes attributable to new traits from GM organisms that are smaller than changes attributable to other factors such as soil type, plant genotype and stand sites (8, 16, 40, 66).

However, transformations leading to deleterious effects on trees (13, 58, 73) or non-target organisms (31, 36) have been reported, but they involved pleiotropic effects that depend on each unique insertion event. Therefore, future impact studies of GM trees will have to test over a long-term period every transgenic line extensively deployed before large-scale propagation to better evaluate the likelihood and the consequences of transgenic trees on mycorrhizal symbioses.

2.8 Acknowledgements

We thank Denis Lachance for the experimental design of the control and transformed poplars and field deployment. We are grateful to Marie-Josée Bergeron and Dr. Philippe Tanguay for insightful comments in the course of this study and on an earlier draft of the manuscript, and we thank the Centre de bio-informatique et de biologie computationnelle (CBBC, Université Laval, QC, Canada) for bioinformatic assistance. This work was supported by grants from the Canadian Biotechnology Strategic fund.

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Table 2.1 Closest sequences recorded from the NCBI GenBank database matching the 84 fungal OTUs (2229 ITS sequences identified) recorded from the root tip (RT) and soil cloning analyses (OH: horizon mineral and MH: mineral horizon).

Blast match[a]	Contigs / singletons	Accession no.	Coverage (%)	Similarity (%)	EM[b]	Number of ITS sequences from		
						RT	OH	MH
Uncultured EM *Cortinarius*	Contig 01	AY748857	100	99	Y	478	395	33
Acremonium (strictum)	Contig 02	AY138846	100	100	N	-	25	440
Cortinarius atrocoeulaeus	Contig 03	AY083178	100	99	Y	102	1	-
Russula emetica	Contig 04	AY061673	100	99	Y	91	-	-
Hebeloma mesophaeum	Contig 05	AB211272	100	99	Y	65	2	1
Laccaria sp.	Contig 06	AJ534899	100	100	Y	34	12	4
Inocybe lacera - Phylotype 1	Contig 07	AB211269	99	96	Y	23	8	3
Inocybe lacera - Phylotype 1	Contig 08	AY750157	99	99	Y	7	1	21
Hymenogaster arenarius	Contig 09	DQ328124	99	91	Y	26	3	10
Uncultured Sebacina isolate	Contig 10	EU668270	100	88	Y	29	3	9
Inocybe radiata	Contig 11	EU819490	100	83	Y	42	2	-
Cortinarius favrei	Contig 12	AF325575	100	99	Y	30	-	-
Uncultured EM *Thelephoraceae*	Contig 13	AJ549971	100	97	Y	28	-	-
Inocybe lacera - Phylotype 2	Contig 14	AB211269	99	94	Y	14	3	1
Cortinarius paleaceus	Contig 15	FJ039709	100	96	Y	-	11	1
Uncultured EM *Thelephoraceae*	Contig 16	AY748885	100	99	Y	23	-	-
Cortinarius parvannulatus	Contig 17	AY669664	100	99	Y	18	-	1
Phialophora finlandia	Contig 18	AF486119	100	98	Y	15	-	4
Saccharomyces cerevisiae	Contig 19	AB212260	100	99	N	-	-	11
Uncultured EM Inocybe	Contig 20	AY310824	99	96	Y	14	5	-
Uncultured EM Pezizales	Contig 21	AY634125	100	100	Y	14	-	-
Leccinum duriusculum	Contig 22	AF454577	100	93	Y	10	-	1
Uncultured EM Inocybe	Contig 23	AY310829	100	85	Y	11	-	-
Uncultured EM *Tomentella*	Contig 24	EF619832	100	98	Y	8	-	-
Uncultured EM *Tuber*	Contig 25	AY748861	100	99	Y	8	-	-
Peziza depressa	Contig 26	DQ200837	100	96	Y	7	-	-
Cryptococcus (fuscencens)	Contig 27	AF145319	100	95	N	-	-	5
Uncultured EM *Tomentella*	Contig 28	EF218833	100	99	Y	6	-	-
Inocybe lacera - Phylotype 3	Contig 29	AB211269	99	94	Y	-	1	1
Uncultured mycorrhizal fungus	Contig 30	AY394904	100	90	N	2	3	-
Peziza ostracoderma	Contig 31	EU819461	100	97	Y	4	1	-
Uncultured EM fungus	Contig 32	AY587740	91	99	Y	5	-	-
Uncultured Pezizales	Contig 33	DQ469743	100	98	Y	-	-	5
Uncultured Thelephoraceae	Contig 34	AM181385	93	99	Y	5	-	-
Cortinarius hemitrichus	Contig 35	AY669680	100	99	Y	-	2	-
Cortinarius cf. *saniosus*	Contig 36	DQ102683	100	94	Y	4	-	-
Mortierella sp.	Contig 37	EU877758	100	100	N	-	4	-
Uncultured fungus (Pezizales)	Contig 38	DQ414728	100	95	Y	-	-	4
Scleroderma bovista	Contig 39	AB211267	100	90	Y	-	-	3
Uncultured fungus	Contig 40	EF434064	100	94	N	-	4	-
Uncultured EM *Tuber*	Contig 41	AY634113	100	99	Y	4	-	-
Hymenogaster glacialis	Contig 42	AF325634	100	99	Y	3	-	-
Inocybe calospora	Contig 43	AF325665	100	94	Y	3	-	-
Tomentella sp.	Contig 44	AB211278	100	93	Y	-	1	1

Uncultured EM Tomentella	Contig 45	EF411108	96	93	Y	3	-	-
Uncultured EM *Tuber*	Contig 46	AY634174	100	99	Y	3	-	-
Hebeloma albocolossum	Contig 47	AY308583	100	98	Y	2	-	-
Inocybe lacera - Phylotype 2	Contig 48	AY750157	99	99	Y	-	-	2
Paecilomyces sp.	Contig 49	DQ191963	100	93	N	-	2	-
Uncultured mycorrhizal fungus	Contig 50	AY656939	100	86	Y	-	-	2
Serendipita vermifera	Contig 51	DQ520096	98	93	Y	2	-	-
Uncultured Sebacina isolate	Contig 52	EU668270	100	90	Y	2	-	-
Mortierellaceae sp.	OH 01F02	FJ025208	87	82	N	-	1	-
Gigaspora margarita	OH 09F18	U15692	100	87	N	-	1	-
Mortierella macrocystis	OH 41F30	AJ878782	35	91	N	-	1	-
Uncultured fungus	OH 41F32	EF619892	100	87	N	-	1	-
Peziza depressa	OH 53F09	DQ200837	100	94	Y	-	1	-
Venturia hystrioides	OH 53F15	EU035459	100	98	N	-	1	-
Hypocrea crassa	OH 53F23	EU280067	100	100	N	-	1	-
Ganoderma applanatum	OH 57F33	AY884179	100	100	N	-	1	-
Uncultured fungus	OH 61F03	AF504878	53	91	N	-	1	-
Lambertella tubulosa	OH 61F10	EF029195	100	98	N	-	1	-
Tetracladium marchalianum	OH 61F11	FJ000360	100	94	N	-	1	-
Uncultured EM fungus	OH 61F16	EF484935	100	99	Y	-	1	-
Fusarium oxysporum	OH 65F14	EU364863	100	100	N	-	1	-
Glomerella lagenaria	OH 69F17	AJ301970	100	95	N	-	1	-
Leaf litter ascomycete	OH 77F05	AF502859	93	98	N	-	1	-
Entophlyctis helioformis	OH 77F21	AY997048	41	82	N	-	1	-
Trichoderma hamatum	OH 81F19	EU595036	100	100	N	-	1	-
Nectria sp.	OH 81F29	DQ317342	100	97	N	-	1	-
Uncultured fungus	MH 34a10	AY969872	98	99	N	-	-	1
Uncultured fungus	MH 34a12	DQ388863	98	92	N	-	-	1
Uncultured fungus	MH 78a25	DQ388863	85	90	N	-	-	1
Geomyces vinaceus	MH 34a19	AJ608972	100	100	N	-	-	1
Uncultured fungus	MH 34a26	AY969871	98	98	N	-	-	1
Wilcoxina mikolae	MH 34a22	DQ069000	100	99	N	-	-	1
Helotiales sp.	MH 38a13	DQ914730	100	93	N	-	-	1
Uncultured ascomycete	MH 78a02	AY969669	90	93	N	-	-	1
Rhodotorula mucilaginosa	MH 86a27	AF444614	100	100	N	-	-	1
Peziza badia	RT A2N49	DQ384574	100	99	Y	1	-	-
Leptosphaeria sp.	RT A3N22	DQ093683	99	99	N	1	-	-
Uncultured EM Pezizales	RT A3O60	AJ893241	98	99	Y	1	-	-
Hebeloma psammophilum	RT A3O95	AY312980	100	96	Y	1	-	-
Leptodontidium orchidicola	RT T5E32	AF486133	100	100	N	1	-	-

a: When different species belonging to the same genus presented exactly the same matches with our environmental sequences, the species name is included in brackets.
b: Y, yes; N, no. For EM identification based on neighbor-joining trees see the supplemental material.

Table 2.2 Impact of the sampling strategy on the fungal (Fg) and ectomycorrhizal (EM) diversities recorded at the Valcartier plantation.

Data set	No. of ITS amplicons		No. of OTUs		EM frequency (%)	Chao index		Bootstrap estimate		H [b]		1 / D [c]	
	Fg	EM	Fg	EM		Fg	EM	Fg	EM	Fg	EM	Fg	EM
Root tips	1150	1146	42	39	99.6	42.2	39.2	43.3	40.5	2.45	2.44	2.45	5.08
Soil cloning [a]	1079	560	58	26	51.9	127	30.1	71.9	31.2	1.63	1.26	2.94	1.75
OH	507	453	39	18	89.3	92.2	25.2	49.6	23.2	1.20	0.77	1.64	1.35
MH	572	107	31	19	61.3	71.0	26.0	37.3	22.0	1.16	2.28	1.66	6.66

a: OH and MH pooled data sets.
b: Shannon diversity index.
c: Simpson reciprocal index. The value starts at 1 for the lowest species diversity.

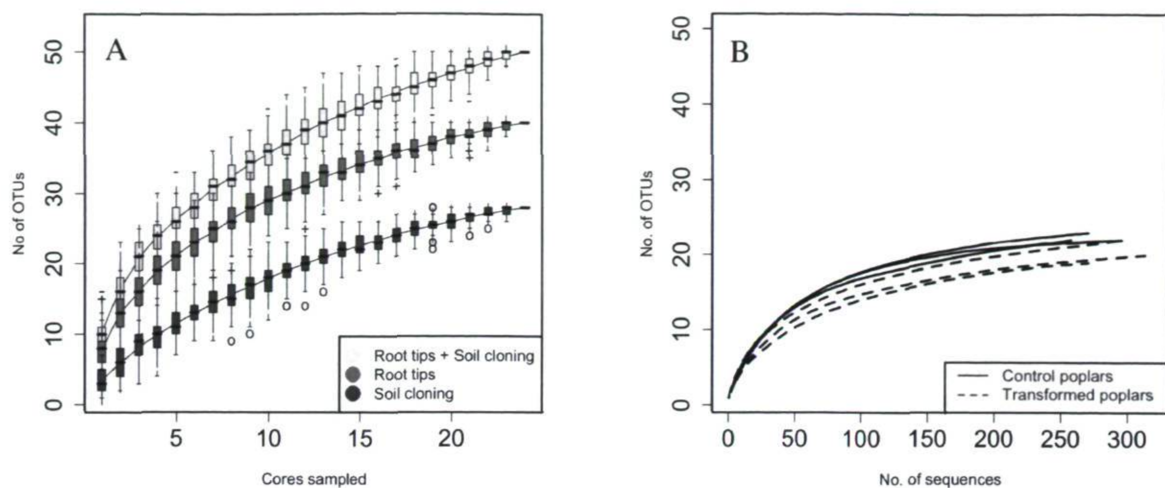

Fig. 2.1 Ectomycorrhizal OTU accumulation curve according to (A) the sampling method (root tip analysis or extraradical mycelium cloning), (B) control (full lines) and transgenic poplars (dashed lines) by pooling data from the root tips data set and soil clone libraries.

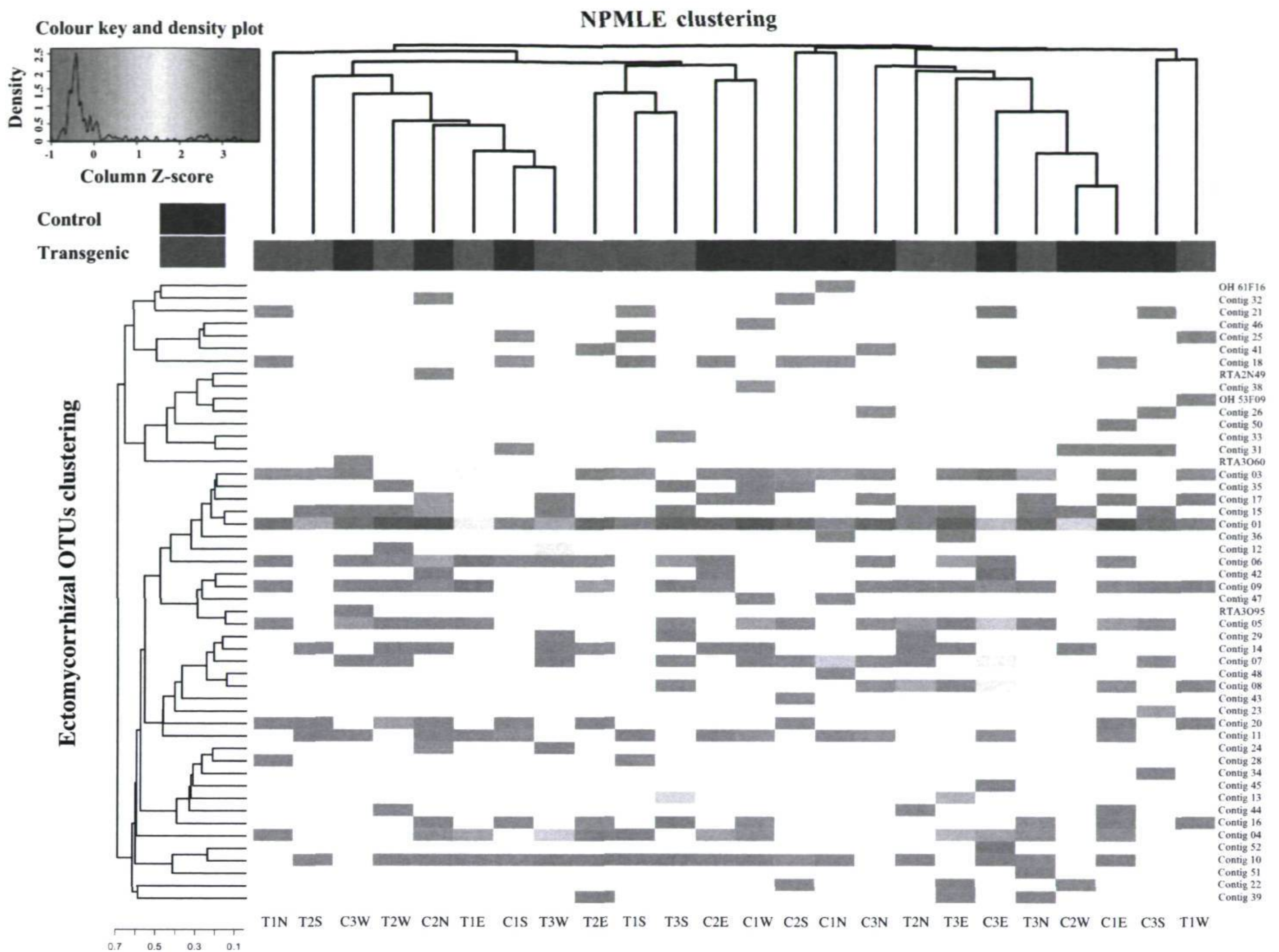

Fig. 2.2 Heatmap distribution based on the relative abundance of the 50 EM OTUs recorded from the root tips data set and soil clone libraries. Hierarchical clustering of the OTUs and of pairwise non-parametric maximum likelihood estimator (NPMLE) between control (blue) and transgenic (red) poplars. Sampled points are identified at the bottom of the figure by a "C" for control and a "T" for transgenic poplars, followed by the number of the tree sampled and the first letter of the corresponding cardinal position.

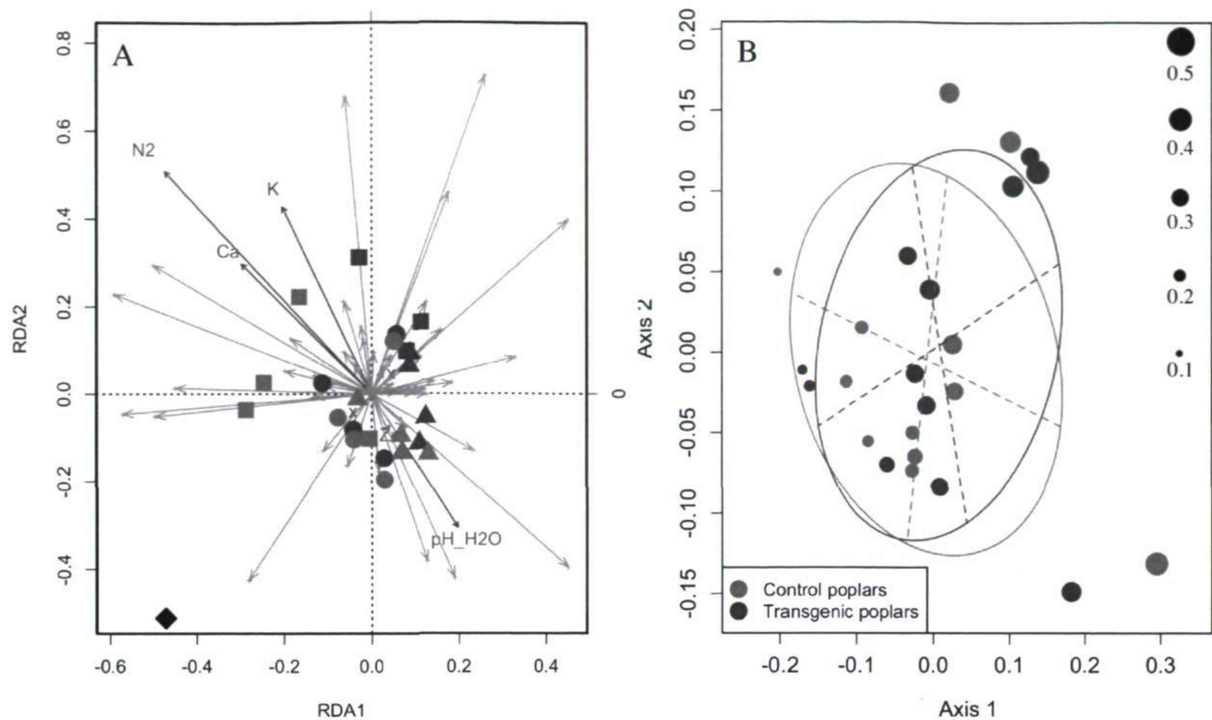

Fig. 2.3 A) Redundancy analysis (scaling 1) of the 24 points sampled in the poplar plantation, based on 1706 EM fungal ITS amplicons identified and six explanatory variables. Points represented by the same plot symbol belong to one of the four cardinal points from a same tree. Blue and red points are associated with control and transformed poplar, respectively. Green and blue arrows represent the 50 EM OTUs identified and the five quantitative explanatory variables. The binary explanatory variable is identified with a black diamond. The first two axes explain 14.6% of the total variation. B) Bubble map from the double principal coordinate analysis (DPCoA). Point size is proportional to the Rao diversity index computed for each point sampled. The diversity scale is shown on the right side of the figure. Blue and red points represent cardinal points from control and transgenic poplars, respectively. Ellipses indicate the distribution of soil samples per treatment.

Table S2.1 Geographic position, mean annual precipitation and temperature (Quebec City / Jean Lesage International Airport weather station, elevation 74.4 m, recorded between 1971 and 2000), and pedologic features of the transgenic poplar plantation at the Valcartier research station. Values given for the physical and chemical soil parameters are the means of the values recorded from soil cores collected at the four cardinal points around each tree, from the organic horizon.

Geographic and climatic features				
Site location	Annual precipitation (mm)		Annual temperature (°C)	
46°56'N - 71°56'W	1230		6	
Pedologic features				
H_2O pH	Moisture	OM [a]	C total (%)	CEC [b]
6	2.43	10.5	3.79	9.6
Primary nutrients		N (%)	P (mg/g)	K (mg/g)
		0.2	1.9	3.3
Secondary and micronutrients		Mg (mg/g)	Ca (mg/g)	Zn (mg/g)
		0.5	2.9	0.02
Texture		Sand (%)	Silt (%)	Clay (%)
		14.8	38.7	46.5

a: OM: organic matter.
b: CEC: cation exchange capacity.

A

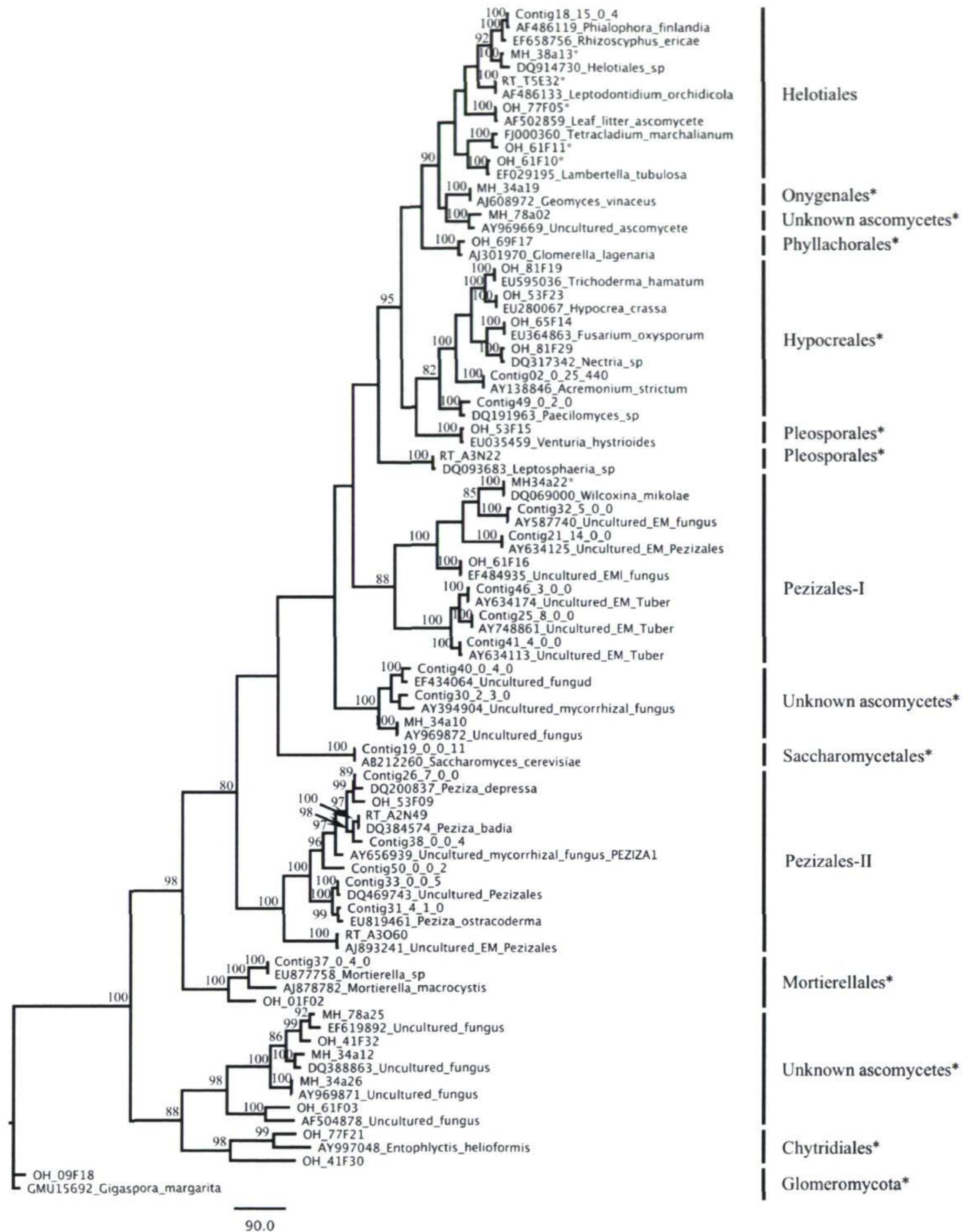

Fig. S2.1 Neighbor-Joining trees based on the analysis of (A) Ascomycetes and (B) Basidiomycetes ITS sequences with their top GenBank hits. For each contig, the three numbers spaced by underscores represent the number of sequences recorded from the root tip, organic and mineral horizon cloned. OTUs ID beginning with OH, MH or RT represent singletons retrieved from the organic and mineral horizons and root tips, respectively. OTUs within clades and fungal class marked with an asterisk were not considered as ectomycorrhizas. Values above branches reflect bootstrap support.

B

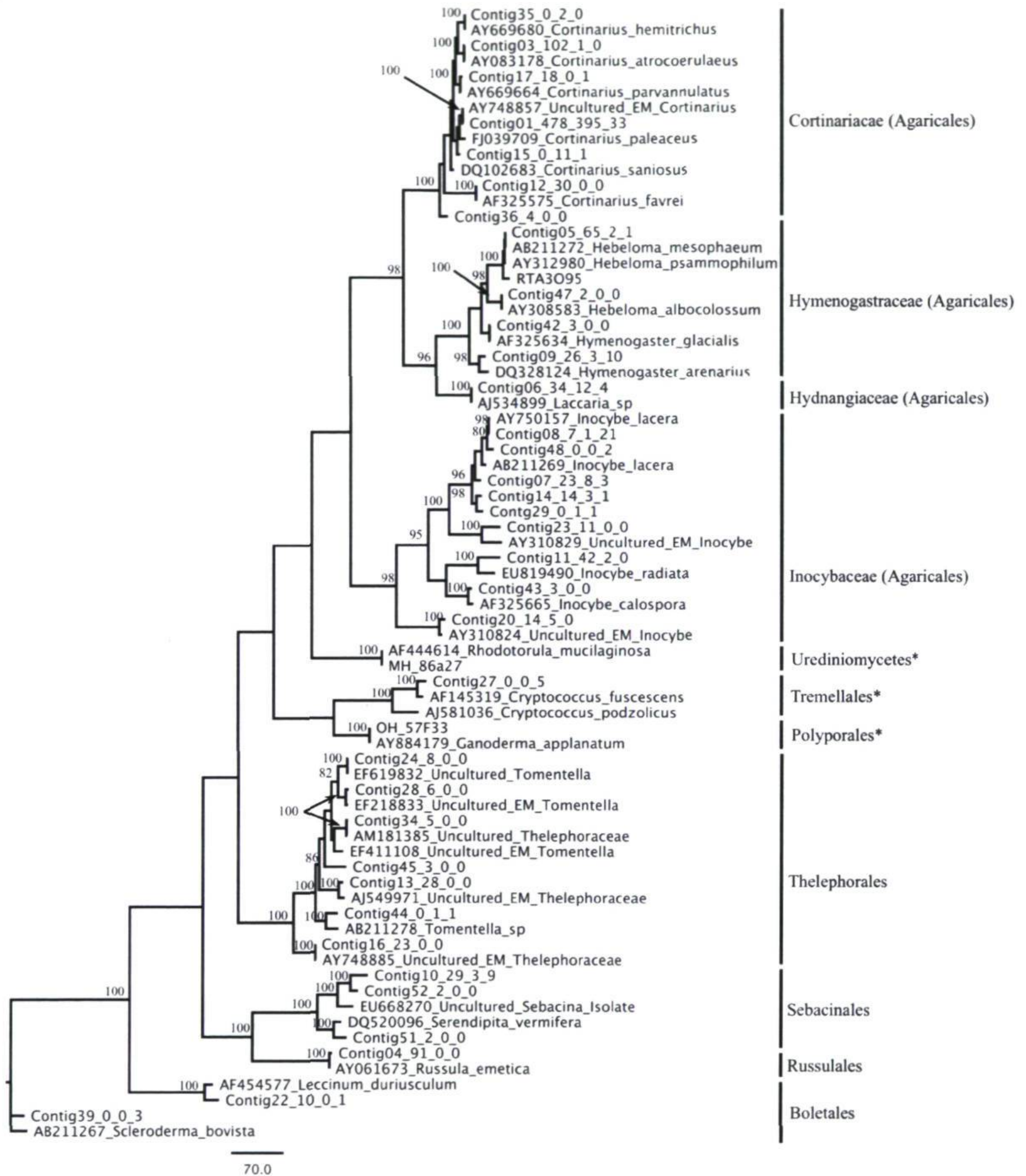

Fig. S2.1 continued.

CHAPITRE III

Impact of endochitinase-transformed white spruce on soil fungal biomass and ectendomycorrhizal symbiosis

3.1 Avant-propos

Ce chapitre a été publié en 2010 dans APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 76 (8) : 2607-2614 (FOP Stefani, P Tanguay, G Pelletier, Y Piché & RC Hamelin). FOP Stefani a défini l'approche méthodologique et réalisé l'ensemble des manipulations excepté la microscopie. Les analyses statistiques ont été réalisées par le premier auteur à l'exception de l'analyse de covariance suggérée et calculée par Michèle Bernier-Cardou. Le premier auteur a également rédigé le manuscrit dans son intégralité. Les amorces ciblant les champignons de l'étude et utilisées en PCR quantitatif ont été dessinées par G Pelletier. Les épinettes blanches transgéniques ont été produites par l'équipe du Dr A Séguin. Philippe Tanguay a formé le premier auteur aux techniques de chromatographie liquide haute pression ainsi qu'à l'ultra-concentration des exsudats racinaires. Philippe Tanguay, G Pelletier, Y Piché et RC Hamelin ont participé aux corrections du manuscrit. RC Hamelin a permis le financement des travaux effectués dans son laboratoire du Service canadien des forêts.

3.2 Résumé

L'impact d'épinettes blanches transgéniques, contenant le gène *ech*42 codant pour la production de l'enzyme endochitinase, a été évalué en serre sur les champignons

ectendomycorhiziens *Wilcoxina* spp. et sur la biomasse fongique du sol. L'activité de l'endochitinase mesurée dans les racines des épinettes génétiquement modifiées était jusqu'à dix fois supérieure à celle mesurée dans les racines des épinettes non transformées. L'activité de l'endochitinase mesurée dans les exsudats racinaires de trois lignées sur quatre était significativement supérieure à l'activité mesurée dans les exsudats des témoins. La quantité d'ergostérols retrouvée dans les échantillons de sol associés aux épinettes témoins était légèrement supérieure à celle relevée dans les échantillons de sol associés aux épinettes transgéniques. Néanmoins cette différence n'était pas significative. Les taux de colonisation mycorhizienne des extrémités racinaires des arbres témoins étaient similaires à ceux des arbres transgéniques. Le séquençage de la région ITS de l'ARNr des extrémités racinaires a montré que les racines étaient colonisées par les champignons ectendomycorhiziens *Wilcoxina* spp. et par le champignon *Phialocephala fortinii*. La colonisation par *Wilcoxina* spp. des extrémités racinaires des épinettes témoins et transgéniques a été quantifiée par PCR en temps réel. Le nombre de molécules de *Wilcoxina* spp. mesuré dans les extrémités racinaires des lignées transformées n'était pas significativement différent de celui mesuré dans les extrémités racinaires des épinettes témoins selon l'analyse de covariance. Les résultats montrent que l'activité de l'endochitinase significativement plus importante des racines et des exsudats racinaires des épinettes transformées avec le gène *ech*42 n'affectait ni le développement de la symbiose ectendomycorhizenne impliquant *Wilcoxina* spp. ni la biomassse fongique du sol.

3.3 Abstract

The impact of transgenic white spruce [*Picea glauca* (Moench) Voss] containing the endochitinase gene (*ech*42) on soil fungal biomass and on the ectendomycorrhizal fungi *Wilcoxina* spp. was tested using a greenhouse trial. The measured level of endochitinase in roots of transgenic white spruce was up to 10 times higher than that in roots of nontransformed white spruce. The level of endochitinase in root exudates of three of four *ech*42-transformed lines was significantly greater than that in controls. Analysis soil ergosterol showed that the amount of fungal biomass in soil samples from control white

spruce was slightly larger than that in soil samples from *ech*42-transformed white spruce. Nevertheless, the difference was not statistically significant. The rates of mycorrhizal colonization of transformed lines and controls were similar. Sequencing the internal transcribed spacer rRNA region revealed that the root tips were colonized by the ectendomycorrhizal fungi *Wilcoxina* spp. and the dark septate endophyte *Phialocephala fortinii*. Colonization of root tips by *Wilcoxina* spp. was monitored by real-time PCR to quantify the fungus present during the development of ectendomycorrhizal symbiosis in *ech*42-transformed and control lines. The numbers of *Wilcoxina* molecules in the transformed lines and the controls were not significantly different ($P > 0.05$, as determined by analysis of covariance), indicating that in spite of higher levels of endochitinase expression, mycorrhization was not inhibited. Our results indicate that the higher levels of chitinolytic activity in root exudates and root tissues from *ech*42-transformed lines did not alter the development of ectendomycorrhizal symbiosis involving *Wilcoxina* spp. or the soil fungal biomass.

3.4 Introduction

White spruce (*Picea glauca* (Moench) Voss) is a tree species with an extensive distribution in boreal and subboreal forests and with significant ecological roles (37, 38). It is also an important commercial species for pulpwood production and for construction-grade lumber. However, in nurseries and plantations, white spruce is sensitive to multiple fungal diseases (23, 29, 42, 62, 76). Climate change scenarios suggest that diseases could result in increased mortality in conifer forests (22, 48). Genetic engineering offers a potential means to mitigate these biotic and abiotic stresses.

During the last two decades, chitinase genes isolated from plants, fungi or bacteria have been studied to transform crops or trees in order to increase their resistance to plant pathogenic fungi. Improving white spruce tolerance to fungal infection through chitinase gene insertion is a potential avenue. Chitin is a biopolymer β-(1-4) linked N-acetyl-glucosamine (NAG), derivative of glucose, and is the primary constituent of the fungal cell wall and arthropod exoskeleton (3, 51). Chitinase enzymes are plant defence pathogenesis-

related (PR) proteins (6, 11) that break down the chitin chain either by the cleavage of internal glycoside bonds (endochitinases), hydrolysis of the non-reducing end of the chitin chain (exochitinases) or hydrolysis of NAG oligomers and trimers into NAG monomers (chitobiases). Endo- and exo-chitinase genes have been well characterized in sugar beet (*Beta vulgaris*; 44) and the filamentous fungal genus *Trichoderma* (14, 24, 69). Chitinolytic genes have been inserted into the genome of cultivated plants and trees in an attempt to boost plant chitinase activity. Among the different genes involved in the production of chitinolytic enzymes, the *ech*42 endochitinase gene from *T. harzianum* has been inserted into plant genomes to successfully enhance their resistance against phytopathogenic fungi. McIntosh apple cultivars transformed with the *ech*42 gene had limited attack by the apple scab fungus *Venturia inaequalis* (5). Transgenic black spruce (*Picea mariana*) expressing the *ech*42 gene was more resistant to the root rot pathogen *Cylindrocladium floridanum* (45).

However, field deployment of crops and trees genetically transformed to improve non-specific resistance against phytopathogenic fungi has raised concerns about the impact on non-target fungi, including potentially beneficial symbionts. This is particularly worrisome when non-specific constitutive promoters control the expression of the resistance gene and the gene becomes expressed in all tissues from roots to leaves. As a consequence, the natural colonization of such transformed plants by endophytic or mycorrhizal fungi can be altered.

Mycorrhizal fungi play a key role in plant nutrition (55) by mobilizing and transferring nutrients to the host through an intimate and highly organized association with plant roots (52, 63). Furthermore, their involvement in soil nutrient recycling (56) makes mycorrhizal symbiosis a major ecological process for the health of soil and forest ecosystems. Crops, fruit and forest trees exhibit mycorrhizal colonization by arbuscular, ecto- and ectendo-mycorrhizae (EEM). While numerous studies have addressed the impact of transgenic plants on arbuscular mycorrhizae (10, 26, 64, 68, 72, 73) and ectomycorrhizae (32, 43, 50, 60), no study has focused on EEM.

Ectendomycorrhizal fungi can be distinguished from ectomycorrhizae by the occurrence of a thin or fragmented mantle and intracellular penetration in root cortical cells. All EEM

identified so far belong to the Ascomycetes and are represented by several genera of Helotiales and Pezizales (77). EEM are prevalent in nurseries of conifers and deciduous trees (27, 39, 40, 70) and are also very common on seedling root tips in disturbed sites (15, 16, 19). The prevalence of EEM on seedling roots, among which the genus *Wilcoxina* is frequently recovered (16, 67), suggests they can play a significant role for seedling establishment and growth (77) and provide a protection against root diseases (31, 61). Consequently, the potentially negative effects of chitinase-transformed trees on ectendomycorrhizal fungi could be detrimental to plant health.

The present study addresses the issue of the potential impact of *ech*42-transformed white spruce on soil fungal biomass and ectendomycorrhizal symbiosis. It was hypothesized that i) the soil fungal biomass from transgenic white spruce rhizosphere is less abundant than soil fungal biomass from control tree rhizosphere and ii) the development of *Wilcoxina* spp. on root tips from transgenic white spruce is less important than on root tips from control trees. To test these hypotheses, 5-year-old white spruces transformed with the 35S promoter-*ech*42 construct were analysed in a greenhouse trial. Soil fungal biomass was estimated through soil ergosterol measurements. A real-time PCR approach was developed to detect changes in the quantity of ectendomycorrhizal hyphae involved in the colonization of transgenic white spruce root tips.

3.5 Materials and Methods

3.5.1 Plant material and culture

Embryogenic cell lines PG653 of white spruce were transformed by Noël et al. (45). Transformed lines had been obtained by *Agrobacterium tumefaciens* C58/pMP90 strain (35) with derivatives of the binary vector pB1N19ESR containing the complete coding sequence of the *ech*42 endochitinase gene, a duplicated enhancer 35S promoter from 35SCaMV, the alfalfa mosaic virus (AMV) leader sequence, and the neomycin phosphotransferase II (*npt*II) gene for kanamycin selection. Four transformed lines and control white spruces were grown in a greenhouse for 5 years with a photoperiod of 16 h in

pots containing peat-perlite-vermiculite (3:1:1, v/v/v). Growth cycle lasted 17 weeks. Plants were fertilized weekly with 11-41-8 (50 ppm) during the first 3 weeks following the dormancy and with 20-8-20:20-20-20 (3:1, v/v, 100 ppm) for the remaining 14 weeks. Plants were then stored at 4°C for 8 weeks to induce dormancy. Two growth cycles were induced each year.

3.5.2 Endochitinase activity in root tissues and root exudates

Root fragments from each tree were sampled, rinsed in distilled water to remove adherent soil particles, and crushed in liquid nitrogen using a pestle.

To quantify the chitinolytic activity, 70 to 130 mg (fresh weight) of ground root tissues were vortexed for 10 min in 1.6 ml of sodium acetate buffer (96 mM sodium acetate, 0.1% SDS, 0.1% Triton X-100, 10 mM Na_2EDTA) as described by Bolar et al. (4). Samples were centrifuged twice at 13,000 rpm for 5 min, and the supernatant was transferred into new tubes. One hundred µl of the extraction solution was incubated for 30 min at 37°C with 50 µl of 0.2 mM 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 100 mM sodium acetate solution. The reaction was stopped by adding 200 µl of 0.2 M sodium carbonate. Enzymatic activity was read at 365 nm excitation / 450 nm emission with a fluorolite 1000 microtiter-plate reader (Dynatech Laboratories, Chantilly, VA, USA). A standard curve of 4-methylumbelliferone (MU) (Sigma-Aldrich Co.) was used to convert fluorescence in nM of MU released per minute per gram of root.

Root exudates from three trees per transformed line or control were collected by soaking 3-5 living roots in 10 ml of Murashige and Skoog basal salt (MSS) mixture (Sigma-Aldrich Co.) for 7 days. The MSS solution was supplemented with sucrose (15 g l^{-1}), protease inhibitors (one Complete®mini tablet of protease inhibitors for every 50 ml, Roche, Indianapolis, IN, USA), benomyl (5 ppm), and streptomycin (100 ppm). Root exudates were filtered on Acrodisc syringe filters with PTFE membrane (0.2 µm) (Ultident Scientific, Saint-Laurent, QC, Canada) and 10X concentrated by ultrafiltration on Amicon PM-30 membranes (Millipore Corp., Bedford, MA, USA). Endochitinase activity

in root exudates was determined as previously described for root tissues. Dry weight of root segments soaked in MSS solution was determined after drying at 70°C for 72 h.

3.5.3 Ergosterol extraction and high performance liquid chromatography

For each tree, two soil samples per pot were collected using a metal punch and mixed together in a coffee mill. Five grams (wet weight) of soil was placed into 50-ml polypropylene falcon tubes. Ergosterol extraction was modified from the microwave-assisted extraction (MAE) technique of Montgomery et al. (41). Eight ml of methanol and 1 ml of 2 M NaOH were added to each sample. The internal standard 7-dehydrocholesterol (0.1 mg ml^{-1}, Sigma-Aldrich Co.) was added to each soil sample. Samples were then homogenized using a vortex and heated until boiling in a domestic microwave oven. Samples were cooled down at room temperature for 15 min and heated a second time in a microwave oven. After cooling for 15 min, samples were neutralized with 1 ml of 1 M HCl and supplemented with 5 ml of methanol. Solutions were homogenized and supplemented with 3 ml of pentane. Samples were vortexed and centrifuged at 3000 rpm for 5 min. Supernatants were transferred in 15 ml polypropylene falcon tubes. Pentane extraction was repeated three times. The organic phase was passed through 0.2 μm nylon syringe filters (Chromspec, Brockville, ON, Canada) and evaporated under N_2 atmosphere for 30 min. Each sample was then re-dissolved in 1 ml of methanol, incubated for 10 min, vortexed, and filtered through 0.2 μm nylon syringe filters. Samples were analyzed with a HPLC system equipped with a Waters 1524 binary pump, a Waters 717 plus autosampler, and a Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA). Ergosterol was separated from other organic soil compounds on a 4.6 x 250 mm Zorbax Rx-C18 reverse-phase column packed with an ODS 5 μm preceded by a Zorbax guard-column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was methanol, acetonitrile (55:45, v/v), at a flow rate of 2 ml min^{-1}. The experiment was performed at room temperature. Absorbance was read at 283 nm. Ergosterol content was determined using a standard curve based on the ergosterol/7-dehydrocholesterol (Sigma-Aldrich Co.) area ratio. Data were processed on Waters Breeze v.3.3 software (Waters Corporation, Milford, MA, USA).

3.5.4 Mycorrhizal fungi identification and colonization

The level of mycorrhizal colonization of four trees per transformed line and control was estimated by a visual scan of 1 g of fine roots (fresh weight) under a stereomicroscope, according to the gridline intersection method (8, 21). Mycorrhizal root tips were then sorted out based on their morphology and identified by sequencing internal transcribed spacers (ITS) regions of the ribosomal RNA (rRNA). For genomic DNA (gDNA) isolation, root tips were crushed individually in liquid nitrogen using a polypropylene micropestle. Ground tissues were resuspended in 30 μl of ultra pure™ DNAse/RNAse-free distilled water (Gibco, New York, NY, USA) and 150 μl of 15% Chelex 100 (Bio-Rad Laboratories, Richmond, CA, USA) suspension with 1.6 $\mu g\ \mu l^{-1}$ of proteinase K (Invitrogen, Carlsbad, CA, USA). Samples were incubated for 2 h at 65°C and then for 20 min at 95°C to inactivate proteinase K, and they were centrifuged at 1000 rpm for 5 min. One microlitre of the supernatant was used as template for PCR. The ITS regions were then amplified using the ITS1-F (20) and ITS4 (75) primer pair. PCR mixture was made up of 1X PCR buffer, 1.6 mM $MgCl_2$, 1.25 mM of each deoxynucleoside triphosphate (GE HealthCare Bio-Sciences, USA), 25 μg of bovine serum albumin (BSA) (Sigma-Aldrich Co.), 0.5 μM of each primer, and 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), in a total volume of 25 μl. Thermal cycling conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 37 cycles at 94°C for 45 s, 58°C for 45 s, 72°C for 45 s, and a final elongation step at 72°C for 10 min. PCR reactions were performed on an MJ research PTC-200 (MJ Research Inc., Waltham, MA, USA).

3.5.5 Real-time PCR assays

To quantify the level of root tip colonization by the ectendomycorrhizal fungus *Wilcoxina* spp., specific primers were designed to target the ITS2 region, beta-tubulin and translation elongation factor 1-alpha coding genes. *Wilcoxina*-specific primers were designed with OligoAnalyzer v1.2 (http://www.genelink.com), based on sequence alignments made of the closest fungi recorded from preliminary root tip sequencing. A research in the GenBank public database using the BLASTN tool (2) was performed to ensure the specificity of all the primers designed. The quantity of plant gDNA within each sample was also estimated

by amplifying three housekeeping genes (Table 3.1). Real-time PCR conditions were optimized for each primer pair by the mean of a gradient of temperature performed in an Opticon-2 DNA Engine (MJ Research, Cambridge, MA). Five root tips per tree colonized by *Wilcoxina* spp. were pooled together for gDNA extraction. Briefly, root tips were ground in liquid nitrogen with micropestles, incubated for 1 h at 65°C with 400 µl of Carlson lysis buffer and 2 µl of β-mercaptoethanol (9) and vortexed every 15 min. Four hundred μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the aqueous phase was collected after centrifugation (13,000 rpm for 10 min). Nucleic acids were precipitated by incubating samples for 1 h at –20°C with 70 µl of 7.5 M sodium acetate and 600 µl of isopropanol. Pellets were obtained by centrifugation (13,000 rpm for 10 min), washed with 800 µl of ethanol 75%, dried on a thermoblock for 10 min at 55°C, and resuspended in 25 µl of TE-8 (Tris EDTA buffer, pH 8). DNA was diluted to 10 ng μl^{-1}. Real-time PCR mixture was made up of 1X QuantiTect SYBR® Green mixture (Qiagen, Valencia, CA, USA), 600 nM of each primer, 1 or 10 ng of gDNA depending on whether *Wilcoxina* spp. or *Picea glauca* gDNA was targeted, in a total volume of 10 µl.

Thermal cycling conditions were as follows: an initial denaturation step at 95°C for 15 min, followed by 40 cycles at 94°C for 5 s, annealing temperature (see Table 3.1) for 30 s, and 65°C for 1 min 30 s. All reactions were performed on a Stratagene Mx3000P cycle engine (Stratagene, La Jolla, CA, USA). Double fluorescent readings were taken at the end of each cycle and each run was followed by a melting curve analysis that confirmed specificity of amplification as well as lack of primer dimer formation. Fluorescence threshold value was set at 500 and filter gain setting was positioned at 8X. Fluorescence and Ct values were exported and analyzed in Excel spreadsheets (Microsoft Excel VER.9.0.3821 SR-1, Redmond, WA).

Amplification efficiency was determined by linear regression of efficiency analysis (57). Gene stability and normalization factor were determined using the geNorm VBA applet for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm). To minimize sampling variations and obtain normalized data, individual raw quantities (Q) were multiplied by the relative DNA quantification $(1+E)^{-CT}$.

3.5.6 Microscopy

For light microscopy, root tips were fixed in 4% paraformaldehyde in 0.1M cacodylate (pH 7.3) at room temperature for 24 h and subsequently washed and dehydrated in a graded ethanol series. Root tips were transferred in toluene before infiltration in paraffin. Five micron sections were cut with a sliding microtome, mounted on slides and soaked in toluene to remove paraffin. These sections were rehydrated in a descending ethanol series, stained with hematoxilin-eosine, dehydrated gradually in ethanol and mounted permanently in Eukitt. Microscope observations were made with an Olympus BX-51.

3.5.7 Statistical analyses

The experimental set-up was made of three blocks, with one non-transformed and four transformed lines per block, and four trees per treatment. Data on endochitinase activity, colonization rate and ergosterol level were separated by Waller-Duncan k-ratio t-test ($P \leq 0.05$). All the data were checked for normality with the Shapiro-Wilk test and for homoscedasticity with Levene's test.

Data on enzyme activity within root tissues were square-root transformed, the logarithmic transformation *log(1+x)* was applied to the values of endochitinase activity recorded from root exudates, and ergosterol data were reciprocal-transformed to meet normality and homoscedasticity assumptions. The experiment was arranged in a randomized complete block analysis.

Potential effect of *ech*42-transformed white spruce on the ectendomycorrhizal fungus *Wilcoxina* spp. was evaluated by covariance analysis (ANCOVA). The ANCOVA was calculated as a linear mixed-effects model (*lme*). Variables tested in the ANCOVA were the five treatments (four transformed lines plus the control), the three blocks, and their interactions. The absolute number of white spruce molecules was considered as covariate. A *log(1+x)* transformation was used on the absolute number of *Wilcoxina* spp. and *Picea glauca* molecules to meet the assumption of homogeneity of variance. Statistical analyses were performed using the R statistical language (53) and SAS (58).

3.6 Results

3.6.1 Endochitinase activity

Endochitinase activity was first measured in root tissues isolated from eight trees per treatment. The means of endochitinase activity measured showed a significant difference between the control and transformed spruces (Fig. 3.1A). Average endochitinase activity in control roots was 3.8 ± 0.8 (standard error mean) nM min^{-1} g^{-1} while it was 24.3 ± 1.1 nM min^{-1} g^{-1} in transformed lines. The strongest endochitinase activity was measured in roots from transformed spruce line 1, with an average activity of 29.3 ± 3.3 nM min^{-1} g^{-1}. This was significantly higher than the level of activity recorded in roots of the other transformed lines.

Endochitinase activity was also determined for concentrated root exudates from four trees per treatment. The level of endochitinase activity was significantly increased in all transformed lines, except line 3 (Fig. 3.1B). Endochitinase activity recorded in concentrated root exudates from transformed lines was 2 to 10 times higher than the level recorded in control spruces. The highest enzyme activity was recorded in exudates from trees belonging to line 4 (83.8 ± 27.9 pM min^{-1} g^{-1} of root dry weight).

3.6.2 Fungal biomass in the rhizosphere

The amount of fungal biomass present in the rhizosphere of control and transgenic white spruces was measured with a standard curve based on the ratio between ergosterol and the internal standard 7-dehydrocholesterol. Ergosterol ranged between 0.38 ± 0.002 and 0.46 ± 0.037 μg g^{-1} of soil dry weight. The highest soil fungal biomass was measured in soil samples from the rhizosphere of control trees; nevertheless, the differences in soil ergosterol means recovered in pots from each treatment were not significant (Fig. 3.2A).

3.6.3 Fungal and mycorrhizal community characterization

The mycorrhizal colonization rate (Fig. 3.2B) ranged between 46.4 ± 2.8% (line 4) and 54.7 ± 1.7% (line 2). Control roots showed a mycorrhizal colonization of 47.7 ± 4.2%. No significant difference in mycorrhizal colonization intensity was found between the five

treatments. Root tips observation under a dissection microscope revealed the occurrence of two main morphological groups. The ITS sequence of the most abundant morphological group (MG1, Fig. 3.3A) was similar to *Wilcoxina mikolae* var. *mikolae* (UAMH 6694) at 92% (548 bp, 100% of coverage) while the second most prevalent group (MG2, Fig. 3.3B) was homologous to the ITS sequence of *Phialocephala fortinii* (UAMH 5524). Root tips colonized by *Wilcoxina* spp. were single to pinnate, 1-4 mm in length, 0.2-0.3 mm in diameter, straight, smooth, glossy, mainly yellowish-brown with tips white to pale-yellow. Root tips colonized by *P. fortinii* were 3-15 mm in length, 0.2-0.3 mm in diameter, single, straight or slightly beaded, smooth, brown or darkish-brown, sometimes with dark-reddish tips.

Inconsistent sequencing results were observed in the MG2. Fifty percent of the recovered ITS sequences corresponded to *Wilcoxina* spp. instead of *P. fortinii* as expected. All ITS sequences recovered from the MG1 corresponded to *Wilcoxina* spp. as expected.

Total DNA of 12 root tips characteristic of the two morphotypes was extracted and amplified with primer pairs specific to the ITS2 region of *Wilcoxina* spp. and the ITS1 region of *P. fortinii* in real-time PCR. The number of molecules at cycle threshold (Ct-No) recorded for the 12 samples characteristic of MG1 (Fig. 3.3C) was on average 10621 ± 1839 and 14 ± 5 with primer pairs specific to *Wilcoxina* spp. and *P. fortinii*, respectively. Conversely, the number of molecules at cycle threshold (Ct-No) recorded for the 12 samples characteristic of MG2 (Fig. 3.3D) was on average 1392 ± 935 and 689 ± 113 with primer pairs specific to *Wilcoxina* spp. and *P. fortinii*, respectively.

3.6.4 Quantification of *Wilcoxina* spp. symbiosis in root tips from control and *ech*42-transformed white spruces

The absolute number of *Wilcoxina* spp. molecules was normalized based on data from beta-tubulin and translation elongation factor 1-alpha. The ITS data set was not included as it displayed the highest gene instability. The three housekeeping genes were considered to normalize the absolute number of plant molecules. The results of the ANCOVA (Fig. 3.4) showed the absolute number of *Wilcoxina* molecules to be significantly correlated with the absolute number of plant molecules ($r = 0.6$, $F = 14.4$; $P = 0.0004$). The absolute mean

number of *Wilcoxina* molecules recovered was 66 (min = 42, max = 103) for controls. This number was the highest in line 3 (78, min = 50, max = 122) and the lowest in line 4 (54, min = 35, max = 83). The results of the ANCOVA showed that variation in the absolute number of *Wilcoxina* molecules was not affected by the different levels of chitinase recorded in the four transgenic lines ($F = 0.44$; $P = 0.77$).

3.6.5 Light microscopy

Figure 3.5 shows a microscope transverse section of an MG1 root tip from transformed tree line 1. The entire cortex up to the stele is colonized by *Wilcoxina* spp. hyphae to form a Hartig net (arrow) while intra-cellular structures were occasional (double arrowheads).

3.7 Discussion

3.7.1 Chitinase overexpression impact on ectendomycorrhizal symbiosis

The objective of our study was to determine if mycorrhizal colonization would be inhibited in spruce that was genetically modified to overexpress endochitinase. Our analysis shows that an increase in chitinase activity of up to ten times in root tissues did not impede the inter- and intracellular space colonization of root tips by ectendomycorrhizal hyphae. These results are in accordance with previous studies that investigated the effect of chitinase-transformed plants on symbiotic fungi. Vierheilig et al. (72) showed that an increase of 5-16 times the level of chitinase in transgenic *Nicotania sylvestris* enhanced its resistance against *Rhizoctonia solani* but did not alter its susceptibility to be colonized by the endomycorrhizal fungus *Glomus mosseae*. In the case of *Betula pendula* overexpressing the sugar beet chitinase, resistance against the leaf spot disease *Pyrenopeziza betulicola* and against *Melampsoridium betulinum* (birch rust) was improved in greenhouse and in field trials, respectively (46, 49). However, when the impact of these silver birch transformed lines was further assessed in vitro on the EM *Paxillus involutus* fungus, all but one transformed line showed similar levels of root mycorrhizal colonization (50). The authors attributed the difference observed in one of the transformed lines to a pleiotropic effect due

to transgene insertion. This *B. pendula* transformed line also showed a significant decrease in root fresh weight compared with the other transgenic lines and controls. Here, no evidence of pleiotropic effect due to the random insertion of the 35S-*ech*42 construct was found. All transformed lines were phenotypically similar to controls and were equally susceptible to mycorrhizal colonization.

The impact of chitinase-transformed plants has been mainly monitored on phytopathogenic fungi on which detrimental effects have been demonstrated. For example, attempts to increase tobacco and rice resistance through chitinase gene insertion were successful against *Rhizoctonia solani* (7, 34). Jayaraj and Punja (28) showed that the level of infection from the two foliar pathogens *Alternaria radicicola* and *Botrytis cinerea* was reduced by up to 40-50% in transgenic carrots expressing a barley chitinase (*chi*-2) protein. The infection was reduced by up to 90-95% when transgenic carrots co-expressed the *chi*-2 protein plus a wheat lipid transfer protein (*ltp*). Hybrid poplars (*Populus nigra* × *P. maximowiczii*) were transformed with the insertion of the *ech*42 gene from *T. harzianum* controlled by the 35S promoter (45). The level of endochitinase recorded in foliar tissues increased 4-65 times according to the different transformed lines, thus enhancing their resistance against the leaf rust pathogen *Melampsora medusae* f. sp. *deltoidae*. The same construct was used to transform black spruce (*Picea mariana*) and the endochitinase activity measured in embryogenic tissues increased 2-8 times compared with the controls. *ech*42-transformed black spruces were more resistant to the root rot pathogen *Cylindrocladium floridanum* than untransformed controls.

The release of higher levels of endochitinase in the rhizosphere from the exudates of *ech*42-transformed white spruce roots did not affect soil fungal biomass in pots, as determined by ergosterol measurements with the HPLC. Although efforts were made to select young and healthy looking roots for each repetition, the variation observed in endochitinase activity was probably due to differences in vigour of the roots soaking in the MSS mixture. Nevertheless, the level and variation of endochitinase activity recorded in *ech*42-transformed white spruce root exudates were comparable to those observed by Tesfaye et al. (65). They transformed alfalfa with an APase signal peptide region from

white lupin fused to the N-terminal region of the *ech*42 protein controlled by the cassava vein mosaic virus (CsVMV) promoter to increase transgenic protein exudation.

Vauramo et al. (71) quantified the fungal biomass using ergosterol measurements in leaf litter from transgenic silver birches transformed with sugar beet chitinase. They found no significant difference in the fungal biomass between litter samples from transformed lines and controls. The level of ergosterol they recorded was 1000-2000 times higher compared to ours. This can be explained by a potential limited production of extraradical mycelium of *Wilcoxina* spp. associated to very low mycorrhizal and non-mycorrhizal fungal diversity.

3.7.2 Real-time PCR to quantify mycorrhizal root tip colonization

Real-time PCR provides useful tools for studying and quantifying mycorrhizal fungi interactions and biomass (33, 47, 54, 59). Unlike in previous studies, we did not use the ITS rRNA region to quantify the number of *Wilcoxina* molecules. The expression of beta-tubulin and translation elongation factor 1-alpha coding genes proved to be the most stable among repetitions and, as a consequence, more accurate for quantification than the ITS rRNA region. This is not surprising considering that rRNA genes occur in several dozens of copies within eukaryotic genomes (close to 150 copies; 17), while the beta-tubulin and translation elongation factor 1-alpha coding genes are supposed to be in single or few copies (12, 66, 74). Also, we showed that determining the plant number of molecules for each root tip is a valuable approach to compare the magnitude of root tip colonization without weighing root tips or *a priori* determining the relation between fungal biomass and the number of molecules.

3.7.3 *Wilcoxina* spp. and *Phialocephala fortinii* interaction

Phialocephala fortinii is part of the *Mycelium radicis atrovirens* (MRA) complex (25), which later became known as dark septate endophyte (DSE). DSE is a prevalent group of Ascomycetes colonizing a wide spectrum of hosts (30) either as fungal root endophyte or ectendomycorrhizal fungi (77). The nature of the association between *P. fortinii* and host plants remains unclear as it can result in negative, neutral or positive benefits, and varies according to experimental conditions and host plants (1, 31). Real-time PCR analysis of

root tips belonging to the MG2 showed a dual colonization by *Wilcoxina* spp. and *Phialocephala fortinii*. It may explain why the direct ITS rRNA sequencing of MG2 with the fungal specific primer pair ITS1-F / ITS4 recovered either *Wilcoxina* spp. or *P. fortinii*. Menkis et al. (39) characterized the fungal communities associated with conifer roots in nurseries and observed that an individual taxon could be found in 2.4 different morphotypes on average. The detection of *P. fortinii* ITS molecules in MG2, while *Wilcoxina* spp. was dominant, suggests that the root tips from this morphological group were initially colonized by *Wilcoxina* spp., and later by *P. fortinii*. The under-representation of *Wilcoxina* spp. ITS molecules in MG2 compared with MG1 (7.5X less) might potentially be explained by the mycoparasitism of *Wilcoxina* spp. by *P. fortinii*. Krasowski et al. (36) observed a reverse relationship between the relative abundance of *Wilcoxina* spp. and DSE on healthy versus senescent root systems in white spruce seedlings. The occurrence of DSE such as *P. fortinii* has been observed on senescent roots from other conifers as well (1, 30).

3.7.4 Conclusion

The level of endochitinase recorded in roots of *ech*42-transformed white spruce did not alter the establishment and development of the ectendomycorrhizal association. So far, there is no evidence in the literature that chitinase-transformed crops, fruits and forest trees have a negative impact on mycorrhizal and non-pathogenic fungi. However, the number of studies assessing the impact of chitinase-transformed plants on non-pathogenic fungi remains insufficient to accurately predict their potential effects in nature. Moreover, greenhouse trial studies are only the first step in assessing the harmlessness of chitinase transgenic trees and this could not necessarily reflect what would happen in the field. For example, Pasonen et al. (49) observed a contrasting response between sugar beet chitinase-transformed silver birches against leaf spot disease and trees grown in the greenhouse or field. Long-term field deployments are required to achieve the assessment of chitinase-transformed trees on non-phytopathogenic fungi and to assess the impact of a higher level of chitinase root exudation on soil mycorrhizal reserve. Finally, the real-time PCR technique is an accurate approach to better understand the intimate mycorrhizal association and the dynamics of root tip fungal colonization.

3.8 Acknowledgements

We thank A. Séguin for the production of transgenic white spruces and D. Stewart for providing housekeeping genes primers for plants. We also thank M. Bernier-Cardou for helping with statistical analyses and M-J. Bergeron for a critical review of the manuscript. This work was supported by a grant from the Canadian Regulatory System for Biotechnology Fund.

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Table 3.1 Sequence and list of organisms and locus targeted by the primer pairs used in real-time PCR analysis.

ID	Organism and locus targeted	Sequence	Ta [a] (°C)	Length (bp)	Reference
ITSWil F2	*Wilcoxina* spp. / ITS2	TCATGGAAGATGAGTATGGTTGCAT	60.4	120	This study
ITSWil R2		GTCAACGGCAGGACAATAACACACA	61.4		
ITSPForti F	*Phialocephala fortinii* / ITS1	GTCAACGGCAGGACAATAACAC	60.5	130	This study
ITSPForti R		CTCTGGCGGGCACACACGAGCAGA	62.4		
tef F353	*Wilcoxina* spp. / Translation	GGAGGGTGGCAAGTCTAGC	65	155	This study
tef R488	elongation factor 1-α	CCAGTCTCGACACGTCCGACA	65		
btub F340	*Wilcoxina* spp. / Beta-tubulin	AGGAGTTGTTCAAGCGTGTCGGA	64.8	119	This study
btub R434		ATCATAGCACAAGTTGGGAAACTCAC	62.8		
Pg PTSR K1	*Picea glauca* / Peroxisomal	TGGGAATTGATATAAGTGTTCTTGTGGAGGGTCT	70.2	371	Friedmann
Pg PTSR K2	targeting signal reception	ACACCAAACCAGTAACCTGAGAAGGAAACA	70.1		et al. (18)
Pg EXP 46630 F1	*Picea glauca* / Translation	GCTAGTCTGTCACAAGGTGCTTTCAAGT	69.7	234	Czechowski
Pg EXP 46630 R1	elongation factor 1-α	TCCGAGTTTCTTTTCACAAGGAGTTGGC	70.1		et al. (13)
Pg ACT2 F1	*Picea glauca* / Actin 2	GTTTCCTGGTATTGCTGACCGTATGAGC	70.4	450	Czechowski
Pg ACT2 R1		GTGCTGAGAGATGCCAAAATAGAACCTCC	70.1		et al. (13)

a: The temperature of primer annealing was determined by a gradient PCR assay on an Opticon II DNA engine(MJ Research, Cambridge, MA, USA).

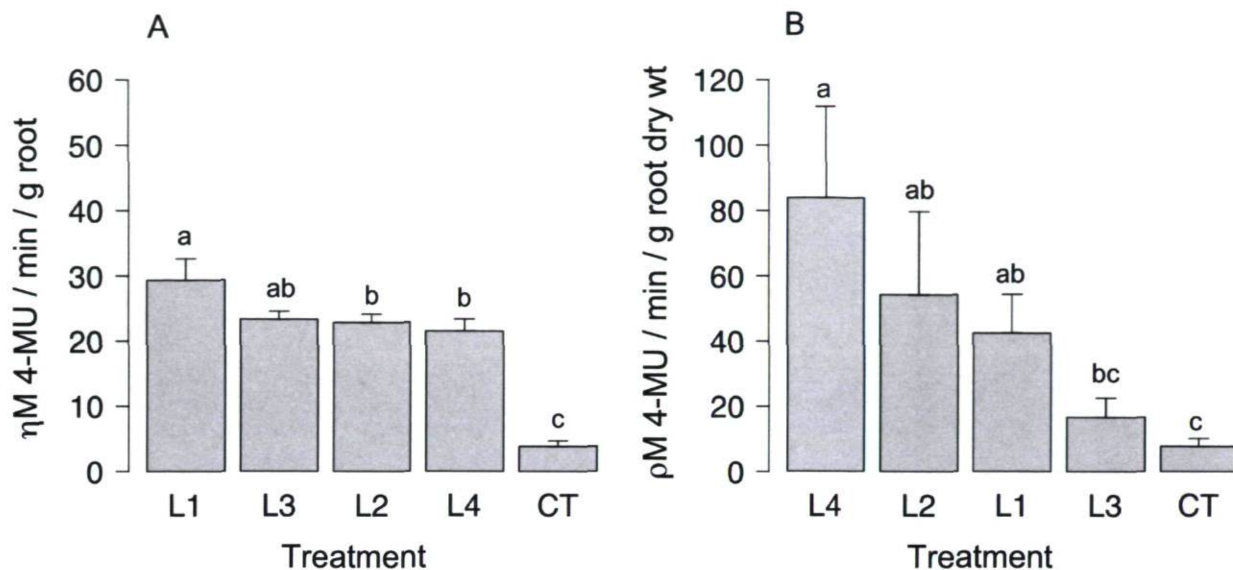

Fig. 3.1 Level of endochitinase activity measured A) in root tissues and B) in root exudates, recorded for the four *ech*42-transformed lines and the control white spruces. Columns identified by the same letter are not significantly different according to Waller-Duncan's multiple range test ($P \leq 0.05$). Error bars represent standard error of the mean.

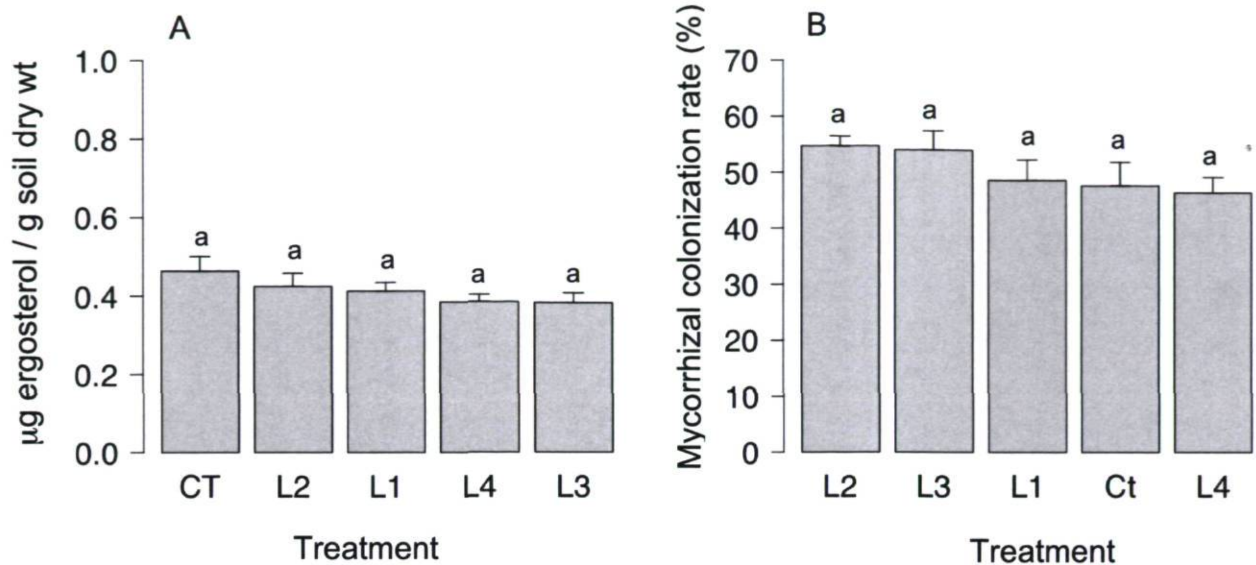

Fig. 3.2 A) Level of fungal biomass in the pots based on ergosterol measurements for the controls and the four transformed lines. B) Level of mycorrhizal colonization recorded in the four *ech*42-transformed lines and the control white spruces. Columns identified by the same letter are not significantly different according to Waller-Duncan's multiple range test ($P \leq 0.05$). Error bars represent standard error of the mean.

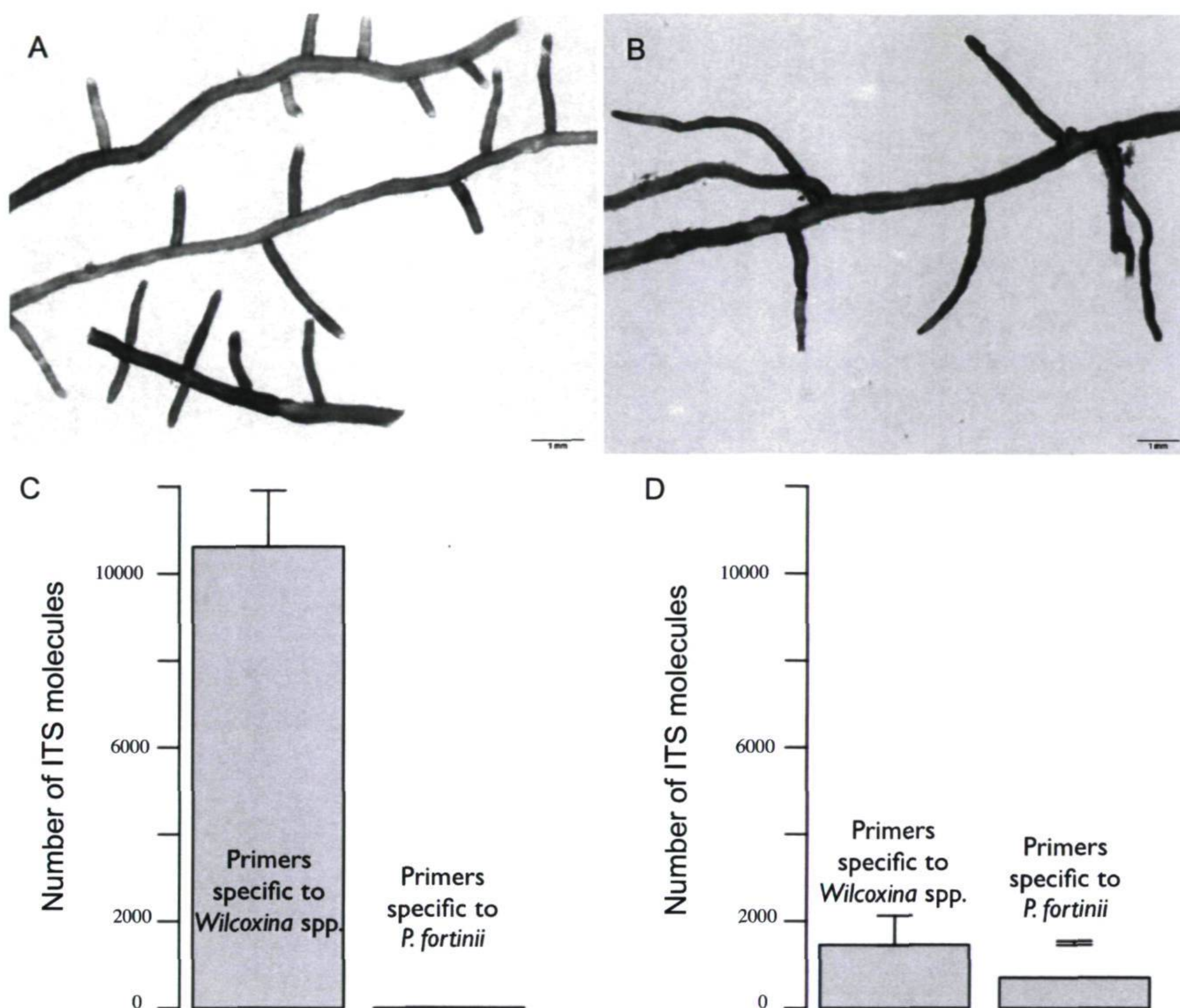

Fig. 3.3 Stereomicroscope view of A) root tips colonized by *Wilcoxina* spp. (MG1) and B) by both *Wilcoxina* spp. and *Phialocephala fortinii* (MG2). Scale bar = 1 mm. Barplots in C and D show the variation in *Wilcoxina* spp. and *P. fortinii* ITS molecules in MG1 and MG2, respectively, according to whether they were amplified in real-time PCR with primer pairs specific to one fungus or the other.

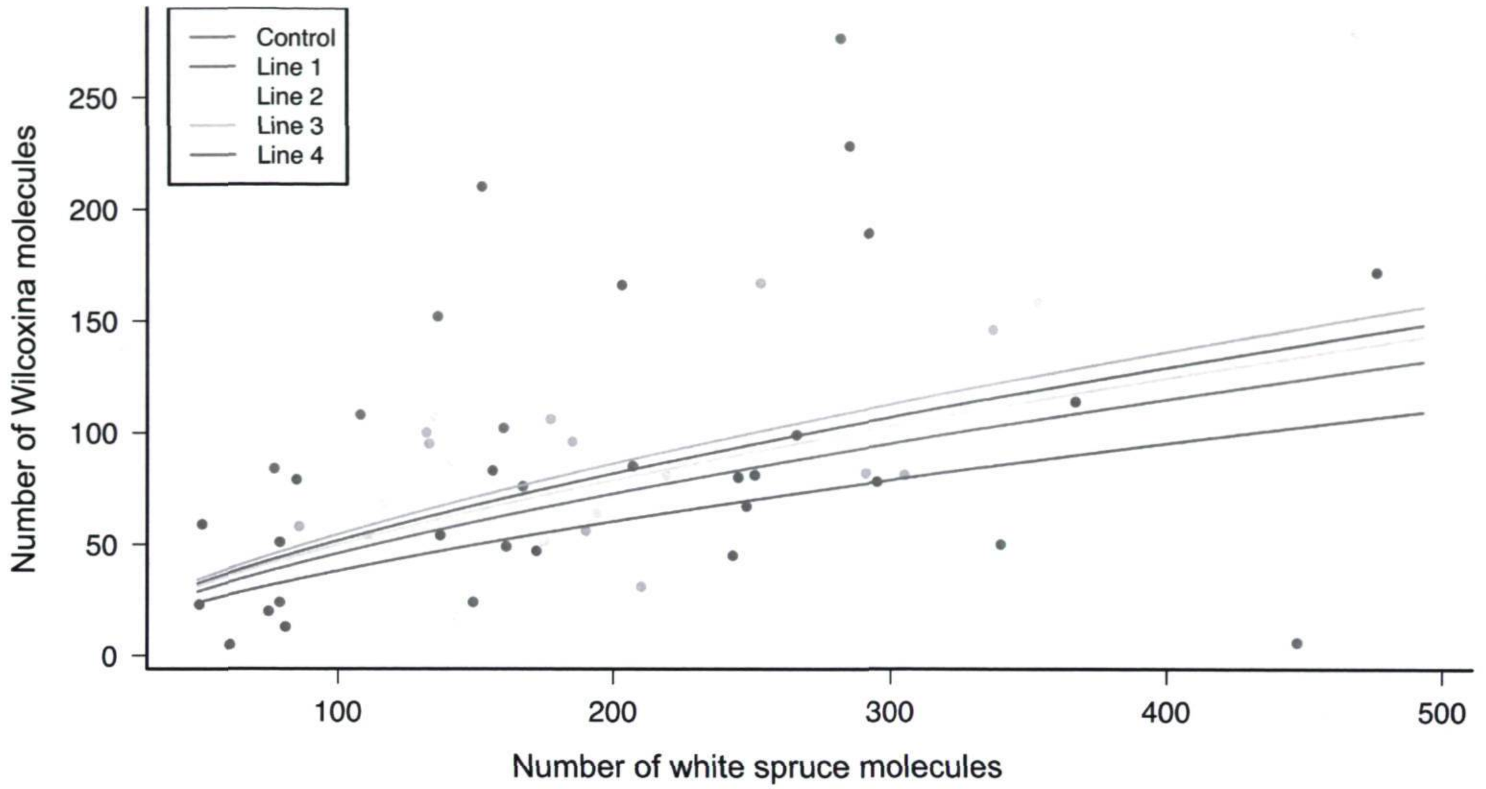

Fig. 3.4 Results of the ANCOVA performed on the real-time PCR data. Points represent the observed values and the fitted lines represent the predicted values based on the ANCOVA model.

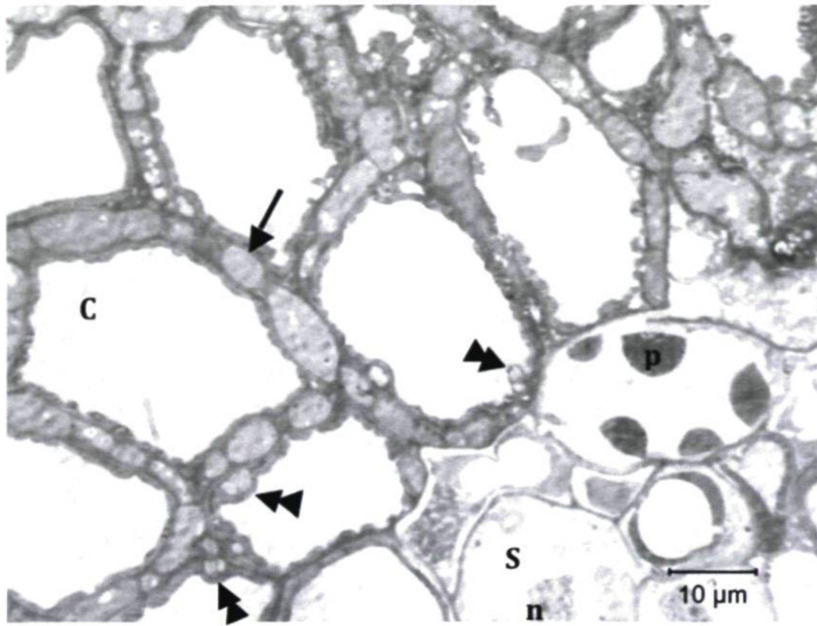

Fig. 3.5 Microscope transversal slide of a root tip belonging to MG1 collected from *ech*42-transformed line 1. Single arrowhead shows Hartig net and double arrowheads show intracellular hyphae. C, cortical cell; p, tannin-filled cell; S, stele; n, nucleus.

CHAPITRE IV

Impact of endochitinase-transformed white spruce on soil fungal communities under greenhouse conditions

4.1 Avant-propos

Ce chapitre a été soumis à la revue Applied and Environmental Microbiology, le 06 avril 2010 (J Lamarche, FOP Stefani, A Séguin & RC Hamelin). Josyanne Lamarche et FOP Stefani ont contribué de façon égale aux analyses et à la rédaction du manuscript. Josyanne Lamarche a eu l'idée originale d'importer deux types de sol forestier afin d'y transplanter les épinettes transformées endochitinase. Le premier auteur a également réalisé le dispositif expérimental ainsi que l'ensemble du travail moléculaire. Les séquences d'ADN ont été analysées par le premier et second auteur. Le second auteur a déterminé la biomasse fongique des échantillons de sol par HPLC, réalisé les analyses de raréfaction, de DPCoA et de structures phylogénétiques des communautés fongiques. Les épinettes blanches transgéniques ont été produites par l'équipe du Dr Armand Séguin. Armand Séguin et RC Hamelin ont corrigé le manuscrit. RC Hamelin a permis le financement des travaux effectués dans son laboratoire du Service canadien des forêts.

4.2 Résumé

Les gènes produisant des chitinases sont couramment utilisés en génie génétique pour transformer les plantes agricoles et les arbres forestiers afin d'accroître leur résistance aux

maladies d'origine fongique. Les champignons non pathogènes (non cibles) ont un rôle important dans la nutrition et la protection des arbres, ainsi que dans le recyclage des nutriments du sol. On peut s'inquiéter de l'impact potentiel des arbres génétiquement modifiés par l'insertion de gènes produisant des chitinases sur ces communautés de champignons non cibles. Le but de cette étude est d'évaluer l'impact sur les communautés fongiques du sol d'épinettes blanches génétiquement modifiées par l'insertion du gène *ech*42 produisant une endochitinase. Des épinettes transgéniques et non transgéniques ont été transplantées dans des sols provenant de deux milieux forestiers, et cultivées pendant huit mois en serre. Des carottes de sol ont été échantillonnées au moment de la transplantation et après huit mois. La quantité de biomasse fongique des carottes de sol provenant de la rhizosphère des épinettes transgénique n'était pas significativement différente de celle mesurée dans la rhizosphère des épinettes témoins. La richesse spécifique fongique et les valeurs de l'indice de Shannon ne présentaient pas de différence significative entre les banques de clones de séquences fongiques provenant des épinettes témoins et des épinettes transgéniques. Seules les valeurs de l'indice de Chao, estimant la richesse spécifique totale attendue, étaient significativement supérieures dans les échantillons de sol provenant de la rhizosphère des épinettes contrôles que dans ceux associés aux épinettes transgéniques. La structure phylogénétique des communautés fongiques associées aux deux types d'épinettes était la même après huit moins d'interaction. Le type de sol et la présence ou pas de semis avaient plus d'impact sur la structure des communautés fongiques que la présence des épinettes blanches génétiquement modifiées. Les résultats suggèrent que l'insertion dans le génome des épinettes blanches du gène *ech*42 et son expression n'affectent pas de manière significative la biomasse, la diversité et la structure des communautés fongiques des deux sols analysés en serre.

4.3 Abstract

Chitinase genes isolated from plants, bacteria or fungi have been widely used in genetic engineering to enhance the resistance of crops and trees to fungal pathogens. Since fungi play such an important role in tree nutrition, protection and nutrient cycling, there are

concerns about the possible effect of chitinase-transformed tree on non-target fungi. This study aimed at evaluating the impact of endochitinase-transformed white spruce on soil fungal communities. Endochitinase white spruce and untransformed controls were transplanted in soils imported from two natural forests and grown for 8 months in a greenhouse. Soil samples were cored at the time of transplantation and 8 months later. Soil fungal biomass was not significantly different from the control. Fungal diversity was not different in soil fungal clone libraries obtained from transgenic and control tree rhizospheres when measured by the number of OTUs and the Shannon diversity index. Only the Chao index predicted that the total fungal species richness expected in the control rhizosphere would be significantly higher than in endochitinase-transformed white spruce rhizosphere. The fungal phylogenetic community structure was the same in soil samples from control and transgenic white spruces after 8 months. The soil type and the presence of seedlings had a much more important impact on fungal community structure than the insertion and expression of the *ech*42 transgene within the white spruce genome. The results suggest that insertion and constitutive expression of the *ech*42 gene in white spruce did not significantly affect the biomass, diversity and structure of soil fungal communities.

4.4 Introduction

White spruce (*Picea glauca* [Moench] Voss) is an important native forest species with a wide geographic distribution, ranging from Newfoundland to Alaska. It is an important commercial species for pulpwood production, softwood lumber and reforestation (13). However, when grown in nurseries and plantations, white spruce seedlings can be affected by a variety of fungal pathogens causing root rots (19, 22) with adverse effects on their survival.

One research avenue to limit losses caused by fungal pathogens is to increase tree resistance by the introduction of resistance genes through genetic modification (5, 6). One particularly interesting approach consists in using fungal genes that are inhibitory to fungal pathogens. Chitinases are digestive enzymes that break down glycosidic bonds in chitin, a linear homopolymer of β-1,4 linked N-acetylglucosamine. Since chitin is an important

component of the cell walls of fungi, organisms producing high levels of chitinolytic enzymes were used as biocontrol agents against fungal plant pathogens. Previous work on the biocontrol agent *Trichoderma harzianum* demonstrated the strong inhibition effect of its chitinases against a broad range of fungal plant pathogens (17, 30). Among the different chitinase genes, the *ech42* gene produces an endochitinase that randomly hydrolyses the chitin polymer chain and it was shown to have the strongest antifungal activity (30). Moreover, the *ech42* gene product is more effective than plant encoded chitinases (20, 29). As a consequence, the *ech42* gene was used to genetically transform some crops, fruit and forest trees to increase their resistance against fungal pathogens (6, 15, 30, 33, 34, 47). Black spruce and hybrid poplar were the first forest tree species to be transformed with the *ech42* gene (34). Transformed black spruce and hybrid poplar showed, *in vitro*, an increased resistance to the root pathogen *Cylindrocladium floridanum* and to the foliar rust *Melampsora medusae*, respectively.

However, since all true fungi possess chitin in their cell wall, exogenoeous chitinase overexpression in genetically modified organisms (GMOs) could result in negative impacts on non-targeted fungi, such as saprophytes and symbionts. Random integration of the *ech*42 transgene into the genome may also have pleiotropic effects leading to the alteration of physiological processes such as metabolism and plant development. Such pleiotropic effects have been observed in apples transformed with *ech*42. Expression of *T. harzianum* endochitinase gene in apple resulted in reduced growth in *ech*42 transgenic lines (5), suggesting an impact on some metabolic pathways. Also, apple trees transformed with *T. harzianum* endochitinase had significantly higher peroxidase and glucanase activities; these enzymes are involved in plant disease response to fungal pathogens (12). In both cases, the expression of *T. harzianum ech*42 gene apparently affected apple metabolism. Therefore, the up- or down-regulation of certain metabolic pathways might be harmful to particular fungal groups.

During their lifetime, trees develop intimate relationships with different kinds of fungal communities. Leaves are colonized by fungal endophytes (3, 23), roots are associated with mycorrhizal fungi (42), and a large diversity of saprophytic fungi are present in the soil and litter to decompose dead wood and leaves (28, 46). These fungi play important roles in tree

nutrition and protection, nutrient cycling and humus formation in forest ecosystems. If trees exhibiting antimicrobial activity, such as endochitinase trees, were deployed as part of plantation or reforestation programs, these fungal communities could be affected and this could have a negative impact on entire forest ecosystems.

Investigation of the impact of chitinase-transformed plants on non-target fungi has so far focused on mycorrhizal fungi by means of single-species inoculation, and no negative impact has been reported (37, 50). However, the consequence on the overall fungal communities inhabiting the rhizosphere is an important issue that needs to be addressed. No study has investigated the impact of transgenic plants significantly overexpressing endochitinase on soil fungal diversity at the community level.

The objective of the present study was to assess the impact of endochitinase-transformed (Et) white spruce on soil fungal communities imported from two natural forest soils. To do so, soil fungal libraries were constructed using the ITS1-F / LR5 primer pair to amplify in a single PCR reaction the entire ITS and nLSU fragment. We hypothesized that the fungal diversity recorded in soil samples associated with control white spruce is more diverse than in soil samples associated with Et white spruce after 8 months of interaction.

4.5 Materials and Methods

4.5.1 Experimental design and soil sampling

Embryogenic cell line PG653 of white spruce (*Picea glauca* (Moench) Voss) was transformed by Noël *et al*. (34). Six untransformed (control) and six *ech*42-transformed (Et) white spruce seedlings (transformed lines 1, 2 and 3) were transplanted in 2.5 dm^3 pots after a dormancy period of 2 months. Half of the pots were filled with organic soil and the other half were filled with mineral soil collected in May 2006 near the city of Québec. The organic soil came from a white spruce stand (46°79' N, 71°34' W) and the mineral one from a yellow birch/sugar maple hardwood stand (47°01' N, 71°35' W). Soil samples were removed from the top layer to a depth of 20 cm, transported in coolers, sieved (4-mm mesh), and kept at 4°C until distributed in pots 5 days later. Basic soil chemical analyses

were performed according to protocols by Carter (9). Trees were disposed in a factorial design. The treatment factors tested were soil type (organic or mineral soil), transplantation (no seedling, control or Et white spruce) and sampling time (at the time of transplantation (T0) and 8 months later (T8)). Four soil samples were cored per pot at two different depths. All soil samples from the same pot were then pooled prior to soil genomic DNA (gDNA) extraction. Soil samples were kept at –80°C until processing.

4.5.2 Endochitinase activity

Endochitinase activity was measured using needles as correlation between needle endochitinase activity and systemic resistance has already been demonstrated (34). To quantify endochitinase activity, 25 mg of needles from three trees per treatment were used. Protein extraction and enzymatic activity quantification were performed as in Noël *et al.* (34).

4.5.3 Ergosterol extraction and high performance liquid chromatography

Soil fungal biomass was determined for each repetition using 250 mg (wet weight) of soil. The ergosterol extraction protocol followed the microwave-assisted extraction (MAE) protocol as described by Montgomery *et al.* (32). Samples were analyzed with an HPLC system equipped with a Waters 1524 binary pump, a Waters 717 plus autosampler, and a Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA). Ergosterol was separated from other organic soil compounds on a 4.6 x 250 mm Zorbax Rx-C18 reverse-phase column packed with an ODS 5 μm preceded by a Zorbax guard-column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was methanol, acetonitrile (55:45, v/v), at a flow rate of 2 ml min^{-1}. The experiment was performed at room temperature. Absorbance was read at 283 nm. Ergosterol content was determined using a standard curve based on ergosterol/7-dehydrocholesterol (Sigma-Aldrich Co., St. Louis, MO, USA) area ratio. Data were processed on Waters Breeze v.3.3 software (Waters Corporation, Milford, MA, USA).

4.5.4 DNA extraction and amplification

Total gDNA was extracted from ~250 mg (dry weight) of soil using the PowerSoil™ DNA kit from Mo Bio (Mo Bio Laboratories Inc., Solana Beach, CA, USA) following the manufacturer's instructions. Internal transcribed spacer (ITS) regions and the large 28S subunit (LSU) of the nuclear ribosomal RNA (rRNA) were amplified with the reverse fungal specific primer ITS1-F (14) and the forward universal primer LR5 (34). PCR reactions were performed in a final volume of 25 μl and contained 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP (Invitrogen), 0.2 μM of each primer, 1 U of Platinum® *Taq* DNA polymerase (Invitrogen), and 1 μl of extracted DNA. PCR cycle was as follows: 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final elongation at 72°C for 5 min. PCR reaction was performed in triplicate for each of the 36 samples (2 soils x 2 sampling times x 3 treatments x 3 replicates).

4.5.5 Library construction and sequencing

Three PCR reactions per sample were pooled and then purified using QIAquick PCR purification kit (QIAGEN, Rockville, MD, USA). DNA concentration was measured with a ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). PCR products were cloned with QIAGEN PCR Cloning plus Kit according to the manufacturer's instructions. Five-fold molar excess of PCR products were incubated for 2 h at 14°C with the pDrive Cloning Vector. A total of 12 libraries were constructed. After an overnight incubation at 37°C, 150 white bacterial colonies per library were spiked and transferred into the 25 µl PCR mixture for amplification as described above, except that the annealing temperature was set to 55°C. Both strands' sequencing was performed on a ABI 3730xl (Applied Biosystems, Foster City, CA, USA) using the ITS1-F and LR5 primers.

4.5.6 Bioinformatic analyses

ITS and nLSU sequences were edited and assembled with Sequencher v4.6 (GeneCodes, Ann Arbor, MI, USA). The similarity threshold for ITS-nLSU sequences belonging to the same operational taxonomic unit (OTU) was set to 99%. Consensus sequences of each OTU were identified with the closest sequences found in the NCBI GenBank database

using BLASTN (1). PCR-generated chimeric sequences were determined from BLAST hits displaying conspicuous incongruence between the ITS and nLSU sequences and were excluded from the data sets. Sequences were aligned with MUSCLE software v3.6 (11) with two iterations. For each treatment, the number of OTUs, the Chao index to estimate unseen species and the Shannon diversity index were computed with DOTUR software v1.53 (43) using a 1% distance level. The fungal OTUs turnover between libraries at T0 and T8 and between control and Et white spruce libraries was calculated with the non-parametric maximum likelihood estimator (NPMLE, θ index) based on species proportions (53) using SONS software v1.0 (44). Sequence alignments were converted in distance matrices using DNADIST program (Jukes-Cantor as substitution model) from the PHYLIP package to produce neighbour-joining trees with MEGA 3.1 software (25) and with the R package *ape* v2.3 (36).

4.5.7 Fungal community phylogenetic analyses

To test if Et white spruce influences the phylogenetic structure of the soil fungal community, the phylogenetic relatedness of fungal species found in soil samples from control and Et white spruce was measured by computing the standardized effect size of the mean pairwise distance (SES_{MPD}). The mean pairwise distance was weighed by fungal OTU abundance. The R package *picante* v4.0.1 (24) was used to compute the SES_{MPD} metric for control and Et white spruce libraries, at T0 and T8. The observed phylogenetic relatedness was compared with null communities generated by randomly shuffling (500 times) the tip labels across the tips of the phylogenies. Phylogenetic even dispersion is indicated by significantly positive SES_{MPD} values ($P > 0.95$), while significantly negative SES_{MPD} values ($P < 0.05$) indicate phylogenetic clustering. SES_{MPD} values close to zero associated with non-significant P values means that OTUs are spread randomly across the tree. UniFrac (31) was used to perform a principal coordinate analysis (PCA) using normalized abundance weights as it treats each sample equally instead of treating each unit of branch equally. The similarity of fungal phylotype distribution between soil, control and Et white spruce libraries was also evaluated with a double principal coordinate analysis (DPCoA)

(39) using the R package *ade4* v1.4-11. The DPCoA allows to compare inter- and intra-library fungal phylotype variability and to compute the Rao diversity index.

4.5.8 Statistical analyses

The level of endochitinase activity recorded in control and Et white spruce needles was contrasted with a linear mixed-effects model. The effects of soil type (organic and mineral), transplantation treatment (no seedling, control and Et white spruce) and sampling time (T0 and T8) were tested on fungal biomass with a linear mixed-effects model. Considering data observed at T0 as a covariable, the potential effect, at T8, of transplantation treatments on the number of species was analyzed with a Poisson linear regression. Chao, Shannon and Rao diversity indices were analyzed in an analysis of variance. All the statistical analyses were done in R v2.9 (40) or using SAS software v9.1 of the SAS System for Windows (Copyright ©2002-2003, SAS Institute Inc., Cary, NC, USA). For each soil type, fungal OTU distribution between control and Et white spruce was examined by distance-based redundancy analysis (db-RDA) using PrCoord v1.0 and CANOCO v4.5. Rare OTUs were weighed using the Hellinger distance (27).

4.5.9 Nucleotide sequence accession numbers

Nucleotide sequences were deposited in the NCBI GenBank database and are registered under the accession numbers EU689158 to EU690957, and EU690958 to EU692757, for ITS and nLSU sequences, respectively.

4.6 Results

4.6.1 Soil chemical analyses

The organic (org) and mineral (mnl) soils sampled in this study contrasted by their content in total carbon (org: 48.93%; mnl: 4.87%) and nitrogen (org: 1.68%; mnl: 0.33%). The pH recorded in both soils was similar (org: 3.2; mnl: 3.7). The remaining soil chemical

analyses are presented in Table S4.1 in the supplemental material.

4.6.2 Endochitinase activity

The average endochitinase activity recorded in needles was 152.57 ± 36.79 nM MU/min/mg proteins and 1324.75 ± 303.45 nM MU/min/mg proteins for control and Et white spruce, respectively. Endochitinase activity recorded in Et white spruce was significantly higher (d.f. = 18; $P < 0.001$) than in control white spruce.

4.6.3 Fungal biomass in control and Et white spruce rhizosphere

The average fungal biomass, measured by the level of ergosterol, was 7.5 times greater in organic soil than in mineral soil at T0 (see Fig. S4.1 in the supplemental material). The level of ergosterol at T8 was 1.44 ± 0.172 μg g^{-1} and 1.60 ± 0.315 μg g^{-1} in organic soil samples associated with control and Et white spruce, respectively. In mineral soil samples, the ergosterol content recorded at T8 was 0.19 μg g^{-1} of soil in both the control and Et white spruce. The Et white spruce has no effect on soil fungal biomass (d.f. = 18; $P = 0.79$).

4.6.4 Fungal diversity in organic and mineral soil

The slope of the sequence-based rarefaction curves (Fig. 4.1) was lower for organic soil than for mineral soil at T0 and T8, indicating that the soil fungal diversity was more saturated in organic soil samples. Comparisons between observed and expected (measured by the Chao estimator) fungal OTU richness (Table 4.1) showed that 60.1% of the potential fungal diversity was recorded in organic and mineral soils at T0 and T8. The fungal communities observed at T0 were qualitatively and quantitatively different in organic and mineral soils. The organic soil was primarily colonized by Ascomycetes (82.5%), whereas the mineral soil harboured mainly Basidiomycetes (70.3%). Fungal OTU richness at T0 was 41 and 71 in organic and mineral soils, respectively. In the organic soil, the two most abundant OTUs were an uncultured fungus belonging to the Dothideomycetes (40.4%) and an *Acremonium* sp. (30.4%). In the mineral soil, the two most abundant OTUs were identified as a Tricholomataceae (26.9%) and an Agaricales (8.5%). The fungal community

overlap based on the NMPLE (θ) was only 2.5% between the organic and mineral soil at T0.

4.6.5 Comparison of fungal communities recorded in soil samples from control and Et white spruce

The number of OTUs along with the Chao and Shannon indexes computed for each transplantation treatment at T0 and T8 are displayed in Table 4.1. The fungal species richness recorded in the organic soil had slightly increased for each transplantation treatment after 8 months in the greenhouse (Fig. 4.1). In the mineral soil, fungal species richness was similar between T0 and T8 except for soil samples associated with Et white spruce. For this treatment, the number of OTUs decreased from 47 to 35 between T0 and T8. Nevertheless, the level of fungal species richness observed at T8 in soil samples associated with Et white spruce was similar to the level observed in the other two treatments (Fig. 4.1).

Fungal species richness and the Shannon diversity index were not significantly different between the three transplantation treatments nor between the two soil types according to the Poisson linear regression and the analysis of variance, respectively. The Chao estimator was significantly higher in mineral soil compared with organic soil ($P = 0.005$) and significantly higher in soil samples associated with control trees than in the ones associated with Et white spruce and in soil without seedling ($P = 0.002$). Chao values computed in soil samples with no seedling were similar to those computed in samples from Et white spruce rhizosphere at T8 (Table 4.1).

Analyses of the standardized effect size of the mean pairwise distance (SES_{MPD}, Table 4.2) showed that the structure of the fungal community recorded in organic soil samples from controls was random at T0 and T8. As for that of soil samples from Et white spruce, it was even at T0 and random at T8. In the mineral soil, the observed fungal community was clustered in control and Et white spruce at T0 and random at T8 (see Fig. S4.2A and B in supplemental material).

The PCA (Fig. 4.2) showed the fungal community found in organic soil samples to be highly different from that found in mineral soil samples at T0. At T8, the PCA showed the

fungal communities from the two soil types to cluster together, independent of the level of transplantation treatment (no seedling, control and Et white spruce). Results from db-RDA showed that the fungal phylotypes recorded in soil samples associated with Et white spruce did not differ significantly ($P > 0.05$) from the fungal phylotypes observed in soil samples associated with control white spruce.

The DPCoA showed that 88.7% of the fungal phylotype variability was explained by intra-library differences for all libraries, either from control or Et white spruce, for both soil types. The fungal phylotype diversity estimated by the Rao diversity index was not affected by the presence of Et white spruce in the two soil types according to the analysis of variance. It was significantly higher in libraries from mineral soil than in libraries from organic soil (d.f. = 17; $P = 0.015$). In the organic soil, the fungal community overlap (θ index, Table 4.1) between T0 and T8 in the control and Et white spruce treatments was ca. 33% while at T8 it was 65%. In the mineral soil, the fungal community overlap between T0 and T8 in the control and Et white spruce treatments was ca. 12% while at T8 it was 64%.

4.7 Discussion

This study aimed to examine the possible impact of chitinase-transformed trees on two soil fungal communities. Despite the fact that chitinolytic activity in Et white spruce increased 8.7 times compared with untransformed white spruce, results showed that the two soil fungal communities considered were not significantly affected after 8 months. Soil fungal biomass, fungal OTU richness, Shannon and Rao diversity indexes were not significantly different between control and Et white spruce. Investigating the soil fungal phylogenetic structure by SES_{MPD} analyses showed the organization of the fungal community to be similar in the rhizosphere associated with control and Et white spruce at T8. If endochitinase overexpression had affected some fungal taxa, the fungal species turnover would have resulted in two distinct phylogenetic structures between control and Et white spruce at T8. Results from the UniFrac analysis showed the two fungal communities recorded in organic and mineral soils to be less divergent from each other at T8 than at T0, probably because of the soil colonization by common greenhouse fungi and mycorrhizal

fungi associated with white spruce roots. It also suggests that the soil type and the presence of seedlings had a much more important impact on fungal community structure than the insertion and expression of the *ech*42 transgene within the white spruce genome. This is not really surprising since mycorrhizae are a major constituent of the overall soil fungal community and it has been suggested that they might not persist long in the absence of their host (2, 18, 21). In our study, *Wilcoxina*, *Clavulina*, *Rhizoscyphus*, a Thelephoraceae and an Agaricales, which are all mycorrhizal fungi, were present in control and Et libraries at T0 and T8, but completely absent from the "no seedling" libraries.

Only the analysis of variance performed on Chao values showed significant differences between control and Et white spruce. The Chao index estimates the total species richness expected, including undetected species. It focuses on singletons and doubletons as it considers OTUs only seen once or twice to be related to the proportion of undetected species (10). The ANOVA performed on Chao values showed that the total fungal species richness expected after 8 months should be significantly higher in soil from untransformed white spruce compared with soil from Et white spruce. The differences observed in values of the Chao estimator were the result of a small variation in the number of singletons. It was slightly higher in soil samples associated with control white spruce than in soil from Et white spruce (16 versus 11). This difference only had a minor effect on the total fungal species richness observed as the number of OTUs recorded was not significantly different among the three levels of transplantation treatment.

Results based on fungal biomass and observed fungal diversity are in accordance with previous studies investigating the potential impact of the constitutive overexpression of pathogenesis-related/antifungal proteins in genetically modified plants on non-target fungi. Vierheilig *et al*. (50) showed that chitinase-transformed *Nicotiana sylvestris* was equally colonized by the endomycorrhizal fungus *Glomus mosseae* 8 weeks after inoculation, while tobacco resistance against *Rhizoctonia solani* was enhanced compared with the control. Similarly, the increased activity of chitinase and glucanase measured in roots of different transgenic tobaccos did not affect the time course and level of colonization by *G. mossae* (51). Transformed aubergines (*Solanum melongena*) expressing Dm-AMP1 defensin protein within roots and root exudates showed a reduced growth of the phytopathogenic

fungus *Verticillum albo-atrum* without consequence on root colonization by *G. mossae* (48). Girlanda *et al.* (16) did not find significant differences in species richness of fungal rhizosphere and phyllosphere communities associated with glucanase- and chitinase-transformed tomato (*Solanum lycopersicum*) and wild-type plants after 2 and 8 months of interaction in greenhouse conditions. They showed that the establishment and development of endomycorrhizal symbiosis was similar between transgenic and control tomatoes.

Among the few studies that investigated the potential impact of transgenic trees producing exogenous antifungal protein on non-target fungi, no negative impact have been reported so far. Transgenic silver birch (*Betula pendula*) overexpressing sugar beet chitinase had similar levels of root colonization by the ectomycorrhizal fungus *Paxillus involutus* compared with untransformed silver birch (37). In a previous study (35), these chitinase silver birches were shown to be more resistant to the leaf spot disease of birch caused by *Pyrenopeziza betulicola*. Vauramo *et al.* (49) showed no negative effect on fungal biomass associated with decaying leaf litter by contrasting the ergosterol content of litters made of control and chitinase transgenic silver birch leaves after 8 months of decomposition in the field. Pasonen *et al.* (38) showed that the mycorrhizal colonization rate of a transformed line of silver birch overexpressing chitinase 3.7 times compared with the control was similar to that of untransformed trees and other wild-type clones. They did not find evidence of differences in fungal structure community associated with transgenic and wild-type genotypes clearly related to the expression of sugar beet chitinase IV. Finally, Stefani *et al.* (45) showed that an increase of up to 10 times of the endochitinase levels within root tissues of transformed white spruce lines did not prevent the colonization and development of ectendomycorrhizal symbiosis by *Wilcoxina* sp. under greenhouse conditions. Moreover, they showed that the increased chitinase activity in transgenic root exudates had no effect on soil fungal biomass.

Based on the studies previously mentioned, fungal pathogens seem to be more affected by chitinase overexpression in chitinase-transformed plants than mycorrhizal fungi and other soil fungi, such as saprophytes. The reasons for such a different susceptibility to chitinolytic activity between fungi are not well known. Fungal cell walls are highly complex structures composed of lipids, proteins, polysaccharides and other substances such

as aminopolysaccharides (e.g. chitin and chitosan), neutral polymers (e.g. cellulose, β-glucan, α-glucan, glycogen and mannan) and/or polyuronides (e.g. mucoran) (4). The mere presence of chitin in the cell wall is not a guarantee in itself that chitinase will successfully degrade the polymer. A study on the impact of *Trichoderma harzianum* endochitinase in transgenic tobacco on the survival of nematode (*Meloidogyne hapla*) eggs, mainly composed of chitin, did not detect any difference between non-transgenic and transgenic lines (8). Another study comparing *T. harzianum* endochitinase activity in nine different fungi demonstrated different levels of antifungal activity (29). Furthermore, it has already been demonstrated that rice chitinase exhibits different antifungal activities against the four different pathogenic fungi. This difference in antifungal activity was directly correlated to the surface microstructure and the proportion of chitin in the fungal cell wall (52).

Up until now, studies of transgenic plants' impact on non-target organisms have shown that plant genotype and environmental conditions account for stronger community shifts than transgene insertion and expression (7, 26, 38, 41). The results presented here show that no significant difference was detected between control and endochitinase-transformed white spruce in a greenhouse controlled experiment. Only the Chao index predicted total fungal species richness to be significantly higher in the control rhizosphere than in the Et white spruce rhizosphere. This emphasizes that even though no difference was detected at the community level, it cannot be excluded that possible changes due to endochitinase overexpression in transgenic white spruce were too weak to generate detectable effects on soil fungal diversity. Therefore, before concluding that those genetically modified trees are environmentally safe, impact studies using high-throughput sequencing methods to saturate diversity and increase the level of detection of rare OTUs are required. Moreover, future studies should be performed on a long-term field deployment.

4.8 Acknowledgements

We thank A. Noël and C. Levasseur for providing the genetically transformed white spruce used in this study. We also thank M. Bernier-Cardou for helping us with statistical analyses, and Dr P. Tanguay and I. Lamarre for the revision of the manuscript. We also

acknowledge those who developed the different analytical and statistical tools we used, their work is very valuable. This work was supported by grants from the Canadian Regulatory System for Biotechnology.

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Table 4.1 Fungal species richness and diversity indexes recorded in fungal DNA libraries according to each transplantation treatment.

		Organic soil					Mineral soil				
	Sampling time	No. of sequences	No. of OTUs	Chao [a] (Sp)	Shannon [b] (*H*)	θ [c] (%)	No. of sequences	No. of OTUs	Chao (Sp)	Shannon (*H*)	θ (%)
No seedling	T0	140	21	73.5	1.73	46.9	135	37	54.1	2.78	16.5
	T8	95	21	34.7	2.20		102	36	64.5	3.21	
Control white spruce	T0	142	20	21.4	2.02	33.4	135	33	42.1	2.78	14.2
	T8	133	29	53	2.48		123	36	86.6	2.69	
Et white spruce	T0	138	19	30.2	1.72	33.1	142	47	102	3.02	8.9
	T8	132	23	30.8	2.25		120	35	62.1	2.71	

a: Chao index
b: Shannon index
c: Non-parametric maximum likelihood estimator θ

Table 4.2 Measures of the fungal phylogenetic structure recorded in organic and mineral soil samples associated with control and Et white spruce at T0 and T8.

		Organic soil			Mineral soil		
	Sampling time	SES_{mpd} [a]	*P*-value	Phylogenetic structure	SES_{mpd}	*P*-value	Phylogenetic structure
Control white spruce	T0	1.04	0.87	Random	-2.48	0.001	Clustered
	T8	0.46	0.66	Random	0.66	0.75	Random
Et white spruce	T0	0.53	0.97	Even	-2.01	0.02	Clustered
	T8	0.66	0.74	Random	-1.14	0.1	Random

a: Standardized effect size of the mean pairwise distance

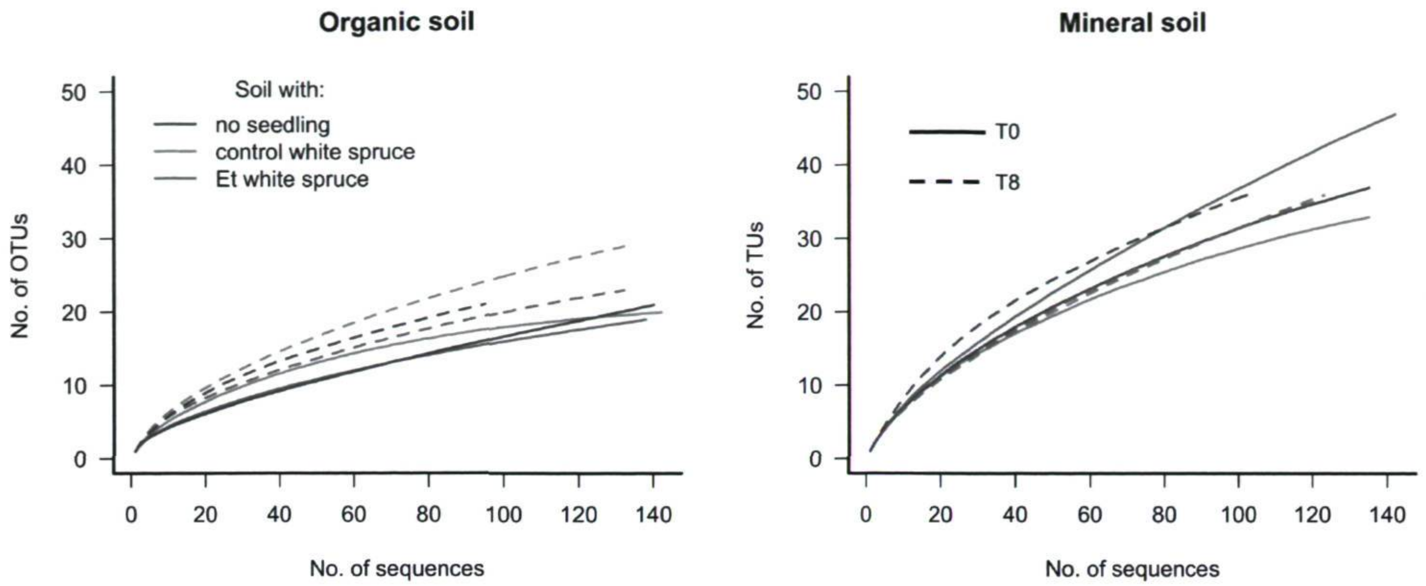


Fig. 4.1 Soil fungal OTU accumulation curves according to the number of sequences identified in fungal DNA libraries from the organic (A) and mineral (B) soil types at T0 (full lines) and T8 (dashed lines).

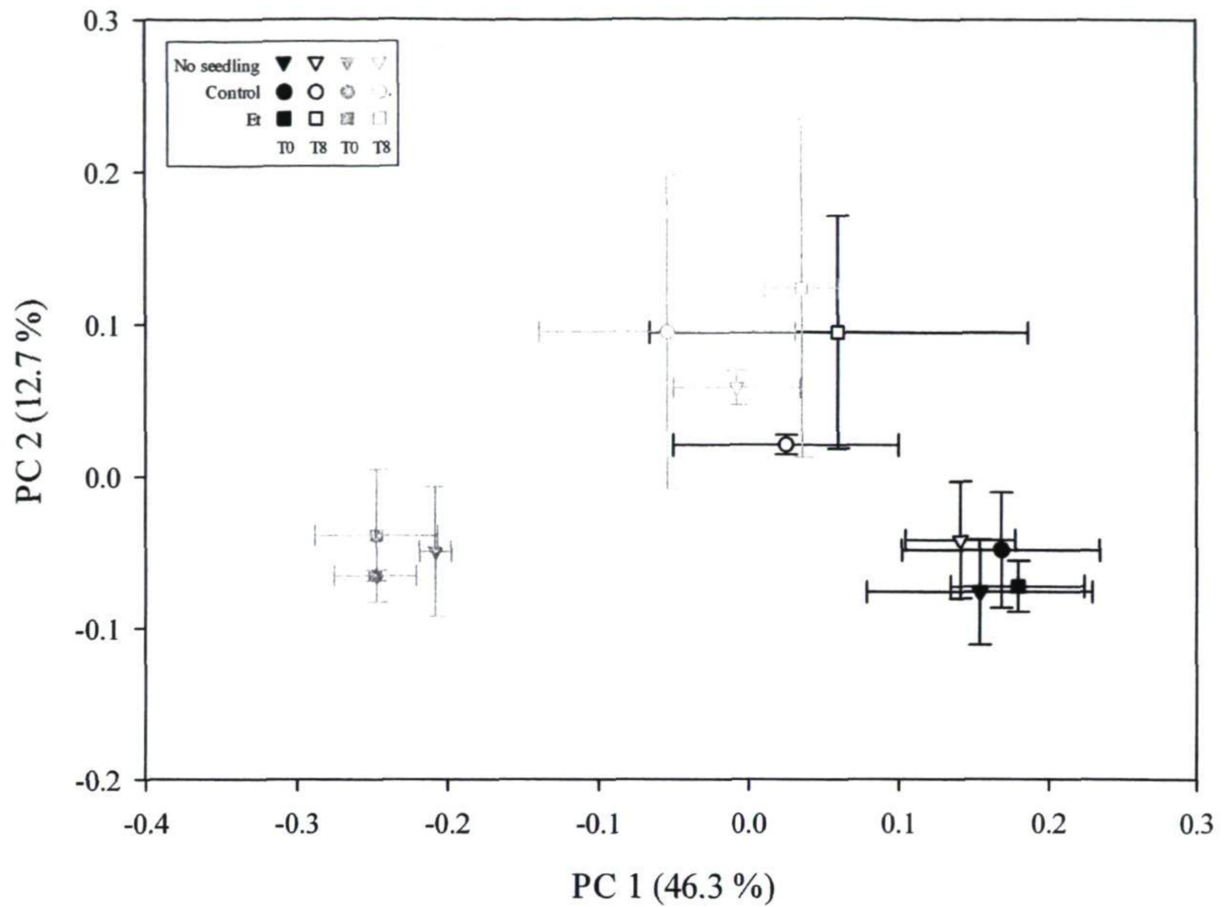


Fig. 4.2 Principal coordinate analysis performed on fungal DNA libraries from soil samples with no seedling and soil samples associated with control and Et white spruce.

Table S4.1 Soil chemical properties.

	Total carbon (% C)	Total nitrogen (% N)	C/N	Ca_{ext} (g/kg)	Mg_{ext} (g/kg)	K_{ext} (g/kg)	CEC (cmol(+)/kg)	pH (H_2O)	pH ($CaCl_2$)
Organic soil	48.93 (1.04) [a]	1.68 (0.03)	29.16 (0.32)	2.24 (0.04)	0.16 (0.01)	0.30 (0.08)	19.16 (1.15)	3.20 (0.19)	2.97 (0.06)
Mineral soil	4.87 (0.11)	0.33 (0.01)	14.95 (0.48)	0.12 (0.01)	0.01 (0.00)	0.06 (0.01)	3.87 (0.12)	3.69 (0.05)	3.81 (0.03)

a: Standard deviations

Table S4.2A Identification of the 97 contigs recorded in the organic and mineral soil samples with the best BLAST match of the ITS (first line) and nLSU sequences (second line) from the NCBI GenBank database.

		BLAST match	Coverage (%)	Similarity (%)	GenBank accession	No. of sequences
OTU 01	*Dothideomycetes*	Uncultured fungus	97	96	AY970243	262
		Gloniopsis praelonga	100	80	EU552133	
OTU 02	*Acremonium*	*Acremonium strictum*	100	100	GQ450275	137
		Acremonium strictum	100	98	FJ176879	
OTU 03	*Tricholomataceae*	*Hygrocybe virginea*	95	86	FM208869	124
		Xeromphalina campanella	100	95	AF261469	
OTU 04	*Wilcoxina*	*Wilcoxina mikolae*	100	91	AY219841	118
		Wilcoxina cf. mikolae	99	97	AF430285	
OTU 05	*Thelephoraceae*	*Thelephora terrestris*	100	100	FN393122	72
		Thelephora sp.	100	96	AF287890	
OTU 06	*Inocybe*	*Inocybe jacobi*	92	99	AM882710	49
		Inocybe petiginosa	99	94	AF261510	
OTU 07	*Clavulina*	*Clavulina cf. cristata*	97	99	EU862212	46
		Clavulina cf. cristata	99	99	AF261553	
OTU 08	*Cryptococcus*	*Cryptococcus podzolicus*	100	100	FN394715	40
		Cryptococcus cf. podzolicus	81	99	FJ743620	
OTU 09	*Agaricales*	*Hygrocybe reidii*	100	92	EU784347	37
		Hygrophorus purpureofolius	100	92	EF535264	
OTU 10	*Hygrocybe*	*Hygrocybe chlorophana*	100	98	EU435148	33
		Hygrocybe chlorophana	100	99	EU435148	
OTU 11	*Rhyzoscyphus*	*Hymenoscyphus ericae*	100	98	AF069439	33
		Rhyzoscyphus ericae	100	99	AM887699	
OTU 12	*Dothideomycetes*	Ectomycorrhizal root tip	100	81	AF476985	32
		Gloniopsis praelonga	100	80	EU552133	
OTU 13	*Cryptococcus*	*Cryptococcus terricola*	100	100	FN298664	31
		Cryptococcus terricola	80	100	AM039670	
OTU 14	*Clavulina*	*Clavulina cf. cristata*	97	98	EU862212	28
		Clavulina cf. cristata	99	99	AF261553	
OTU 15	*Agaricales*	*Hygrocybe splendidissima*	90	96	FM208892	26
		Hygrophorus purpureofolius	100	93	EF535264	
OTU 16	*Wilcoxina*	*Trichophaea cf. hybrida*	100	96	DQ200834	23
		Wilcoxina cf. mikolae	99	99	AF430285	
OTU 17	*Rhizophydium*	*Rhizophydium macroporosum*	100	100	DQ485642	19
		Rhizophydium sp.	100	98	DQ273825	
OTU 18	*Trichoderma*	*Trichoderma asperellum*	100	100	FJ605246	13
		Trichoderma atroviride	99	99	EF591763	
OTU 19	*Sordariomycetes*	*Uncultured fungus*	100	89	GQ159998	13
		Leuconeurospora pulcherrima	100	80	AF096193	
OTU 20	*Fomes*	*Ganoderma* sp.	99	99	AF255097	11
		Fomes fomentarius	100	99	DQ208419	
OTU 21	*Mycena*	*Mycena rubromarginata*	100	87	EF530939	10
		Mycena monticola	100	98	EU669336	
OTU 22	*Geoglossales*	*Uncultured fungus*	96	94	DQ093781	10
		Sarcoleotia globosa	100	91	AY789409	
OTU 23	*Pseudeurotiaceae*	*Pseudeurotium desertorum*	99	94	AY129288	9
		Pseudeurotium zonatum	100	95	AF096198	
OTU 24	*Pezizomycotina*	*Uncultured fungus*	100	96	GQ160019	9
		Geoglossum nigritum	100	79	AY544650	

OTU 25	*Mortierellaceae*	*Mortierella minutissima*	100	95	EU484265	8
		Mortierella verticillata	100	95	DQ273794	
OTU 26	*Mortierella*	*Mortierella humilis*	100	100	AJ878778	8
		Mortierella verticillata	100	99	DQ273794	
OTU 28	*Helotiaceae*	*Clathrosphaerina zalewskii*	100	89	EF029222	8
		Cudoniella sp.	100	95	AY789377	
OTU 30	*Rhyzoscyphus*	*Meliniomyces variabilis*	100	99	EF093171	7
		Rhyzoscyphus ericae	97	100	AM887699	
OTU 31	*Mortierellales*	*Mortierella chlamydospora*	97	88	AB476422	7
		Mortierella verticillata	100	94	DQ273794	
OTU 32	*Emericellopsis*	*Emericellopsis terricola*	100	98	FJ430737	7
		Emericellopsis terricola	99	99	U57082	
OTU 33	*Hyalodendriella*	Helotiales	100	100	EF093148	7
		Hyalodendriella betulae	100	97	EU040232	
OTU 35	*Pezizomycotina*	Ectomycorrhizal root tip	100	84	AF476985	7
		Rhyzoscyphus ericae	100	78	AM887699	
OTU 36	*Pezizomycotina*	Ectomycorrhizal root tip	100	80	AF476985	7
		Rhyzoscyphus ericae	100	78	AM887699	
OTU 37	*Orbiliomycetes*	*Uncultured fungus*	100	100	FJ626931	7
		Drechslerella doedycoides	100	80	EF445992	
OTU 39	*Ascomycota*	*Sebacina incrustans*	31	86	EU819442	6
		Symbiotaphrina kochii	100	71	DQ248314	
OTU 40	*Lecanoromycetes*	*Uncultured fungus*	100	85	GQ160017	6
		Umbilicaria decussata	100	81	EF489960	
OTU 45	*Mortierellaceae*	*Mortierella* sp..	100	99	EU877758	5
		Mortierella verticillata	100	95	DQ273794	
OTU 46	*Mortierella*	*Mortierella minutissima*	97	99	AB476417	5
		Mortierella verticillata	100	99	DQ273794	
OTU 47	*Agaricales*	*Hygrocybe splendidissima*	93	97	EU784441	5
		Hygrocybe coccinea	100	93	EU435146	
OTU 48	*Ascomycota*	*Schizangiella* sp.	25	83	EF392540	5
		Symbiotaphrina kochii	100	71	DQ248314	
OTU 49	*Hyphodiscus*	*Hyphodiscus hymeniophilus*	100	94	DQ227263	5
		Hyphodiscus hymeniophilus	100	97	DQ227263	
OTU 50	*Chalara*	*Chalara microchona*	100	98	DQ093752	5
		Chalara constricta	96	99	FJ176256	
OTU 51	*Rhyzoscyphus*	*Rhizoscyphus ericae*	100	95	AM084704	5
		Rhyzoscyphus ericae	100	98	AM887699	
OTU 52	*Xylariales*	*Plectosphaera eucalypti*	100	84	DQ923538	5
		Subramaniomyces fusisaprophyticus	100	94	EU040241	
OTU 57	*Mycosphaerellaceae*	*Phialocephala fortinii*	100	99	EU888625	4
		Trimmatostroma salicis	100	96	EU019300	
OTU 58	*Hydnotrya*	*Hydnotrya cubispora*	95	94	EU784273	4
		Hydnotrya cubispora	96	99	DQ200845	
OTU 59	*Tomentella*	*Thelephora sp.*	100	99	FN393090	4
		Tomentella botryoides	100	98	AY586717	
OTU 60	*Tomentella*	*Tomentella cf. sublilacina*	100	97	AJ889982	4
		Tomentella botryoides	100	98	AY586717	
OTU 61	*Cladophialophora*	*Cladophialophora* sp.	100	89	EU139127	4
		Cladophialophora carrionii	100	98	AF050262	
OTU 62	*Trimmatostroma*	*Trimmatostroma betulinum*	90	96	EU019299	4
		Trimmatostroma betulinum	100	99	EU019299	
OTU 63	*Sarcostroma*	*Amphisphaeria* sp.	100	98	AF346545	4
		Sarcostroma bisetulatum	100	98	EU552155	

OTU 64	*Helotiales*	*Uncultured Leotiomycetes*	100	96	FJ152529	4
		Rhyzoscyphus ericae	100	94	AM887699	
OTU 65	*Sordariomycetes*	*Cephalotheca sulfurea*	99	78	AB278194	4
		Tectonidula hippocrepida	100	90	FJ617557	
OTU 66	*Geoglossales*	*Uncultured fungus*	96	95	DQ093781	4
		Sarcoleotia globosa	100	91	AY789409	
OTU 67	*Helotiales*	*Cistella acuum*	100	99	U57492	4
		Hyalodendriella betulae	100	96	EU040232	
OTU 68	*Articulospora*	*Articulospora tetracladia*	100	97	EU998923	4
		Articulospora tetracladia	100	99	EU998927	
OTU 73	*Rhyzoscyphus*	*Meliniomyces bicolor*	100	95	EF093183	4
		Rhyzoscyphus ericae	100	98	AM887699	
OTU 75	*Mortierellales*	*Mortierella minutissima*	100	79	EU484265	3
		Mortierella indohii	100	95	EU688966	
OTU 76	*Mucoromycotina*	*Umbelopsis isabellina*	100	89	AB193546	3
		Umbelopsis ramanniana	100	88	DQ273797	
OTU 77	*Corticiales*	*Hyphodontia nespori*	98	82	DQ873622	3
		Hyphodontia nespori	100	93	DQ873622	
OTU 78	*Clavulina*	*Clavulina castaneipes*	100	98	EU669209	3
		Clavulina castaneipes	100	99	EU669262	
OTU 79	*Rhodosporidium*	*Rhodosporidium toruloides*	100	97	AB073266	3
		Rhodosporidium toruloides	95	99	DQ832191	
OTU 80	*Cladophialophora*	*Cladophialophora chaetospira*	100	97	EU035404	3
		Cladophialophora chaetospira	100	99	EU035406	
OTU 81	*Trichoderma*	*Trichoderma hamatum*	100	100	GQ221830	3
		Trichoderma viride	98	100	AY291123	
OTU 84	*Polyporales*	*Nectria mariannaeae*	100	99	AB099509	3
		Fomitopsis feei	100	90	AY515327	
OTU 85	*Leotiomycetes*	*Pseudeurotium desertorum*	98	82	AY129288	3
		Pseudeurotium zonatum	100	93	AF096198	
OTU 96	*Helotiales*	*Hyphodiscus hymeniophilus*	93	80	DQ227263	2
		Hyphodiscus hymeniophilus	100	91	DQ227262	
OTU 97	*Mortierellales*	*Mortierellales* sp.	100	74	EF126343	2
		Mortierella verticillata	100	92	DQ273794	
OTU 99	*Mortierellaceae*	*Mortierella minutissima*	100	96	EU484265	2
		Mortierella verticillata	100	95	DQ273794	
OTU 100	*Mortierellales*	*Mortierella minutissima*	100	94	EU484265	2
		Mortierella verticillata	100	94	DQ273794	
OTU 101	*Mortierella*	*Mortierella exigua*	99	94	FJ161929	2
		Mortierella verticillata	100	94	DQ273794	
OTU 102	*Mortierellales*	*Zygomycete sp.*	100	96	AM292200	2
		Mortierella verticillata	100	92	DQ273794	
OTU 103	*Mortierellales*	*Mortierella macrocystis*	100	99	AJ878782	2
		Mortierella verticillata	100	92	DQ273794	
OTU 108	*Tomentella*	*Tomentella* sp.	100	91	U92537	2
		Tomentella botryoides	100	98	AY586717	
OTU 109	*Hygrocybe*	*Hygrocybe coccinea voucher*	100	94	EU784294	2
		Hygrocybe coccinea	100	98	EU435146	
OTU 110	*Tomentella*	*Tomentella* sp.	100	89	U92537	2
		Tomentella botryoides	100	97	AY586717	
OTU 112	*Agaricales*	*Hygrocybe ceracea*	100	91	EU784289	2
		Hygrocybe cantharellus	100	91	DQ457675	
OTU 113	*Microbotryomycetes*	*Rhodotorula fragaria*	100	87	AF444530	2
		Rhodotorula sp.	100	95	FN400943	
	Sebacinaceae	*Sebacina incrustans* voucher	100	89	EF644113	2

OTU 114	*Sebacinaceae*	*Sebacina incrustans* voucher	100	89	EF644113	2
		Sebacina incrustans	100	95	DQ521406	
OTU 117	*Sebacinales*	*Sebacina incrustans*	100	81	AF490395	2
		Sebacina incrustans	100	91	DQ521406	
OTU 118	*Sakaguchia*	*Sakaguchia dacryoidea*	100	94	AF444597	2
		Sakaguchia dacryoidea	100	97	DQ832205	
OTU 119	*Leotiaceae*	Helotiales	100	99	EF093149	2
		Neobulgaria pura	100	96	DQ257365	
OTU 120	*Verticillium*	*Verticillium bulbillosum*	98	99	AJ292410	2
		Verticillium bactrosporum	97	99	AF339538	
OTU 121	*Hyaloscyphaceae*	*Uncultured Leotiomycetes*	100	98	FJ152529	2
		Haplographium catenatum	100	95	FJ839657	
OTU 122	*Helotiales*	*Holwaya mucida*	100	82	DQ257357	2
		Holwaya mucida	100	90	DQ257356	
OTU 123	*Chalara*	*Chalara microchona*	100	96	DQ093752	2
		Chalara constricta	96	99	FJ176256	
OTU 124	*Nectria*	*Nectria* sp.	100	96	EF029830	2
		Nectria grammicospora	99	99	AF193238	
OTU 125	*Pseudeurotiaceae*	*Satchmopsis brasiliensis*	97	82	DQ195785	2
		Pseudeurotium zonatum	100	95	AF096198	
OTU 126	*Helotiales*	*Scleropezicula alnicola*	100	89	AF141168	2
		Hyalodendriella betulae	100	96	EU040232	
OTU 128	*Bionectria*	*Bionectria ochroleuca*	100	100	AB369487	2
		Bionectria sp.	100	99	DQ327624	
OTU 129	*Pseudeurotiaceae*	*Pseudeurotium desertorum*	97	92	AY129288	2
		Pseudeurotium zonatum	100	95	AF096198	
OTU 130	*Helotiales*	*Leptodontidium elatius*	97	93	AY781230	2
		Hyalodendriella betulae	100	96	EU040232	
OTU 131	*Allantophoma*	*Allantophomopsis lycopodina*	99	98	AB041243	2
		Allantophoma endogenospora	96	99	EU754125	
OTU 132	*Venturia*	*Venturia hystrioides*	100	97	EU035459	2
		Venturia hystrioides	100	99	EU035459	
OTU 133	*Geomyces*	*Geomyces* sp.	100	98	DQ499473	2
		Geomyces sp.	93	99	AB470544	
OTU 134	*Articulospora*	*Articulospora tetracladia*	100	96	EU998918	2
		Articulospora tetracladia	100	99	EU998929	
OTU 136	*Dothideomycetes*	Ectomycorrhizal root tip	100	81	AF476985	2
		Gloniopsis praelonga	100	80	EU552133	

Table S4.2B Identification of the 87 singletons recorded in the organic and mineral soil samples with the best BLAST match of the ITS (first line) and nLSU sequences (second line) from the NCBI GenBank database.

			BLAST match	Coverage (%)	Similarity (%)	GenBank accession
OTU 137	*Chaunopycnis*	BPA-Ctl1-T0-3C	*Chaunopycnis alba*	95	97	AF389192
			Chaunopycnis alba	100	99	AF245296
OTU 138	*Leotiomycetes*	BPA-Ctl1-T0-5D	*Glomus custos*	34	88	GQ205075
			Cudoniella clavus	100	81	AY789373
OTU 139	*Hyphodiscus*	BPA-Ctl1-T0-5F	*Hyphodiscus hymeniophilus*	99	93	DQ227260
			Hyphodiscus hymeniophilus	100	97	DQ227260
OTU 140	*Phoma*	BPA-Ctl1-T0-6H	*Phoma herbarum*	100	99	AY293791
			Phoma herbarum	100	99	AY293791
OTU 141	*Chalara*	BPA-Ctl1-T0-7H	*Chalara microchona*	100	100	DQ093752
			Chalara constricta	95	99	FJ176256
OTU 142	*Hyaloscyphaceae*	BPA-Ctl1-T8-2B	*Cryptosporiopsis radicicola*	100	91	EF413597
			Haplographium catenatum	100	96	FJ839657
OTU 143	*Lophium*	BPA-Ctl1-T8-4F	*Lophium mytilinum*	76	82	EF596819
			Lophium mytilinum	100	97	EF596819
OTU 144	*Pezizomycetes*	BPA-Ctl1-T8-4G	*Cyphelium karelicum*	26	94	AY450585
			Ascodesmis nigricans	100	80	DQ168335
OTU 145	*Exobasidiomycetes*	BPA-Ctl1-T8-5F	*Tilletiopsis oryzicola*	33	93	AB045708
			Tilletiopsis washingtonensis	97	86	AY745714
OTU 146	*Hypomyces*	BPA-Ctl1-T8-7E	*Hypomyces chrysospermus*	100	99	AB027385
			Hypomyces chrysospermus	100	100	AB027385
OTU 147	*Phoma*	BPA-Ctl1-T8-8D	*Phoma herbarum*	100	98	AY293791
			Phoma herbarum	100	98	AY293791
OTU 148	*Phoma*	BPA-Ctl2-T8-1B	*Phoma glomerata*	99	100	EU273521
			Phoma sojicola	100	99	EU167568
OTU 149	*Helotiales*	BPA-Ctl2-T8-2B	*Rhyzoscyphus ericae*	84	90	AM084704
			Rhyzoscyphus ericae	100	93	AM887699
OTU 150	*Galerina*	BPA-Ctl2-T8-3B	*Galerina chionophila*	95	91	AJ585506
			Galerina marginata	100	98	DQ457669
OTU 151	*Capnodiales*	BPA-Ctl2-T8-7C	*Devriesia americana*	98	88	AY251068
			Devriesia americana	100	96	EU040227
OTU 152	*Ramularia*	BPA-GM1-T0-D1	*Ramularia eucalypti*	100	99	EF394861
			Ramularia pratensis	100	99	EU019284
OTU 153	*Lecanoromycetes*	BPA-GM2-T0-1H	*Antennariella placitae*	29	92	GQ303268
			Mycobilimbia hypnorum	100	82	AY533005
OTU 154	*Helotiaceae*	BPA-GM2-T0-3B	*Leptodontidium elatius*	98	98	AY781230
			Rhyzoscyphus ericae	100	96	AM887699
OTU 155	*Auriculariales*	BPA-GM2-T8-6H	*Hericium erinaceum*	30	95	FJ869190
			Basidiodendron caesiocinereum	95	94	AJ406416
OTU 156	*Heyderia*	BPA-GM3-T8-2H	*Heyderia abietis*	86	92	AY789290
			Heyderia abietis	100	98	AY789289
OTU 157	*Penicillium*	BPA-GM3-T8-3C	*Penicillium glabrum*	100	99	DQ682590
			Penicillium glabrum	92	99	AB470560
OTU 158	*Hyaloscypha*	BPA-GM3-T8-4E	*Hyaloscypha daedaleae*	92	97	AY789416
			Hyaloscypha daedaleae	93	99	AY789415
OTU 159	*Cladophialophora*	BPA-Sol1-T0-B8	*Cladophialophora chaetospira*	100	95	EU035404
			Cladophialophora chaetospira	100	99	EU035404
OTU 160	*mitosporic*	BPA-Sol1-T0-C8	*Tritirachium oryzae*	25	91	GQ329853

OTU 160	*Ascomycota*		*Tritirachium oryzae*	25	91	GQ329853
			Tritirachium oryzae	100	85	GQ329853
OTU 161	*Ampulloclitocybe*	BPA-Sol1-T0-F4	*Ampulloclitocybe clavipes*	100	98	AF335448
			Ampulloclitocybe clavipes	99	99	AY639881
OTU 162	*Pholiota*	BPA-Sol1-T0-F8	*Pholiota adiposa*	100	99	AB470888
			Pholiota gummosa	100	99	AF195605
OTU 163	*Mortierellales*	BPA-Sol1-T8-2G	*Mortierella chlamydospora*	100	91	AB476422
			Mortierella indohii	85	94	EU688966
OTU 164	*Exophiala*	BPA-Sol1-T8-7D	*Exophiala bergeri*	84	88	EF025404
			Exophiala salmonis	100	98	AF050274
OTU 165	*Helotiaceae*	BPA-Sol2-T0-3D	*Cudoniella acicularis*	100	93	DQ202512
			Cudoniella clavus	100	96	AY789373
OTU 166	*Herpotrichiellaceae*	BPA-Sol2-T0-7F	*Cladophialophora scillae*	100	88	EU035412
			Cladophialophora hostae	100	95	EU035407
OTU 167	*Russula*	BPA-Sol2-T8-2F	*Russula emetica*	100	100	AY061673
			Russula emetica	100	99	DQ421997
OTU 168	*Athelia*	BPA-Sol2-T8-4H	*Athelia epiphylla*	100	94	AEU85793
			Athelia epiphylla	100	98	AY586633
OTU 169	*Bionectria*	BPA-Sol2-T8-5E	*Bionectria ochroleuca*	100	97	EU273558
			Bionectria ochroleuca	98	99	AY489716
OTU 170	*Sakaguchia*	BPA-Sol3-T8-4C	*Sakaguchia dacryoidea*	100	94	AF444597
			Sakaguchia dacryoidea	100	98	DQ832205
OTU 171	*Agaricales*	MW-Ctl1-T0-1C	*Hygrocybe punicea*	93	93	FM208876
			Hygrocybe coccinea	100	93	EU435146
OTU 172	*Geoglossaceae*	MW-Ctl1-T0-2C	*Geoglossum fallax*	42	96	AY789311
			Geoglossum nigritum	99	96	AY544650
OTU 173	*Lactarius*	MW-Ctl1-T0-7C	*Lactarius theiogalus*	97	99	AF349716
			Lactarius leonis	100	98	AF506411
OTU 174	*Inocybe*	MW-Ctl1-T8-3C	*Inocybe petiginosa*	100	95	AM113952
			Inocybe petiginosa	98	100	AF261510
OTU 175	*Geomyces*	MW-Ctl2-T8-2D	*Geomyces pannorum*	100	99	AJ938166
			Geomyces sp.	91	99	AB470567
OTU 176	*Umbelopsis*	MW-Ctl2-T8-6D	*Umbelopsis ramanniana*	97	97	DQ888724
			Umbelopsis vinacea	76	99	AB090305
OTU 177	*Mortierellales*	MW-Ctl2-T8-7H	*Mortierella chlamydospora*	99	99	AB476422
			Mortierella indohii	100	94	EU688966
OTU 178	*Microbotryales*	MW-Ctl3-T0-3B	*Rhodotorula glacialis*	100	95	EF151250
			Rhodotorula hordea	100	93	AY631901
OTU 179	*Hygrocybe*	MW-Ctl3-T0-7H	*Hygrocybe coccinea*	100	93	EU784294
			Hygrocybe coccinea	100	98	EU435146
OTU 180	*Chytridiomycetes*	MW-Ctl3-T8-6D	*Phlyctochytrium planicorne*	30	88	AY997070
			Phlyctochytrium planicorne	100	84	DQ273813
OTU 181	*Mucorales*	MW-GM1-T0-A10	*Umbelopsis ramanniana*	36	88	AB193544
			Umbelopsis ramanniana	100	83	DQ273797
OTU 182	*Agaricales*	MW-GM1-T0-A11	*Hygrocybe punicea*	84	89	FM208876
			Hygrocybe coccinea	100	92	EU435146
OTU 183	*Tricholomataceae*	MW-GM1-T0-B4	*Collybia cirrhata*	67	90	AF361317
			Sarcomyxa serotina	98	96	EU365678
OTU 184	*Inocybe*	MW-GM1-T0-C3	*Inocybe cf. grammata*	99	94	GQ166895
			Inocybe albodisca	100	98	EU307819
OTU 185	*Cystoderma*	MW-GM1-T0-C5	*Cystoderma amianthinum*	100	97	DQ192177
			Cystoderma amianthinum	100	100	DQ154108
OTU 186	*Cortinariaceae*	MW-GM1-T8-1E	*Inocybe relicina*	100	85	AF325664
			Inocybe relicina	100	96	AY038324

OTU 187	*Russula*	MW-GM1-T8-3A	*Russula emetica*	100	98	AY061673
			Russula emetica	100	98	DQ421997
OTU 188	*Aquapoterium*	MW-GM1-T8-4A	*Aquapoterium pinicola*	80	92	FJ172275
			Aquapoterium pinicola	99	98	EU183121
OTU 189	*Sarcosomataceae*	MW-GM2-T0-2B	*Urnula craterium*	66	87	EU834221
			Urnula craterium	99	95	AY945851
OTU 190	*Hyaloscyphaceae*	MW-GM2-T0-3D	*Phialophora finlandia*	84	91	AF486119
			Haplographium catenatum	100	95	FJ839657
OTU 191	*Articulospora*	MW-GM2-T0-4B	*Articulospora tetracladia*	100	96	GQ152144
			Articulospora tetracladia	100	99	EU998929
OTU 192	*Amanita*	MW-GM2-T0-4D	*Amanita fulva*	96	98	FJ596775
			Amanita fulva	96	98	AF097373
OTU 193	*Candida*	MW-GM2-T0-5A	*Candida paludigena*	92	100	DQ911451
			Candida paludigena	95	100	DQ438194
OTU 194	*Mycena*	MW-GM2-T0-5H	*Mycena sanguinolenta*	97	99	FJ596764
			Mycena sanguinolenta	95	99	AY207257
OTU 195	*Glomeromycetes*	MW-GM2-T0-6B	*Glomus cerebriforme*	99	85	GQ205037
			Glomus intraradices	100	88	FM865549
OTU 196	*Helotiales*	MW-GM3-T0-2C	*Meliniomyces bicolor*	100	97	EF093183
			Hyalodendriella betulae	100	96	EU040232
OTU 197	*Glomeromycetes*	MW-GM3-T0-2D	*Glomus intraradices*	100	81	AY035641
			Glomus intraradices	100	89	FM865549
OTU 198	*Fulvoflamma*	MW-GM3-T0-4G	*Fulvoflamma eucalypti*	94	92	DQ195779
			Fulvoflamma eucalypti	98	98	DQ195791
OTU 199	*Occultifur*	MW-GM3-T0-4H	*Occultifur externus*	100	96	AF444643
			Occultifur externus	99	97	AY745723
OTU200	*Tylopilus*	MW-GM3-T0-5H	*Tylopilus felleus*	99	99	EU819449
			Tylopilus felleus	100	99	AY586723
OTU201	*Trechisporales*	MW-GM3-T8-3E	*Trechispora alnicola*	42	90	DQ411529
			Trechispora kavinioides	100	94	AF347086
OTU202	*Pleosporales*	MW-GM3-T8-3H	*Ochrocladosporium frigidarii*	80	88	FJ755255
			Ochrocladosporium elatum	100	96	EU040233
OTU203	*Clavaria*	MW-GM3-T8-4F	*Clavaria acuta*	99	99	AY228353
			Clavaria straminea	95	99	EF535267
OTU204	*Rhodotarzetta*	MW-GM3-T8-5B	*Scutellinia colensoi*	27	97	AY220838
			Rhodotarzetta rosea	95	98	DQ220413
OTU205	*Agaricales*	MW-Sol1-T0-C11	*Hygrocybe flavipes*	69	98	EU784303
			Hygrocybe aff. conica	99	92	AY684167
OTU206	*Phialea*	MW-Sol1-T0-G7	*Phialea strobilina*	100	90	EF596821
			Phialea strobilina	100	97	EF596821
OTU207	*Rhyzoscyphus*	MW-Sol1-T8-7C	*Rhizoscyphus ericae*	100	98	AM084704
			Rhyzoscyphus ericae	100	98	AM887699
OTU208	*Trichoderma*	MW-Sol1-T8-7F	*Trichoderma oblongisporum*	100	99	DQ083020
			Trichoderma atroviride	99	98	EF591763
OTU209	*Helotiales*	MW-Sol2-T0-1H	*Hyphodiscus hymeniophilus*	93	80	DQ227263
			Hyphodiscus hymeniophilus	100	92	DQ227262
OTU210	*Mortierellales*	MW-Sol2-T0-3A	Zygomycetes	100	97	EF152531
			Mortierella verticillata	100	92	DQ273794
OTU211	*Agaricales*	MW-Sol2-T0-4B	Uncultured Agaricomycetes	100	87	FJ554094
			Pachylepyrium carbonicola	100	94	DQ986294
OTU212	*Saccharomycetales*	MW-Sol2-T0-5G	*Candida subhashii*	100	100	EU836707
			Candida dubliniensis	100	92	FM992695
OTU213	*Dothideomycetes*	MW-Sol2-T8-4D	*Uncultured fungus*	97	96	AY970243
			Gloniopsis praelonga	100	80	EU552133
OTU214	*Helotiales*	MW-Sol2-T8-5B	Uncultured Pezizomycotina	100	83	FJ554184

			Hyalodendriella betulae	100	92	EU040232
OTU215	*Rhodotorula*	MW-Sol3-T0-2E	*Rhodotorula* sp.	99	91	AM922291
			Rhodotorula sp.	100	97	FN400943
OTU216	*Cortinariaceae*	MW-Sol3-T0-4B	*Inocybe lacera*	97	84	AB211269
			Inocybe lacera	100	96	AY038318
OTU217	*Pezizales*	MW-Sol3-T0-4C	Uncultured fungus	100	94	DQ309152
			Phialea strobilina	100	94	EF596821
OTU218	*Venturia*	MW-Sol3-T8-10C	*Venturia hystrioides*	100	97	EU035459
			Venturia hystrioides	100	99	U035459
OTU219	*Fusicladium*	MW-Sol3-T8-11A	*Fusicladium fagi*	100	99	EU035431
			Fusicladium fagi	100	99	EU035431
OTU220	*Mortierellaceae*	MW-Sol3-T8-4G	*Mortierella* sp.	98	98	AJ890432
			Mortierella verticillata	100	95	DQ273794
OTU221	*Pleosporaceae*	MW-Sol3-T8-5B	Uncultured Pleosporales	100	95	FJ552860
			Drechslera erythrospila	100	96	EU552124
OTU222	*Leotiomycetes*	MW-Sol3-T8-5E	*Gymnostellatospora japonica*	96	76	AF062818
			Pseudeurotium zonatum	100	90	AF096198
OTU223	*Helotiaceae*	MW-Sol3-T8-6H	*Clathrosphaerina zalewskii*	100	88	EF029222
			Cudoniella sp.	100	95	AY789377

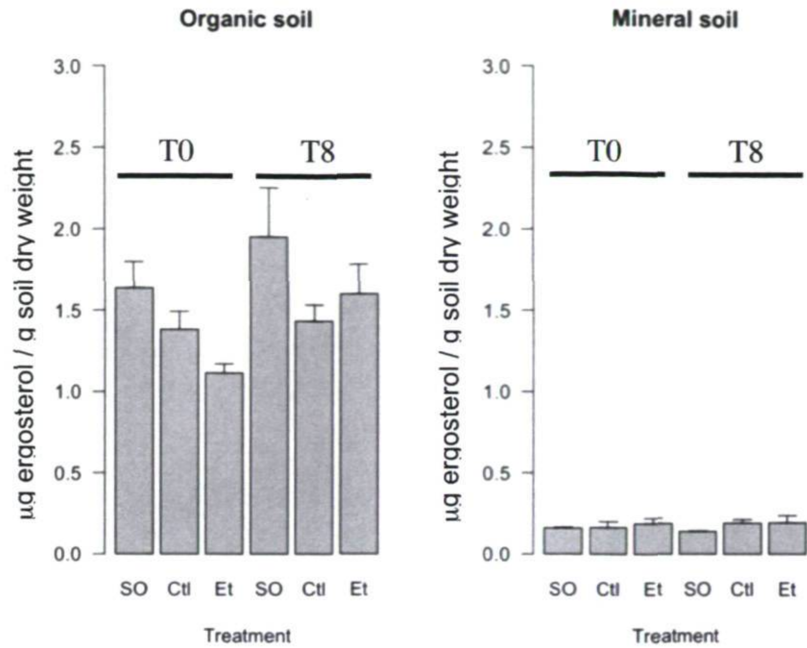


Fig. S4.1 Soil ergosterol content. SO: soil without seedling, Ctl: control white spruce, Et: endochitinase white spruce.

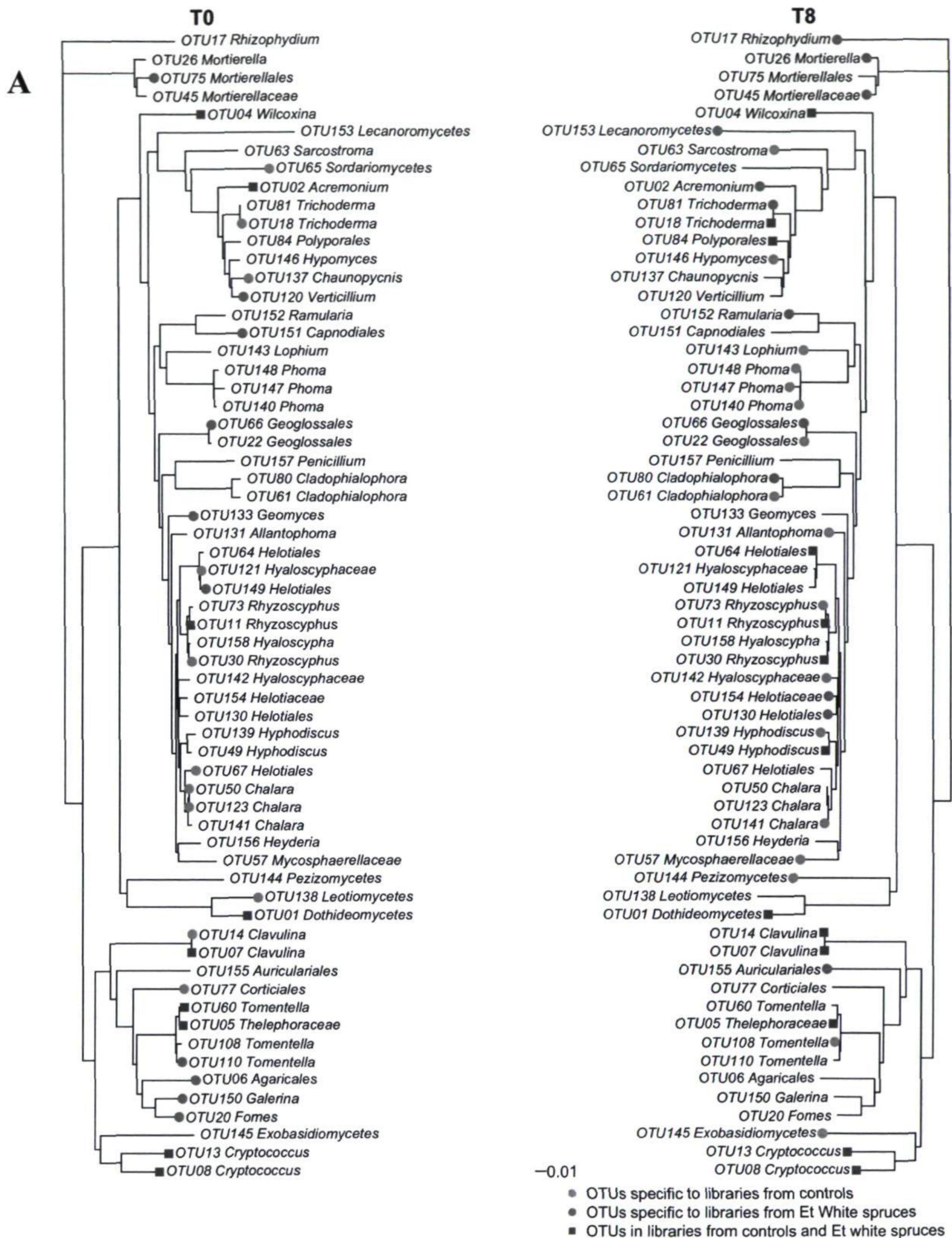
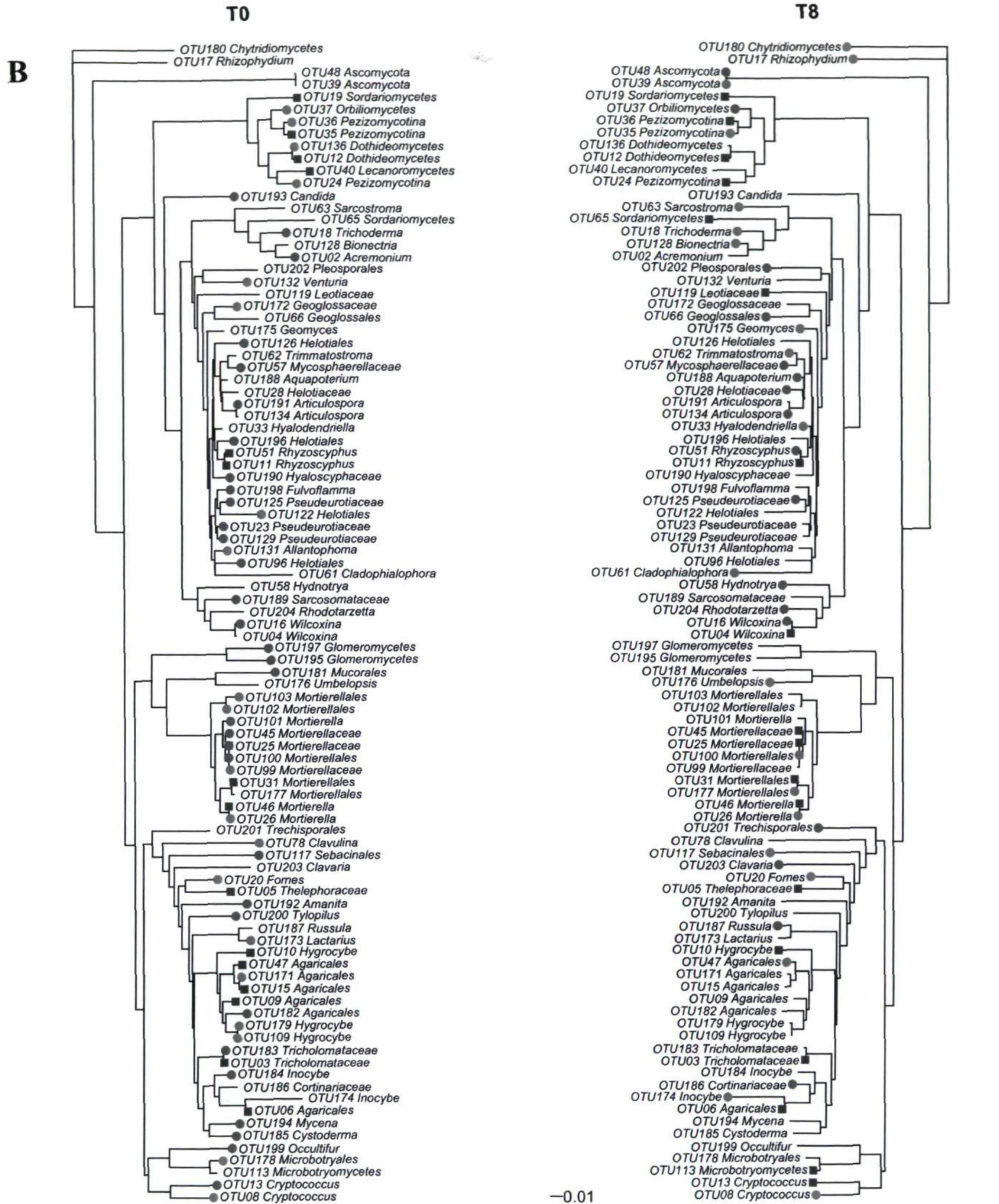


Fig. S4.2 Phylogenetic structure of the fungal communities found in samples from control and Et white spruces between T0 and T8 in the organic (A) and mineral (B) soil types.



- OTUs specific to libraries from controls
- OTUs specific to libraries from Et White spruces
- ▪ OTUs in libraries from controls and Et white spruces

Fig. S4.2 Continued

CONCLUSION GÉNÉRALE

L'objectif de la thèse était d'évaluer l'impact potentiel des AGM sur les champignons non cibles du sol en général, et les champignons mycorhiziens en particulier. Contrairement à l'agriculture, la sylviculture est intergénérationnelle et ce, même à l'époque des biotechnologies. En dépit d'une forte augmentation du nombre d'essais au champ d'arbres transgéniques ces dernières années, l'application des biotechnologies au secteur forestier ne se compare pas, en terme de développement et de commercialisation, au secteur agricole. Il était donc impératif, dans un premier temps, de faire le point sur les interactions des champignons avec les cultures et les arbres transgéniques afin d'avoir une vision intégrale de l'impact des PGM sur les champignons.

Alors, qu'en est-il ? L'analyse de la littérature scientifique publiée depuis une vingtaine d'années sur l'interaction champignons / plantes transgéniques montre qu'il y a un décalage entre les PGM testées et celles couramment cultivées aujourd'hui en agriculture ou en foresterie, aussi bien en terme d'essences que de caractères transgéniques exprimés. À la lecture de ces études présentées dans le chapitre 1, on s'aperçoit également que la plupart des expériences évaluent les effets des PGM sur les champignons en conditions contrôlées. Enfin, les études s'intéressant aux conséquences sur les champignons non ciblés par le nouveau caractère exprimé chez les PGM représentent 25 % et 50 % des expériences impliquant des plantes agricoles et forestières, respectivement. En résumé, environ cinq travaux de recherche par année s'intéressent, en général loin des conditions naturelles, aux effets sur les champignons non cibles de PGM qui ne sont pas représentatives en terme d'essences et de caractères transgéniques de celles qui sont ou seront utilisées en agriculture et foresterie. En 2000, la revue *Science* publiait un article de Jose Domingo[3] au sujet des risques sanitaires des OGM intitulé : « Risques sur la santé : beaucoup d'opinions mais peu de données » (traduction libre). Il en va de même des risques sur la flore fongique et donc sur l'environnement, en 2010.

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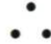
[3] Domingo, J. 2000. Health risks of GM foods: many opinions but few data. Science 288: 1748-1749.

Parmi les études évaluant l'impact des PGM sur les champignons non cibles, 30% rapportent des changements significatifs par comparaison aux témoins. Par changement, il faut entendre une diminution ou une augmentation de la colonisation et du développement du ou des champignon(s) non cible(s) étudié(s) en contact direct ou indirect avec les PGM. Cependant, plusieurs de ces expériences, réalisées en conditions extérieures, montrent que les changements induits par la culture des PGM sur la diversité des communautés fongiques et leur structure sont moindres que ceux résultant d'un effet de site (type de sol), d'un changement de cultivar (génotype), du stade de développement des plantes ou des conditions environnementales. Ces études ont en commun de recourir aux techniques moléculaires telles que DGGE, PCR-RFLP, clonage et séquençage de l'ADN fongique du sol. L'avènement des méthodes de séquençage direct et massif devrait permettre un suivi encore plus fin et systématique des communautés fongiques dans le cas des études de l'impact des PGM en milieu naturel sur les champignons non cibles. Lorsqu'on regarde la nature des caractères transgéniques exprimés par les PGM impliquées dans les études rapportant des changements significatifs sur les champignons non cibles, on constate qu'aucun de ces caractères nouveaux ne pouvaient laisser entrevoir *a priori* une quelconque causalité de leur expression avec les changements observés sur les champignons non cibles. Mieux, bien souvent les effets fluctuent d'une lignée transformée à une autre jusqu'à s'opposer et peuvent ne pas être corrélés avec les niveaux d'expression du transgène. L'insertion aléatoire du transgène dans le génome de la plante-hôte est la plus souvent mise en cause. Cette incertitude explique pourquoi les PGM sont soumises à l'application du principe de précaution dans certains pays. Le philosophe du risque, François Ewald, définit le principe de précaution comme « *la mise en place par les États d'outils juridiques pour la gestion des risques environnementaux afin de faire face à des situations où manque la connaissance d'une relation causale certaine entre une pollution et une substance* ».

La revue de littérature du chapitre 1 montre bien le déficit de données concernant l'impact des AGM sur les champignons non cibles. Les chapitres 2, 3 et 4 de cette thèse apportent de nouvelles données aussi bien au sujet de l'impact des AGM sur les champignons que sur les méthodes d'investigation des communautés fongiques du sol.



Les résultats du chapitre 2 proviennent de l'analyse du premier essai au champ autorisé au Canada de peupliers transgéniques. Ce dispositif offrait l'opportunité d'évaluer les effets potentiels sur la communauté des ectomycorhizes (EM) colonisant des peupliers transgéniques après huit années d'interaction. Les peupliers du dispositif étaient génétiquement modifiés pour exprimer un gène marqueur (*npt*II) et un gène rapporteur (*GUS*), ce dernier sous le contrôle d'un promoteur inductible par blessure. De ce fait, l'hypothèse testée ne s'attendait pas à voir de différence entre la communauté des EM associée aux arbres non transformés et celle associée aux peupliers transgéniques. Cette hypothèse nulle fut vérifiée et confirmait que le produit de l'expression du gène *npt*II en particulier, couramment utilisé en transgénèse végétale, était sans danger pour les EM. Par ailleurs, dans le cas de la lignée étudiée, l'insertion des transgènes dans le génome des peupliers n'a pas généré d'effets pléiotropiques aux conséquences délétères pour les EM, ni pour les arbres transformés.

L'effort d'échantillonnage et l'effort de séquençage ont permis d'atteindre le niveau de saturation de la diversité des EM, fait rare parmi les études s'intéressant à la diversité des champignons du sol. La saturation de la diversité est un prérequis important (mais pas toujours réalisable) quand il s'agit de comparer deux ensembles. La comparaison de la diversité des EM colonisant les extrémités racinaires avec la diversité des EM obtenue par clonage et séquençage de l'ADN fongique du sol montre que les deux méthodes d'échantillonnage sont complémentaires. Le fait que, prises séparément, les deux approches d'échantillonnage présentaient une diversité saturée, montre clairement le biais méthodologique de la méthode sur le résultat. Lorsque le jeu de données des séquences ITS des extrémités racinaires a été fusionné avec celui des banques de clones, le nombre d'unités taxonomiques opérationnelles d'EM (défini au seuil de dissimilitude de séquence de 2%) augmentait de 22% et 48% par rapport aux nombres d'UTO obtenus par chacune des deux méthodes d'échantillonnage, respectivement. La combinaison des deux approches nous a permis d'estimer à 50 le nombre d'espèces d'EM potentiel colonisant le dispositif expérimental, une richesse spécifique supérieure à celles observées dans de nombreuses autres études sur la diversité des EM. La comparaison de l'efficacité des deux approches

pour identifier la diversité des EM présente dans le sol de la plantation étudiée, montre que l'analyse des extrémités racinaires est plus performante. En effet, l'identification des EM colonisant les extrémités racinaires des peupliers a défini une richesse spécifique des EM plus importante que l'approche du clonage et séquençage des régions ITS amplifiées à partir de l'ADN fongique du sol. De plus, quatre UTO d'EM communément retrouvées sur les extrémités racinaires des peupliers étaient absentes des banques de clones fongiques. Ainsi, la détection de certaines espèces d'EM est sensible à la méthode utilisée.

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La revue de littérature du premier chapitre le met clairement en évidence, la majorité des études faisant le suivi de l'interaction champignons / PGM, se limitent à vérifier si des plantes transformées avec des gènes conférant une activité antifongique ou antimicrobienne sont plus résistantes aux champignons pathogènes. Peu d'études se sont intéressées à leur impact sur les champignons non pathogènes, que l'on peut qualifier de non cibles. Or, dans le chapitre 3, l'impact potentiel d'épinettes blanches transformées pour surexprimer de l'endochitinase a été étudié sur la biomasse fongique du sol et, particulièrement sur la symbiose ectendomycorhizienne. Cette symbiose joue un rôle important dans l'établissement des semis de conifères, surtout en milieu perturbé. Les épinettes ont été cultivées pendant cinq années en serre dans un sol artificiel à base de tourbe avant que ne soient collectées les données. L'hypothèse de recherche proposait que la biomasse fongique ainsi que la symbiose ectendomycorhizienne seraient limitées par l'activité enzymatique endochitinase des épinettes transformées. Cette activité mesurée dans les racines et les exsudats racinaires était significativement plus élevée dans les lignées transformées que dans les arbres témoins. Si la quantification de la biomasse fongique du sol s'est faite par la classique méthode de mesure des ergostérols en HPLC, une approche originale basée sur la quantification de l'ADN par PCR en temps réel a été développée afin de mesurer et comparer le développement de la symbiose ectendomycorhizienne sur les extrémités racinaires des épinettes transformées et non transformées. Les résultats des expériences ont rejeté l'hypothèse d'un effet délétère sur la biomasse fongique du sol et la symbiose ectendomycorhizienne, alors même qu'il a été observé que le développement de champignons pathogènes était limité pour des niveaux comparables d'activité de

l'endochitinase[4]. Jusqu'ici, aucune étude n'a rapporté des effets négatifs sur les champignons non cibles impliquant des plantes transformées afin d'améliorer leur résistance aux maladies d'origine fongique.

D'un point de vue méthodologique, l'analyse du développement de la symbiose ectendomycorhizienne sur les extrémités racinaires par PCR en temps réel s'est révélée être une approche précise afin d'évaluer l'effet des traitements. Pour chaque échantillon d'extrémités racinaires, le nombre de molécules de *Wilcoxina* spp. a été comparé au nombre de molécules d'épinettes, ces deux valeurs étant logiquement corrélées positivement. Peu d'études ont eu recours au PCR en temps réel pour quantifier le développement des symbioses mycorhiziennes et toutes n'utilisaient que des amorces ciblant les régions ITS de l'ADNr. Or, trois régions du génome nucléaire ont été ciblées pour quantifier *Wilcoxina* spp. : la région ITS et les gènes codant pour le facteur d'élongation-1 alpha et la beta-tubuline. Les données de quantification obtenues avec l'ITS ont été rejetées car trop variables et donc moins précises que celles obtenues par l'amplification des gènes codants. Enfin, l'analyse par PCR en temps réel du morphotype 2, le plus abondant observé sur les extrémités racinaires, a révélé la présence de *Wilcoxina* spp. et *P. fortinii* alors qu'aucune molécule de ce dernier n'avait été mesurée sur le morphotype 1, clairement identifié comme *Wilcoxina* spp. La forte diminution du nombre de molécules correspondant à *Wilcoxina* spp., l'abondance relative de *P. fortinii* et l'aspect sénescent du second morphotype appuient l'hypothèse que *P. fortinii* agirait comme un mycoparasite de *Wilcoxina* spp. D'autres études ont également permis d'observer la présence de *P. fortinii* sur des racines sénescentes de conifères. Ainsi, l'utilisation du PCR en temps réel offre l'opportunité de mieux comprendre la dynamique des champignons mycorhiziens colonisant les extrémités racinaires des plantes.

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Les résultats du chapitre 3 ont permis de conclure que la surexpression de l'endochitinase au niveau des tissus et des exsudats racinaires des épinettes transgéniques n'affectait ni la

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[4] Noël, A., C. Levasseur, V. Q. Le, and A. Seguin. 2005. Enhanced resistance to fungal pathogens in forest trees by genetic transformation of black spruce and hybrid poplar with a *Trichoderma harzianum* endochitinase gene. Physiol. Mol. Plant Pathol. 67:92-99.

biomasse fongique du sol artificiel dans lequel les épinettes étaient cultivées, ni le développement de la symbiose ectendomycorhizienne. Du fait du déroulement de l'expérience en conditions contrôlées, le spectre des champignons interagissant avec les épinettes transgéniques était très réduit par rapport à un dispositif en milieu naturel. Quels seraient les effets potentiels de ces épinettes sur les communautés fongiques complexes que l'on peut retrouver dans des sols forestiers ? C'est à cette question que les travaux présentés au chapitre 4 ont tenté de répondre. Ainsi, les épinettes transgéniques et les témoins de l'étude présentée dans le chapitre 3 ont été transplantés dans deux types de sol prélevés en milieux forestiers. La biomasse et la diversité fongique des échantillons de sol ont été évaluées au moment de la transplantation puis après huit mois d'interaction avec les arbres, par HPLC et clonage / séquençage des régions ITS de l'ADNr. Seules les valeurs de l'indice de Chao des échantillons de sol associés aux témoins étaient significativement supérieures à celles des échantillons de sol associés aux épinettes transgéniques, après huit mois en serre. L'indice de Chao est une estimation de la richesse spécifique totale attendue qui considère le ratio des singletons sur celui des doubletons comme représentatif des espèces non détectées. La différence du nombre de singletons était mineure dans la mesure où il n'y avait pas de différence significative concernant les valeurs de la richesse spécifique fongique, les valeurs des indices de Shannon et de Rao ainsi qu'entre la structure phylogénétique des communautés provenant des échantillons de sols associés aux épinettes transgéniques et ceux associés aux témoins. Il n'est pas possible de déterminer si cette différence observée dans les valeurs de Chao relève simplement d'aléas expérimentaux ou bien s'il y a une explication biologique. La diversité fongique provenant des échantillons du sol organique était plus proche de la saturation que celle des échantillons du sol minérale. Est-ce que les valeurs de Chao auraient été différentes entre transgéniques et témoins si la diversité fongique des deux sols avait été complètement saturée ? Quels auraient été les résultats avec un dispositif au champ ?

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Les évidences d'effets délétères des AGM sur les champignons sont clairsemées. Les trois chapitres de recherche présentés dans cette thèse ne rapportent l'observation d'aucun effet sur la flore fongique incriminant les AGM, dans les conditions expérimentales propres à

ces études. Cependant, les expériences rapportant des changements significatifs sur les champignons non cibles induits par des cultures agricoles transgéniques montrent que les caractères d'origine transgénique exprimés étaient *a priori* sans effet sur les champignons non cibles. Cela témoigne de l'incertitude qui caractérise les conséquences environnementales des PGM ; de l'incertitude naît le risque.

L'ensemble des expériences réalisées à ce jour sur l'interaction entre champignons et PGM montre un certain nombre d'insuffisances qui ne permettent pas d'établir clairement les effets des PGM sur les champignons. Ainsi des recommandations peuvent être formulées afin de palier à ces insuffisances et augmenter la portée des résultats :

- Il semble évident que les études d'impact des PGM sur les champignons devraient principalement se faire sur les plantes transgéniques commercialisées ou ayant un avenir en agriculture ou foresterie. Un outil de coordination internationale des recherches sur l'impact des PGM sur les organismes non cibles serait utile en ce sens.
- La caractérisation des conséquences potentielles des PGM sur les champignons (ou tout autre organisme non cible) doit suivre nécessairement un processus se déclinant en deux phases, avec des expériences en conditions contrôlées mais également en conditions naturelles. Par ailleurs, chaque lignée doit être étudiée.
- Un suivi à long terme, en conditions naturelles, doit être réalisé afin d'étudier les effets potentiels des PGM sur les champignons non cibles lorsqu'elles sont confrontées aux aléas des facteurs environnementaux.
- Afin d'établir clairement le lien de causalité entre les variations éventuelles observées sur les champignons non cibles et la présence des PGM, mais aussi afin de relativiser ces variations, les dispositifs expérimentaux doivent prévoir d'inclure l'étude de variétés végétales génotypiquement proches des témoins.

ANNEXE

Evaluation of foliar fungal endophyte incidence in field-grown transgenic *Bt* white spruces trees

Avant-propos

L'article suivant a été publié dans la revue Botanique (anciennement revue canadienne de botanique) en 2006. Cette étude demeure la seule publiée à ce jour sur l'impact des plantes génétiquement modifiées sur la communauté des champignons endophytes foliaires. Les échantillons ont été récoltés durant l'été 2002. Les données ont été analysées et l'article a été rédigé au cours de la première année de thèse (hiver 2004 - automne 2005).

Evaluation of foliar fungal endophyte incidence in field-grown transgenic *Bt* white spruce trees

F.O.P. Stefani and J.A. Bérubé

Abstract: A total of 770 transgenic *Bt* white spruce needles were collected and plated on potato dextrose agar to determine their foliar endophyte diversity. The ribosomal internal transcribed spacer regions for 310 foliar endophytes were amplified by polymerase chain reaction (PCR) and digested using *Cfo*I and *Msp*I, which created 21 restriction groups. Isolates from each restriction group were sequenced and compared with reference sequences in GenBank. Eighteen sequence groups were obtained, of which five were identified at the species level. The most common endophytic fungi identified by PCR-RFLP was *Lophodermium piceae* (incidence of 74.5%). The second and third most common ones were *Hypoxylon fragiforme* (3.63%) and *Lophodermium nitens* (3.18%). A statistical analysis performed on the most common endophyte groups showed no statistical difference in endophyte frequency or distribution between the control white spruce needles (nontransgenic) and saplings with constructs containing the reporter gene GUS or the *Bt Cry1A(b)* gene and kanamycin.

Key words: endophytic fungi, kanamycin, ITS, *Bt Cry1A(b)*, *Lophodermium piceae*, RFLP.

Résumé : Sept cent soixante-dix aiguilles d'épinette blanche *Bt* ont été échantillonnées et mises en culture sur PDA, afin de définir la diversité des champignons endophytes foliaires. Les régions ITS de l'ADN ribosomique de 310 endophytes foliaires ont été amplifiées par PCR et digérées avec les endonucléases *Cfo*I et *Msp*I. Vingt et un groupes de restriction ont ainsi été créés. Des isolats de chaque groupe de restriction ont été séquencés et comparés à des séquences de référence de GenBank. Nous avons obtenu 18 groupes de séquences, parmi lesquels 5 ont pu être identifiés au niveau spécifique. Les champignons endophytes les plus communs identifiés sont *Lophodermium piceae* (fréquence de 74,5 %), *Hypoxylon fragiforme* (3,63 %) et *Lophodermium nitens* (3,18 %). L'analyse statistique réalisée sur la distribution et la fréquence du champignon endophyte le plus commun ne montre pas de différence significative entre les aiguilles d'épinette blanche témoins (non transformée) et les aiguilles des semis transformés avec le gène rapporteur GUS ou le transgène *Cry1A(b)* et la kanamycine.

Mots clés : champignons endophytes, kanamycine, ITS, *Bt Cry1A(b)*, *Lophodermium piceae*, RFLP.

Introduction

Transgenic trees with genes of interest are beginning to be field tested for efficacy and environmental impacts. Since the first report on genetic transformation of poplar (Fillatti et al. 1987), major advances in genetically engineered trees have been made (Peña and Séguin 2001) and several transgenic tree species are being field tested throughout the world (Doering 2001). Some of the most interesting genetic transformations of trees are the modification of lignin biosynthesis to increase pulp quality (Eriksson et al. 2000; Pilate et al. 2002), growth enhancement (Jing et al. 2004; Shani et al. 2004) and production of trees resistant to insect or fungal pests (Hu et al. 2001; Delledonne et al. 2001; Liang et al. 2001; Pasonen et al. 2004). The use of genetically modified organisms in agriculture has raised some concerns and opposition from the public. The use of genetically transformed trees remains experimental in most countries except in China, which planted over 1 million genetically modified trees in its reforestation initiative (ISIS 2005).

The expression of the *Bacillus thuringiensis* (*Bt*) crystal insecticidal protein (δ-endotoxin) genes (*cry* genes) within tree genomes was largely investigated to confer pest resistance (Tzfira et al. 1998; Schuler et al. 2001). *Bt* toxins produced from *Cry1A(b)* activity are toxic against Lepidopteran, Dipteran, and Coleopteran insects (Höfte and Whiteley 1989). *Picea glauca* (Moench) Voss transformed with *Cry1A(b)* genes has been produced and field deployed to test efficacy (Lachance et al. 2001). The *Cry1A(b)* transgene introduced within the *Picea glauca* genome is the same one used to transform rice (Cheng et al. 1998). *Bt* white spruces are among the first transgenic conifers deployed in a natural habitat and can be used to study the impact of transgenic constructs on nontarget organisms, such as insects, bacteria, mycorrhizae, and fungal endophyte communities. The impact of transgenic white spruce on nontarget organisms has never been measured. We investigated the environmental impact of the insertion of the *Bt* transgene on endophytic fungi living inside needles of *Bt* transgenic white spruce. Endophytic fungi live inside healthy plant tissues

Received 13 April 2006. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 28 November 2006.

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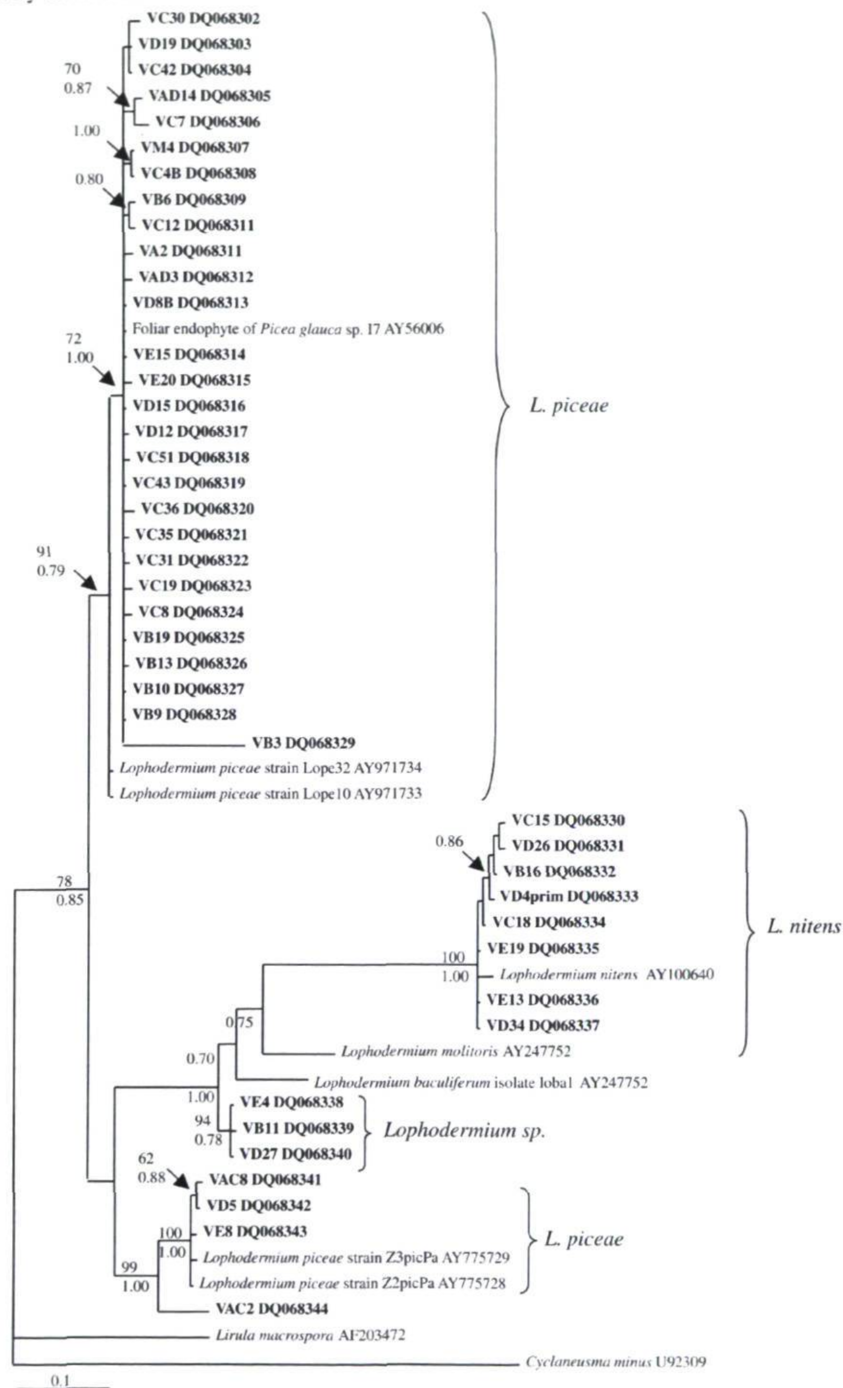
 doi:10.1139/B06-110 © 2006 NRC Canada

Fig. 1. Phylogenetic tree of foliar endophytes of white spruce based on the analysis of ITS1-5.8s-ITS2 rDNA sequences of all strains sequenced and belonging to the Rhytismatales class. Both Bayesian and maximum parsimony analysis was performed. Posterior probability values are given below branches. Bayesian values below 0.70 are not shown. Tree topology and values come from 2 million generations. Sequences from the study are in bold.



and are asymptomatic on the host. Their role and function remains unclear, but they have been shown to provide some protection against pests (Johnson and Whitney 1994).

The purpose of this experiment is to quantify foliar fungal endophyte frequencies and distribution in transformed and nontransformed white spruce saplings planted in a field test

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to determine the impact of the *CryIA(b)* construct on these nontarget organisms.

Materials and methods

Sampling

The sampling site is located at the Canadian Forest Service Valcartier field station (Quebec, Canada), in the balsam fir – white birch bioclimatic domain. The plot is surrounded mainly by a mix of mature white pine (*Pinus strobus*) and red pine (*Pinus resinosa*). The daily mean air temperature is 4.04 °C and the annual total precipitation is 1230.3 mm (Quebec/Jean Lesage station, elevation 74.4 m, 30 years of records between 1971 and 2000). A transgenic plantation of *Picea glauca* was begun in June 2000. There were 240 transgenic saplings at 1 m × 1 m spacing, randomized inside eight different blocks. In June 2002, we sampled 77 five-year-old white spruce saplings from 11 clonal lines, randomly distributed into eight blocks. Two clonal lines were untransformed regenerated *P. glauca* cell lines, one clonal line was transformed with the reporter β-*glucuronidase* (*GUS*) gene and eight clonal lines were transformed with the *Bt CryIA(b)* gene. Each construct also contained the selection gene for kanamycin. One sapling from every randomized block was sampled for each clonal line. From each sapling, 5 healthy needles from 2000 and 5 needles from 2001 were randomly collected, put into sterile tubes, and kept on ice until treated.

Endophyte isolation

White spruce needles were sterilized by dipping them in different sterilizing solutions. The surface sterilization protocol was as follows: 2 min in a 200 mL solution of 5.25% sodium hypochlorite (diluted 1:5) with one drop of Tween 80, 1 min in a 200 mL solution of 70% ethanol (diluted 1:1.25) with one drop of Tween 80, and 1 min in a 200 mL solution of 70% ethanol (diluted 1:5). Then needles were rinsed by dipping them in three different baths of sterilized distilled water for 1 min each (Arnold et al. 2001). Foliar endophytes were then isolated by plating one surface-sterilized needle per Petri dish on potato dextrose agar (PDA; Difco, Detroit, Mich.). All endophytes growing from the same needle were individually subcultured in another Petri dish on PDA. Petri dishes were incubated at room temperature for 8 weeks. All fungal colonies appearing after 7 d were considered to be endophytes. Colonies appearing within 1–6 d after plating, epiphytes, yeast-like colonies, and contaminants with no physical links to the needle were not considered to be endophytes and were subtracted. Then, endophytes were classified into morphological groups based on colour, texture, and morphology features to simplify restriction analysis. These morphological groups were then subjected to DNA extraction, PCR-RFLP, and sequencing.

DNA extraction and PCR

The resulting morphospecies were directly sampled from the Petri dishes. A small piece of agar (10 mm^2) with mycelium was subjected to DNA extraction using the CTAB procedure modified from Zolan and Pukkila (1986). DNA was extracted with CTAB (2% mercapto-ethanol added), purified with 600 μL of phenol – chloroform – isoamyl alcohol, and centrifuged at 10 000 r/min (1 r = 2π rad) for 10 min. Supernatants were transferred into new tubes and precipitated with 600 μL of cold isopropanol, centrifuged at 5000 r/min for 10 min and resuspended with 25 μL of TE-8 buffer. Genomic DNA was diluted 1:10 and stored at –20 °C until PCR amplification.

The internal transcribed spacer (ITS) of the nuclear ribosomal gene was amplified using ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990) primers. The PCR reaction mixture included 14.8 μL of ultra pure distilled water (Dnase, Rnase free, GIBCO), 20 mmol/L TRIS (pH 8.4), 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.25 mmol/L of each dNTP, 1 μmol/L of each ITS primer, and 1 unit of *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). Three microliters of genomic DNA 1:10 were added for a total volume of 25 μL per reaction.

DNA amplification was done on a MJ Research PTC-100 thermocycler (MJ Research Inc., Watertown, Mass.) with a program as follows: 3 min at 95 °C, 40 cycles for denaturation at 92 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and a last elongation step at 72 °C for 10 min. Amplicons were electrophoresed on 1.5% TAE 1× agarose gels, run at 110 V for 1 h. Ethidium bromide was used for coloration and UV light for visualization.

DNA restriction

Each ITS amplicon was directly digested with two endonucleases, *Cfo*I and *Msp*I (Roche Diagnostics), to obtain species-specific patterns. The enzymatic mixture contained 0.05 μL *Cfo*I (2 units per reaction), 0.05 μL *Msp*I (2 units per reaction), 0.5 μL Sure/cut buffer L for restriction enzymes, and 0.4 μL H_2O. One microlitre of the enzymatic mixture was added directly to the PCR reaction mix. Samples were digested for 1 h at 37 °C. Following digestion, fragments were separated on 3.75% TAE agarose gels, and run at 80 V for 1.5–2 h. Ethidium bromide was used for staining and UV light for visualization. Isolates with similar restriction patterns were lumped into restriction groups.

DNA sequencing and analysis

Samples to be sequenced were purified with QIAquick PCR Purification Kit (QIAGEN, Rockville, Maryland) and sequenced on a Genetic Analyzer 3100 (Applied Biosystems) sequencer. Amplicons were sequenced single-stranded. The second strand was sequenced when DNA sequences presented discrepancies. Chromatograms were checked with Chromas version 2.3 (Technelysium Pty. Ltd.) to solve oligonucleotide ambiguities. Using the BLASTn algorithm, we searched for sequences of reference species in GenBank similar to our sequenced isolates to perform a molecular identification for these nonsporulating endophytes.

The most similar sequences found were added to our sequence matrices to align them using the ClustalW algorithm (Thompson et al. 1994) implemented in Megalign version 5.05 (DNASTAR, Madison, Wisconsin), with the default parameter settings. ITS sequence alignments were edited with GeneDoc version 2.6.002 (Nicholas et al. 1997). They were then subjected to a contig analysis using Sequencher version 4.1.4 (GeneCodes, Ann Arbor, Michigan).

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Table 1. Distribution of the 18 endophytic sequence groups identified from molecular analysis among the three control lines (PG-563, PG- and NCBI accession numbers.

	Control white spruce		*GUS*	Transgenic white spruce					
Sequence group	PG-653	PG-99	1-33*	2-19S	2-39W	2-4C	3-3C	1-24C	2-3S
1	15	18	21	10	21	18	17	11	12
2	0	0	0	0	1	0	2	2	1
3	0	2	1	0	1	0	2	0	0
4	0	1	0	0	0	1	4	0	0
5	0	1	1	0	0	0	1	0	3
6	0	1	0	1	1	0	0	0	0
7	0	0	0	0	1	1	0	1	1
8	0	1	0	0	1	0	0	1	0
9	0	0	1	1	0	0	1	0	0
10	0	0	0	0	0	2	0	0	0
11	1	0	0	0	0	0	0	0	0
12	0	0	0	0	1	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	1	0	0	0	0	0
15	0	1	0	0	0	0	0	0	0
16	0	0	1	0	0	0	0	0	0
17	0	0	0	0	0	0	1	0	0
18	0	0	0	0	0	0	1	0	0
Total isolates per clonal line	16	25	25	13	27	22	29	15	17
Average no. of isolates per clonal line	22			19					
Total no. of taxa per clonal line	2	7	5	4	7	4	8	4	4
Average no. of taxa per clonal line	4.7			5.2					

Note: PG-653 and PG-99 lines are nontransgenic trees. The 1-33* line is *GUS* transformed.

Phylogenetic analysis

First, we determined the nucleotide substitution model that best fit with our data using Modeltest (Posada and Crandall 1998). Bayesian posterior probability analyses were performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist 2001). We programmed MrBayes to analyze each contig matrix with the best model of sequence evolution determined by Modeltest, running four MC^3 over 2 million generations, sampling trees every 100th generation (20000 trees saved). Prior probability was equal for all trees. Starting tree was random and "burnin value" was set to 2000 (10% trees excluded). Bayesian analysis was performed three times for each matrix to evaluate the reproducibility of tree topologies and MC^3 values.

Phylogenetic relationships were also investigated using PAUP version 4.8b10 (Swofford 2002). Support for the branching topologies was evaluated using bootstrap analysis (Felsenstein 1985). Parsimony analysis was performed using a heuristic search with the following parameters: characters were unordered and had equal weight, the maximum number of trees was set at 1000, and we selected TBR (tree bisection–reconnection) branch-swapping (multrees in effect). For the Rhytismatales tree, bootstrap analysis was performed using 200 replicates with the maximum number of trees set at 100. *Lirula macrospora* (AF204372) and *Cyclasneusma minus* (U92309) were selected as outgroups. Restriction groups with similar sequences and supported by Bayesian inference were lumped into the same sequence group along with reference species to provide taxonomic identification based on sequence.

Statistical analysis

To compare the number of foliar endophyte isolates per clonal line, number of taxa per clonal line, and Shannon

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99, 1-33*) and the eight transgenic lines (2-19S to 2-26W), incidences of each sequence group, and GenBank identification

1-4C	2-26W	Incidence	GenBank closest sequences	Reference isolate	NCBI accession No.
6	15	74.54	*Lophodermium piceae*	VC-12	DQ068310
1	1	3.63	*Hypoxylon fragiforme*	VK-3	DQ068345
0	1	3.18	*Lophodermium nitens*	VE-19	DQ068335
0	0	2.73	*Lophodermium* sp.	VD-27	DQ068340
0	0	2.73	*Mycosphaerella* sp.	VG-5	DQ068347
1	1	2.27	*Rosellinia quercina*	VI-7	DQ068350
0	0	1.81	*Septoria passerinii*	VL-4	DQ068346
1	0	1.81	*Altenaria* sp.	VH-2	DQ068348
0	0	1.36	*Phialophora* sp.	VT-2	DQ068359
0	0	0.91	*Phomopsis* sp.	VV-2	DQ068349
0	1	0.91	*Hypoxylon* sp.	VBeta-1	DQ068358
1	0	0.91	Ascomycota	VF-6	DQ068356
0	2	0.91	Helotiales	VTeta-2	DQ068357
0	0	0.45	*Nodulisporium* sp.	VC-21	DQ068351
0	0	0.45	*Phaeosphaeria* sp.	VE-10	DQ068352
0	0	0.45	Xylariales	VE-9	DQ068353
0	0	0.45	Hypocreales	VR-4	DQ068354
0	0	0.45	*Phoma* sp.	VR-6	DQ068355
10	21				
5	6				

and Simpson diversity indices between control and transgenic saplings, a mixed generalized linear model was defined according to Brown and Prescott (1999) and the binomial observation unit was the tree. Its parameters were estimated using the maximum likelihood method, with the NLMIXED procedure in SAS (SAS Institute Inc. 1999). Then a nested ANOVA in the MIXED procedure in SAS was performed. As the *GUS*-transformed line can be considered as a control to the *Bt*-transformed lines, the analysis was done twice, once comparing the nontransformed controls with the *Bt*-transformed clonal lines, and the second time adding the *GUS*-transformed line data to the nontransformed controls against the *Bt*-transformed clonal lines.

Distribution of the endophyte species identified using ITS sequences was analyzed for differences within and between the 11 clonal lines using the GENMON procedure on SAS 8.02 (SAS Institute Inc. 1999). A binomial model was built to predict probability of infection by an endophyte and the null hypothesis was that clonal lines had an effect on this probability. The p value was corrected by an over-dispersion coefficient and compared with the uncorrected p value to test the fit of our model to the binomial distribution.

Results

Isolation and grouping

Ten needles were sampled from each of 77 seedlings and plated on 770 Petri dishes. After a growing period of 8 weeks, we recorded 341 fungal isolates with endophytic features. The colonization rate was 38.9% with 297 needles colonized, 258 needles by one endophyte and 39 by two or more morphologically different endophytes. Among these 341 isolates, 310 isolates were successfully amplified and digested with restriction enzymes. A total of 206 isolates

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Table 2. Shannon and Simpson diversity indices.

	Control white spruce			Transgenic white spruce							
	PG-653	PG-99	1-33	2-19S	2-39W	2-4C	3-3C	1-24C	2-3S	1-4C	2-26W
Shannon diversity index	0.233	1.082	0.661	0.793	0.927	0.663	1.419	0.857	0.885	1.227	1.044
Average	0.66			0.98							
Simpson diversity index 1-D	0.117	0.467	0.288	0.390	0.386	0.318	0.623	0.435	0.463	0.6	0.471
Average	0.29			0.46							

were distributed within 21 restriction groups and 104 isolates presented a unique restriction pattern. The number of isolates for each restriction group varied between 2 and 118. On average, 40% of the isolates from each restriction group were randomly chosen to be sequenced and 25% of the isolates with unique restriction patterns were randomly chosen to be sequenced. In total, 69 isolates were sequenced. Finally, the combination of restriction analysis and sequencing allowed us to perform a molecular identification for 220 isolates.

Molecular identification

The sequenced isolates were classified into 18 sequence groups. Each sequence group was identified using a Bayesian–PAUP dendrogram as demonstrated for the genus *Lophodermium* in Fig. 1. Among these 18 sequence groups, 5 were identified to the species level, 9 were identified to the generic level, and 4 were identified only to order or higher levels (Table 1).

The most common endophyte sequence group with an incidence of 74.5% showed great similarity to the *Lophodermium piceae* sequence from GenBank (Fig. 1). The second and third most common fungal endophytes were *Hypoxylon fragiforme* (with an incidence of 3.63%) and *Lophodermium nitens* (incidence of 3.18%) followed by isolates with similarity to *Lophodermium* sp. (2.73%), *Mycosphaerella* sp. (2.73%), *Rosellinia quercina* (2.27%), *Septoria passerinii* (1.81%), and *Alternaria* sp. (1.81%). Finally, 10 other taxa were found with an incidence below 1.5% (Table 1).

Statistical analysis

Table 1 shows the distribution of the 18 endophyte taxa among the 11 clonal lines. The analysis comparing the controls with and without the *GUS* added gave similar values (results not shown), hence we present here only the data of the controls with *GUS* against the *Bt*-transformed clonal lines. The number of isolates per clonal line (Table 1) varied between 10 and 29, with an average of 22 for controls with *GUS* and 19 for *Bt* transgenic spruces, which is not a statistically significant difference (F = 0.4, df = 1, p = 0.54). Similarly the number of taxa per clonal line (Table 1) varied between 2 and 8, with an average of 4.7 for controls with GUS and 5.2 for *Bt* transgenic spruces, again not a statistically significant difference (F = 0.22, df = 1, p = 0.649).

Shannon (F = 2.55, df = 1, p = 0.145) and Simpson (F = 4.09, df = 1, p = 0.0737) diversity indices were also not statistically different between controls-GUS and *Bt* transgenic saplings (Tables 2 and 3).

Statistical analysis of endophyte distribution using the GENMON procedure for the 11 seedling lines was only possible for endophyte isolates with sequence similarity to *L. piceae*. The frequencies of the other endophytes found in this study were too low and their distribution too patchy to allow statistical analysis. The observed presence of endophytes with sequence similarity to *L. piceae* in a seedling line varied from 0.084 to 0.284, and we did not observe a significant difference between lines (p = 0.3075) (Table 3). The uncorrected p value was similar (p = 0.283), indicating an effective binomial distribution.

Table 3. Nested ANOVA for number of isolates and taxa per clonal line, Simpson and Shannon indices, and GENMON analysis of *L. piceae* distribution.

	F	df	p
No. of isolates/clonal line	0.40	1	0.54
No. of taxa/clonal line	0.22	1	0.649
Simpson indices average	4.09	1	0.0737
Shannon indices average	2.55	1	0.145
Endophyte distribution difference (*L. piceae*), GENMON analysis	—	—	0.3075

Discussion

The endophyte species found inside needles of *Bt* transgenic white spruce and the observed incidence correspond to results from a similar study done on natural white spruce populations (Stefani and Bérubé 2006). In that study, *L. piceae* was found to be the most common endophyte with an incidence of 75.15%; many minor species were found in both studies.

Two main differences were observed between endophyte diversity from *Bt* transgenic white spruce saplings and diversity from natural white spruce populations. The first is a variation of 14.3 percentage points between the colonization rate observed for needles from natural white spruce stands (53.2%) and the colonization rate from the present study (38.9%). This difference may be explained by the fact that transgenic and control white spruces, produced from somatic embryogenesis, had been in contact with a natural environment for just 2 years when the needles were sampled. Natural endophytic flora probably takes several years to establish within needles. The second difference is the presence of *L. nitens* in *Bt* white spruce. This *Lophodermium* species is usually only found in pine needles and never in spruce needles (Stefani and Bérubé 2006; Sokolski 2005). We suspect that its presence inside needles of the *Bt* and control white spruce saplings is due to plantation features. A mature forest composed mainly of white pines and red pines surrounds the plot. These pines must have served as a source of inoculum for these young saplings, introducing a foreign endophyte flora that would likely disappear as the white spruce saplings mature.

We did not observe a significant difference in the coloni-

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zation rate of endophyte isolates, the number of taxa, and the distribution of endophytes among the transgenic *Bt* lines, the transgenic *GUS* line, and the control lines. For the sampling effort and the analysis scale used in this study, we showed that the insertion of the *CryIA(b)*–kanamycin or the *GUS*–kanamycin constructs had no effect on the colonization rate of endophytic fungi inside needles from transgenic white spruce. This was the expected result as none of the transgenic expression products are believed to have antifungal activity and were not aimed at nontargeted organisms like fungal endophytes.

To our knowledge, this is the first forestry-related study to evaluate transgenic tree impacts on nontarget organisms. We are awaiting field trials of transgenic conifer seedlings with antifungal activity (such as endochitinase gene expression) aimed at fungal tree diseases to test their impact on foliar endophytic fungi and other symbiotic fungal communities such as mycorrhizae, which are known to be naturally associated with the homologous natural trees.

Acknowledgements

We express our gratitude to Dr. Armand Séguin for making *Bt* white spruce undergoing field trials available for our experiment. We are very grateful to Michèle Bernier-Cardou and Sophie Périgon for statistical analyses. We thank Jean-Philippe Doyon, Camille Lauret, and Julie Spiry for their help during needle sampling. We also thank the Territorial Collectivity of Corsica.

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