

No Evidence of an Impact on the Rhizosphere Diazotroph Community by the Expression of *Bacillus thuringiensis* Cry1Ab Toxin by Bt White Spruce^{∇†}

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Received 11 April 2007/Accepted 17 July 2007

Nitrogen fixation is one of the most important roles played by soil bacterial communities, as fixation supplies nitrogen to many ecosystems which are often N limited. As impacts on this functional group of bacteria might harm the ecosystem's health and reduce productivity, monitoring that particular group is important. Recently, a field trial with Bt white spruce, which constitutively expresses the Cry1Ab insecticidal toxin of *Bacillus thuringiensis*, was established. The Bt white spruce was shown to be resistant to spruce budworm. We investigated the possible impact of these genetically modified trees on soil nitrogen-fixing bacterial communities. The trial consisted of untransformed controls, GUS white spruce (transformed with the β -glucuronidase gene), and Bt/GUS white spruce (which constitutively expresses both the Cry1Ab toxin and β -glucuronidase) in a random design. Four years after planting, soil samples from the control and the two treatments from plantation as well as from two natural stands of white spruce were collected. Diazotroph diversity was assessed by extracting soil genomic DNA and amplifying a region of the nitrogenase reductase (*nifH*) gene, followed by cloning and sequencing. Analysis revealed that nitrogen-fixing communities did not differ significantly among the untransformed control, GUS white spruce, and Bt/GUS white spruce. Nevertheless, differences in diazotroph diversity were observed between white spruce trees from the plantation site and those from two natural stands, one of which grew only a few meters away from the plantation. We therefore conclude, in the absence of evidence that the presence of the *B. thuringiensis cry1Ab* gene had an effect on diazotroph communities, that either site and/or field preparation prior to planting seems to be more important in determining diazotroph community structure than the presence of Bt white spruce.

Plant genetic engineering is a field of biotechnology that has experienced significant development over the last decades. Genetic modifications have been performed with several different plant species, mainly crops (e.g., maize, cotton, wheat, and rice), with different goals such as resistance to insect pests or herbicides, increased growth, and increased nutritional quality. However, plant genome modification might affect some important ecosystem components, such as soil microbial communities. Recent studies have been conducted to assess the potential beneficial and/or detrimental effects of genetically engineered plants on soil microbiota. Particular attention was paid to studying the impact of Bt plants, which constitutively express the Cry1Ab insecticidal toxin of *Bacillus thuringiensis*, on rhizosphere microbial communities. Although studies demonstrating a negative impact of Bt crops on soil microbiota exist (7, 44), more studies showing no effect have been published (1, 3, 4, 10, 12, 23, 26, 32).

Developments with regard to tree genetic engineering have made it possible to produce transgenic conifers. In one instance, Bt/GUS white spruce [*Picea glauca* (Moench) Voss]

(which constitutively expresses both the Cry1Ab toxin and β -glucuronidase), resistant to the spruce budworm, *Choristoneura fumiferana* (Clemens), was produced and tested in the laboratory and in an experimental plantation (D. Lachance and A. Séguin, cited in reference 27). Compared with crops, trees can have a long-lasting impact on their ecosystems: their root network is more widespread, they are generally present in the ecosystem for several decades, and they naturally harbor an important microbial community (17, 38), which might be more or less sensitive to the presence of the *Bacillus thuringiensis cry1Ab* gene. Thus, genetically modified trees could have impacts on soil microbial diversity that are different from those studied in agricultural crops, and it is crucial to investigate further these effects before their extensive release.

Studies of microbial diversity have generally used the 16S rRNA genes. However, to globally understand the microbial ecology of a particular ecosystem, the study of genes with important functions in an ecosystem is necessary, since that approach provides more information about the biological and/or ecological functions carried out by microbial communities. Soil microorganisms are responsible for different key functions in ecosystems as they are involved in many decomposing processes as well as in all major biogeochemical cycles, in the recycling of essential elements. Studies of the impact of genetically modified organisms should therefore also focus on microbial community functions as they are key elements in a healthy ecosystem.

One crucial function carried out by soil microorganisms is

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

[∇] Published ahead of print on 27 July 2007.

nitrogen fixation, which is the major source of nitrogen for many natural ecosystems. It is important primarily because nitrogen often is the limiting nutrient in many terrestrial ecosystems (43). Moreover, nitrogen fixation is a function performed by a wide diversity of bacteria belonging to many different taxa (46, 47). Hence, the study of one of the most conserved genes involved in nitrogen fixation, the *nifH* gene (29), is an interesting tool with which to evaluate microbial functional diversity, as it represents the diversity of a group of microorganisms, the nitrogen fixers, that are crucial to ecosystem productivity. Moreover, it has been demonstrated that *nifH* phylogenies are generally congruent with 16S rRNA gene phylogenies (6, 46).

The present study reports on diazotroph communities from the white spruce rhizosphere, and it is the first study to assess the impact of genetically modified organisms, for instance, Bt white spruce, on N₂-fixing communities. The objectives of this study were (i) to evaluate the functional diversity of the diazotroph community in the rhizospheres of genetically modified (GM) and non-GM spruce and (ii) to determine if genetic transformation has an effect on the composition of the diazotroph community in spruce rhizospheres.

MATERIALS AND METHODS

Study site, experimental design, and soil sampling. Rhizosphere soil samples were collected in August 2004 at Valcartier (approximately 25 km north of Québec City), from the only transgenic tree plantation in Canada. The Bt/GUS white spruce [*Picea glauca* (Moench) Voss] plantation was established in June 2000 and comprises eight replicates in a randomized complete block design of three different treatments—untransformed control, GUS white spruce, and Bt/GUS white spruce—surrounded by two guard rows of Norway spruce [*Picea abies* (L.) Karst.]. GUS white spruce contained the gene *uidA* encoding β -glucuronidase, a selective marker. Bt/GUS white spruce contained the *uidA* and *cryIAb* genes, the latter encoding the CryIAb toxin. GUS and Bt/GUS constructs also contained the neomycin phosphotransferase gene (*nptII*), which is a widely used selective genetic marker in plant transformation. Soil samples were also collected from the rhizospheres of white spruce growing around the plantation site (less than 50 m away) (Wild-VC; 3.72% total C; 0.20% total N; and pH 6.08) and from white spruce from the Base de plein air de Sainte-Foy (approximately 35 km from the plantation site) (Wild-SF; 48.93% total C; 1.68% total N; and pH 3.20). Three trees were sampled, and two subsamples per tree were taken and pooled for each of the two treatments and the control from the plantation and from the two natural stands. Approximately 5 to 10 g (wet weight) of soil from the rhizosphere was collected, placed in coolers with ice packs, and transported to the Laurentian Forestry Centre (Québec City), where samples were lyophilized and kept at -80°C until further analysis.

DNA extraction and PCR amplification. Total DNA was extracted from approximately 250 mg (dry weight) of rhizosphere by using a PowerSoil DNA kit from MoBio (MoBio Laboratories Inc., Solana Beach, CA) according to the manufacturer's instructions. Universal *nifH* primers PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3') (28) were used to amplify a 360-bp portion of the *nifH* gene. PCRs were performed with a final volume of 25 μl and contained 1 \times PCR buffer (QIAGEN), 1.5 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate, 0.6 μM of each primer, 1 U of *Taq* platinum DNA polymerase (Invitrogen), and 1 μl of extracted DNA. The PCR program was as follows: initialization at 94 $^{\circ}\text{C}$ for 3 min and 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 56 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 1 min. Final extension was performed at 72 $^{\circ}\text{C}$ for 5 min. For each DNA sample, PCR was repeated twice. To confirm amplification, aliquots (5 μl) of community *nifH* amplicons were run in a 1.5% agarose gel in 1 \times Tris-acetate-EDTA and stained with ethidium bromide. PCRs from the same sample were then pooled, purified using a QIAquick PCR purification kit (QIAGEN), and quantified using a Picogreen double-strand DNA quantitation kit (Molecular Probes, Eugene, OR).

Cloning and sequencing. Five clone libraries were constructed (control, GUS, Bt/GUS, Wild-VC, and Wild-SF) by cloning purified products using a QIAGEN PCR CloningPlus kit. Transformants were screened by enzymatic digestion with EcoRI (2 U per reaction; 37 $^{\circ}\text{C}$ for 2 h and 20 min for inactivation at 80 $^{\circ}\text{C}$).

Ninety transformants (three sublibraries by 30 transformants) were selected for each treatment. Transformant plasmid DNA was purified using the standard "lysis-by-alkali" protocol (31) and sequenced on a model CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, CA) with an M13 reverse universal primer.

DOTUR software analysis. Alignments of *nifH* sequences were performed using a ClustalW algorithm, version 1.4 (13), and were converted into a distance matrix using DNADIST from PHYLIP software. The operational taxonomic units (OTUs) for each library were determined using DOTUR software version 1.51 (33) (<http://www.plantpath.wisc.edu/fac/joh/dotur.html>). A 3% distance level between sequences was considered to be the cutoff to consider distinct OTUs. Bias-corrected Chao1 (8) and bootstrap (37) richness estimator values as well as Shannon's (*H*) and Simpson's (1-*D*) diversity indices were calculated for each replicate (the sublibraries, consisting of the library from one sampled tree). Analysis of variance and post hoc Duncan's multiple range tests were performed to evaluate statistical significance among the control, the treatments (GUS and Bt/GUS), and the natural stands (SAS Institute Inc., Cary, NC).

Dendrogram analysis. *nifH* DNA sequences included in one specific OTU were aligned using a ClustalW algorithm, version 1.4 (13), and a consensus sequence was derived. All consensus OTU DNA sequences of the *nifH* gene were then aligned with the *nifH* sequences from relevant known, described diazotrophs obtained from the NCBI GenBank database. Following the alignment of all consensus OTUs, Kimura two-parameter distances (19) between pair-wise sequences were calculated, and a neighbor-joining tree was reconstructed using MEGA 3.1 software (22). Clade stability was assessed using 1,000 bootstrap replications performed with the resulting tree.

SONS software analysis. SONS software version 1.0 (34) (<http://www.plantpath.wisc.edu/fac/joh/sons.html>) was used to obtain an OTU distribution and to evaluate the percentage of shared OTUs between libraries.

J-LIBSHUFF analysis. J-LIBSHUFF is a computer program derived from LIBSHUFF (36) which uses the exact and integral form of the Cramer-von Mises statistic instead of the approximated form. Pair-wise comparisons between libraries were performed using J-LIBSHUFF, following the directives of the authors (35), and *P* values were corrected accordingly.

UniFrac analysis. UniFrac is a recently developed phylogenetic method for comparing many samples simultaneously and for measuring the distance between communities by using the lineages they contain (24). We first performed significance tests using the UniFrac metric (UniFracP), which measures the differences between environments in terms of the branch length that is unique to each environment. We used UniFracP to test the significance of three different aspects: (i) the overall differences, testing whether the sequences from all of the different environments in the tree were significantly different from each other; than the individual differences, testing whether a particular environment had a more unique branch length than expected by chance (ii) the rest of the tree; and (iii) the pair-wise differences, testing whether each pair of environments differed from one another. The UniFrac *P* value represents the probability that there are more unique branch lengths than expected by chance (24). We also performed a *P* test (PhylogeneticTestP, implemented in UniFrac) as described by Martin (25), which uses parsimony. We tested the significance of overall differences and pair-wise differences. The distance matrix derived from the UniFrac metric calculation can also be used with standard multivariate statistics. Therefore, we also used the ones implemented in the UniFrac package: the unweighted-pair group method using average (UPGMA) linkages, the robustness of which was tested using jackknifing (with 75% of the sample size as the minimum sequence to keep), and principal coordinate analysis (UniFracPCA) (<http://bayes.colorado.edu/unifrac.zip> [accessed and downloaded 12 December 2005] and <http://bmf.colorado.edu/unifrac/index.psp> [accessed 18 May 2006]).

Nucleotide sequence accession numbers. Nucleotide sequences were registered with the NCBI GenBank database under the accession numbers DQ776309 to DQ776758.

RESULTS

DOTUR analysis. Using DOTUR software, we calculated richness estimators (bias-corrected Chao1 and bootstrap values) and diversity indices (Shannon and Simpson), and they are presented in Table 1. Ninety clones were analyzed for each library. By performing DOTUR analysis at the 3% distance level, we obtained a much lower abundance of OTUs with wild (VC and SF) stands than with the plantation stand (including the control, GUS, and Bt/GUS white spruce), supported by

TABLE 1. Comparison of diazotroph diversity^a

Treatment	No. of DNA sequences	No. of OTUs	Richness estimator ± SD		Diversity index ± SD	
			Bias-corrected Chao1	Bootstrap value	Shannon (<i>H</i>)	Simpson (1- <i>D</i>)
Control	90	53	77.17 ± 37.95 b	26.81 ± 4.31 b, c	2.85 ± 0.23 b	0.958 ± 0.022 b
GUS	90	52	59.70 ± 13.58 a, b	29.66 ± 1.40 c	2.98 ± 0.12 b	0.970 ± 0.014 b
Bt/GUS	90	55	66.28 ± 38.74 a, b	28.31 ± 5.83 b, c	2.91 ± 0.29 b	0.963 ± 0.030 b
Wild-VC	90	34	36.50 ± 20.32 a, b	16.42 ± 7.48 a, b	1.84 ± 0.66 a, b	0.726 ± 0.193 a, b
Wild-SF	90	23	15.94 ± 16.73 a	11.74 ± 10.32 a	1.34 ± 1.27 a	0.508 ± 0.439 a

^a Comparisons of microbial diversity were done by sampling the *nifH* genes in white spruce rhizospheres (transgenic and nontransgenic). Numbers of DNA sequences, OTUs, richness estimators (bias-corrected Chao1 and bootstrap values), and diversity indices (Shannon and Simpson) are shown for each treatment. Values ± standard deviations (SD) were obtained by use of the DOTUR program, using a 3% distance level. Groups with the same letters, a, b, and c, are not significantly different at α = 0.05.

bias-corrected Chao1 and bootstrap richness indicators. Moreover, the diversity indices (Shannon and Simpson) indicate that the overall diversity was also much lower in the wild stands.

Dendrogram analysis. We performed a dendrogram analysis using the *nifH* OTU sequences and the *nifH* sequences from known organisms to infer the possible affiliations of our different OTUs (see Fig. S1 in the supplemental material). The majority of clones (>75%) were related to the classes *Alpha*- and *Betaproteobacteria*, including genera such as *Methylocella*, *Xanthobacter*, and *Methylosinus*, as well as members of the rhizobia genera (e.g., *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium*) of *Alphaproteobacteria* and the genera *Burkholderia* and *Herbaspirillum* of *Betaproteobacteria*. Even though we could not clearly separate those two classes of proteobacteria, we observed that the OTUs found in that cluster were more closely related to *Alphaproteobacteria*. The second most well-represented group to which clones were related was the class *Deltaproteobacteria*, which includes the genera *Geobacter* (in cluster A) and *Desulfovibrio* and *Desulfomicrobium* (in cluster B) (Table 2 and see Fig. S1 in the supplemental material). According to the recent classification (11, 30), the majority of OTUs (>80%) belong to the *nifH* group I gene, which corresponds to typical molybdenum-iron (Mo-Fe) nitrogenase. Cyanobacteria were absent from wild stands, and there were more clones related to *Deltaproteobacteria* cluster B in the Wild-VC stand. No major trend was observed for the control and the two treatment groups from the plantation stand.

SONS analysis. We used SONS software to obtain OTU distribution across libraries (Fig. 1). The control, GUS, and Bt/GUS white spruce stands shared approximately 25% of their OTUs, whereas none of the OTUs from either the

Wild-VC or the Wild-SF stands were shared with any other library.

J-LIBSHUFF analysis. Clone libraries were compared in a pair-wise manner between treatments. Comparisons between wild stands (Wild-VC and Wild-SF) of white spruce and the control, GUS, and Bt/GUS white spruce yielded small *P* values (<0.0001) (Table 3). Wild-VC and Wild-SF were also significantly different from each other (Table 3). The significance of those differences was supported by the Monte Carlo procedure (*P* < 0.001).

UniFrac analysis. We first used UniFrac to perform significance tests (Table 4). The UniFrac *P* value is the proportion, out of the 1,000 randomized trees, that had at least as many unique branch lengths as the true tree, whereas the phylogenetic *P* value is the proportion, out of the 1,000 randomized trees, that had at least as many parsimony changes as the true tree. Both the UniFrac and phylogenetic tests gave significant *P* values for overall differences. The UniFrac test, investigating individual differences, shows that the control, GUS, and Bt/GUS white spruce have significantly more unique branch lengths than expected by chance, whereas for pair-wise differences, both the UniFrac and the phylogenetic tests present significant differences between all pairs of libraries, except for control versus Bt/GUS white spruce and GUS versus Bt/GUS white spruce. Both wild white spruce libraries (VC and SF) were significantly different from all other libraries.

We then used the clusterEnvs function to compare diazotroph communities from each library. Samples were clustered using UPGMA (Fig. 2). Diazotroph communities originating from the rhizospheres of both natural stand sites (Wild-VC and Wild-SF) were on a completely different branch than those

TABLE 2. Comparison of *nifH* diversity distribution among bacterial groups in GM and non-GM white spruce rhizospheres^a

Treatment	<i>Cyanobacteria</i>		<i>Alpha- or Betaproteobacteria</i>		<i>Gammaproteobacteria</i>		<i>Deltaproteobacteria</i>		<i>Firmicutes</i>	
	% of clones	No. of DNA sequences	% of clones	No. of DNA sequences	% of clones	No. of DNA sequences	% of clones	No. of DNA sequences	% of clones	No. of DNA sequences
Control	0		58.9	53	3.3	3	37.7	34	0	
GUS	2.2	2	31.1	28	3.3	3	63.3	57	0	
Bt/GUS	3.3	3	50.0	45	0		45.5	41	1.1	1
Wild-VC	0		22.2	20	1.1	1	75.5	68	0	
Wild-SF	0		83.3	75	4.4	4	12.2	11	0	

^a Percentages of clones (total of 90 per treatment) in each bacterial group are shown. There was one Wild-VC OTU that was not included (it is in the uncultured bacterial clade).

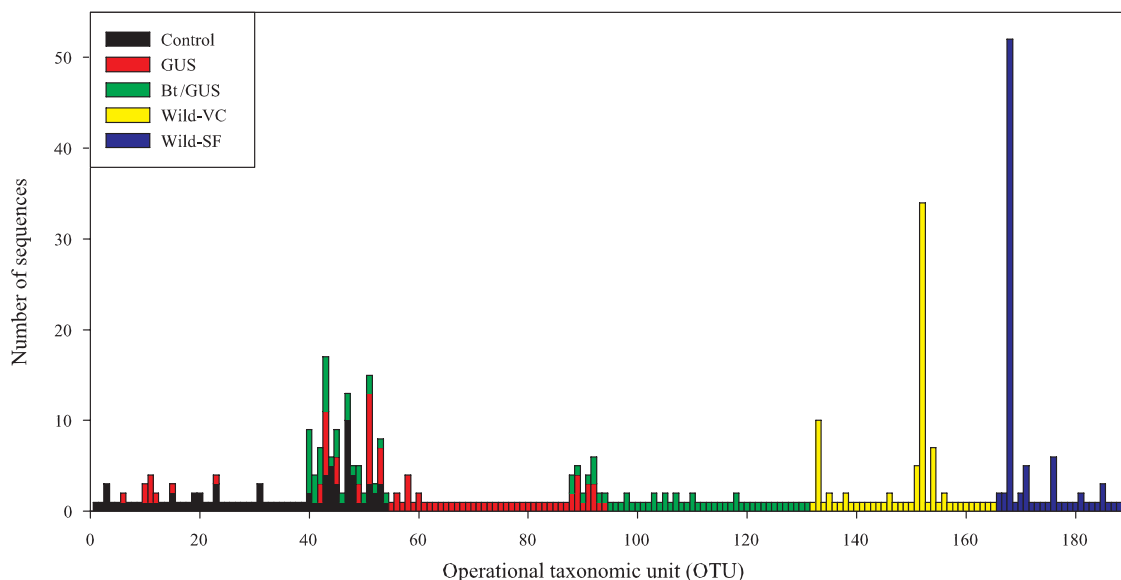


FIG. 1. OTU distribution for each treatment.

of the control, GUS, and Bt/GUS white spruce trees, located in plantation, all with high jackknife values. Finally, we performed a principal coordinate analysis (UniFracPCA) showing the mean coordinate values with standard deviations of the three sublibraries (i.e., from the three trees sampled) (Fig. 3). The first two principal coordinates for the analysis including all five libraries (Fig. 3a) explained 15.0 and 14.2% of the variance. The first principal component separates samples originating from the rhizospheres of white spruce in plantation versus those originating from natural white spruce stands. As wild samples had an important impact, we also performed the analysis by including only the control, GUS, and Bt/GUS white spruce stands to have a closer look at the possible impact of Bt/GUS white spruce (Fig. 3b). No clear segregation was observed.

DISCUSSION

Our study describes diazotroph communities present in white spruce rhizospheres. This study is the first to evaluate the impact of Bt white spruce, or any other Bt plant, on nitrogen-fixing communities. No effect associated with the presence of the *B. thuringiensis* transgene was observed in this study, which

is in agreement with other recent studies evaluating the impact on overall bacterial diversity (1, 3, 4, 10, 12, 23, 26, 32). On the other hand, significant differences were found between diazotroph communities associated with white spruce in natural stands and those associated with trees in a plantation. This result is specifically in agreement with those obtained in a previous study we carried out (J. Lamarche and R. C. Hamelin, submitted), where terminal restriction fragment length polymorphism profiles of 16S rRNA genes showed that the tree's growing site had a greater impact on bacterial diversity than the treatment of soil.

One main objective was to characterize the N_2 -fixing community of the white spruce rhizosphere. To do so, we used tree reconstruction with sequences of well-known N_2 fixers (see Fig. S1 in the supplemental material). It revealed that the vast majority of OTUs were clustering within the group I nitrogenase. Group I nitrogenases are typical Mo-Fe nitrogenases and are found predominantly in proteobacteria and cyanobacteria, whereas group II nitrogenases are anaerobic Mo-Fe nitrogenases, predominantly found in methanogens and anaerobic bacteria (30). There exist three other nitrogenase groups according to Raymond et al. (30), but none of the clones we obtained seemed to belong to those groups. Moreover, the results showed that most of the OTUs belonged to the *Alpha-*, *Beta-*, or *Deltaproteobacteria* class (see Fig. S1 in the supplemental material and Table 1), and very few were members of the phyla *Cyanobacteria* and *Firmicutes*. According to the latest nitrogenase phylogeny (11, 30), species within the class *Deltaproteobacteria* could have nitrogenase genes that belong either to group I or group II, thus explaining why they cluster in two different clades (see Fig. S1 in the supplemental material). In addition, 45% of the *nifH* OTUs identified in this study belonged to *Deltaproteobacteria* cluster A, where the only known sequences are those from *Geobacter metallireducens* and *Geobacter sulfurreducens*. This phenomenon has already been reported in other studies (cluster A in the study by Hamelin et al. [14], *nifH* cluster 3 in Bürgmann et al. [6], and cluster

TABLE 3. Comparison of *nifH* gene libraries between GM and non-GM white spruce rhizospheres, done using *f*-LIBSHUFF^a

Treatment	Significant difference (<i>P</i> value)				
	Control	GUS	Bt/GUS	Wild-VC	Wild-SF
Control		<0.0001*	0.0119	<0.0001*	<0.0001*
GUS	0.0388		0.0367	<0.0001*	<0.0001*
Bt/GUS	0.1869	0.0063		<0.0001*	<0.0001*
Wild-VC	0.0010*	<0.0001*	<0.0001*		<0.0001*
Wild-SF	<0.0001*	<0.0001*	<0.0001*	<0.0001*	

^a Correction for multiple comparison (20) tests: for a family-wise error rate of 0.05, the minimum *P* value is 0.0026 (significant results are followed by an asterisk). Monte Carlo error (10,000 permutations), 95% margin of error for minimum *P* value is <0.0001.

TABLE 4. Comparison of *nifH* gene libraries, done using UniFracP and PhylogeneticTestP^a

Treatment	Significant difference (<i>P</i> value) ^b									
	Control		GUS		Bt/GUS		Wild-VC		Wild-SF	
	UnifracP	Phylogenetic-TestP	UnifracP	Phylogenetic-TestP	UnifracP	Phylogenetic-TestP	UnifracP	Phylogenetic-TestP	UnifracP	Phylogenetic-TestP
Control										
GUS	<0.001*	<0.001*								
Bt/GUS	0.014	0.003	0.007	<0.001*						
Wild-VC	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*				
Wild-SF	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*		

^a Comparisons of *nifH* gene libraries from GM and non-GM white spruce rhizospheres were made, using UniFracP and PhylogeneticTestP implemented in UniFrac. A *P* test (PhylogeneticTestP, implemented in UniFrac) was also performed as described by Martin (25).

^b *P* values were calculated using 1,000 permutations. Correction for multiple comparison (20) tests: for a family-wise error rate of 0.05, the minimum *P* value is 0.0026 (significant results are followed by an asterisk). Values for overall treatments were <0.001 for both tests, whereas UniFrac *P* values for each treatment were 0.022 (control), 0.043 (GUS), 0.047 (*B. thuringiensis*), 0.780 (Wild-VC), and 0.998 (Wild-SF). The minimum *P* value for the latter test is 0.05 as there is no multiple comparison.

NF5 in Yeager et al. [45]). As the diversity within that group is quite important and is noticeable in many different ecosystems (47), thus suggesting it might be a major nitrogen-fixer group, it should be investigated further. More sequences of known species belonging to that group would be required for a better characterization.

As for the effect of Bt/GUS white spruce, the dendrograms as well as the distributions of the different OTUs throughout the different bacterial groups did not appear different between libraries. We also looked at diversity and more specifically at richness, one of the two major components of diversity, and we observed that both were much lower and more variable in the rhizospheres of white spruce in natural stands. Lower diversity is the result of unevenness, as there is some redundancy in cloned sequences, with some OTUs being represented by up to 53 clones in Wild-SF but only 10 clones per OTU in plantation. Since the natural stands were around 25 and 65 years old for Wild-VC and Wild-SF, respectively, diazotroph communities might have reached equilibrium with their environment. Selection toward the “best performers” may have occurred, resulting in the overrepresentation of those species or bacterial groups. Diazotroph communities in plantation (which were approximately 4 years old when sampled) might still be in their “adaptation period,” with a greater abundance of species and

no species dominating the communities. There was also a large variability among the plots in the natural stands compared to that of samples in plantation. Two main hypotheses might explain that result: (i) soil under plantation treatment has been plowed before tree transplantation, therefore homogenizing the site, and (ii) a greater abundance of soil microsites is present in natural stands; each microsite could then have various degrees of diversity.

The overall distribution of OTUs according to each library was analyzed using SONS (34), and results indicate that many sequences from a particular library are attributed to each OTU (OTU number in Fig. 1). We clearly see that some OTUs from samples in plantation (control, GUS, and Bt/GUS white spruce) are shared, whereas Wild-VC and Wild-SF have a completely distinct sets of OTUs, which was quite unexpected. Even though the sites were quite different, we were expecting to observe at least some shared OTUs. These results suggest that diazotroph groups are very diverse and that they may be site specific. It also demonstrates that the soil environment in plantation is quite different from that of both of the wild sites. The Wild-VC site was only a few meters away from the plantation, but the immediate surroundings were quite different; there was the presence of understory vegetation as well as other tree species growing around. As for the Wild-SF site, it was in a completely different environment (as described in Materials and Methods), approximately 35 km from the plantation site in the St. Lawrence Plain.

To further investigate the differences in diazotroph communities, and more specifically that of Bt/GUS white spruce, we used three different microbial community analysis tools (*f*-LIBSHUFF, UniFracP, and PhylogeneticTestP). In general, they led us to the same conclusions, with some minor disparities. Nevertheless, the major conclusions drawn from those analyses were that (i) there is an absence of evidence that *cryIAb* insertion (as well as *nptIII* and *uidA* insertion) in white spruce has an effect on nitrogen-fixing bacterial communities and that (ii) the differences between treatments in plantation and natural stands are highly significant, which is consistent with the results previously discussed.

The absence of evidence of the impact of Bt/GUS white spruce suggests that there is no direct impact of the toxin itself even if bacteria can utilize the *B. thuringiensis* toxin as a source

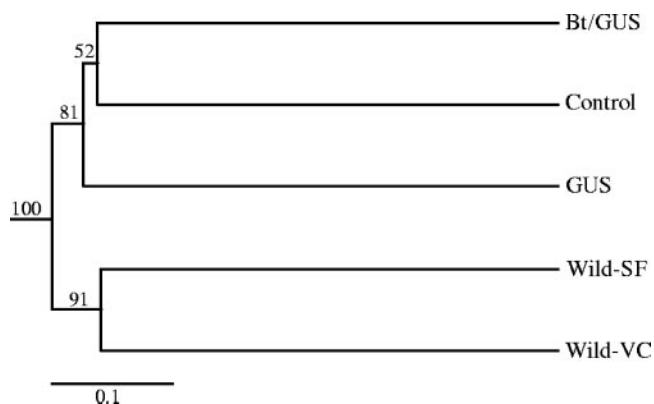


FIG. 2. UPGMA cluster of the different treatments as obtained by UniFrac (clusterEnvs). Jackknifing values (percentages) are presented for each branch.

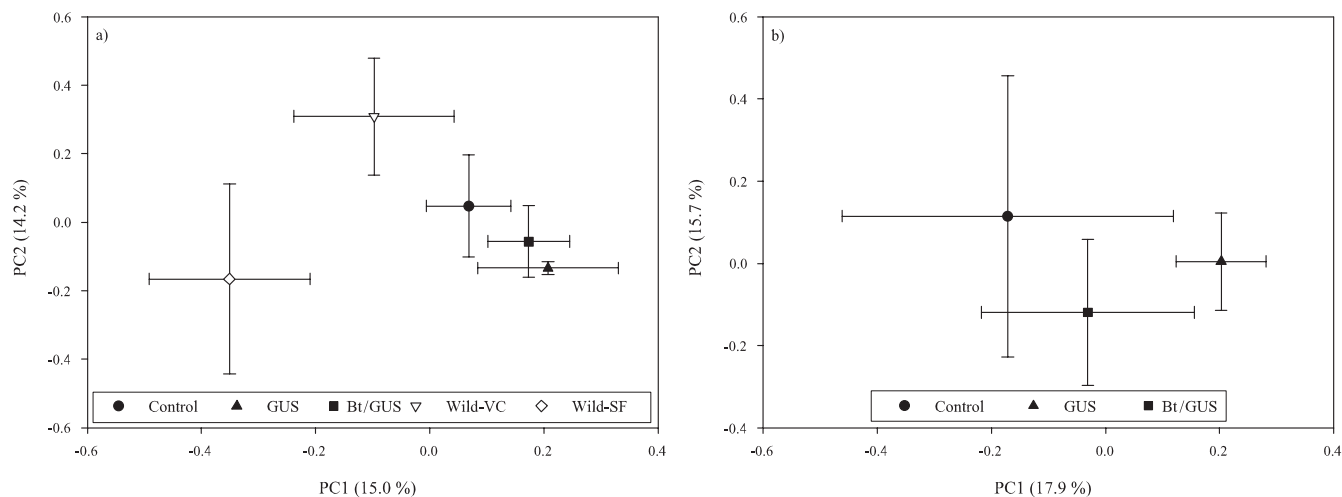


FIG. 3. Principal coordinate analysis as obtained by UniFrac (UniFracPCA). Each treatment is represented by the means and standard deviations of three replicates. Percentages in parentheses in the axis labels represent the percentages of variation explained by the principal coordinate. Analysis was performed with all five treatments (a) and with treatments in plantation only (b).

of carbon and/or nitrogen (21). It is in agreement with the demonstration that the toxin itself does not have any microbicidal or microbiostatic activity (20). No indirect impact triggered by *B. thuringiensis* gene insertion within the white spruce genome was detectable in the present study. This absence of evidence of Bt white spruce impact, however, is not the result of low or no *B. thuringiensis* toxin production by trees, as the expression of the toxin was confirmed in 2004 by Northern blotting and enzyme-linked immunosorbent assay (D. Lachance and A. Séguin, personal communication). Nonetheless, the available concentrations of free toxins in the soil might have been low due to the presence of humic acids and/or clay particles to which the toxin is known to adhere (9, 39–42). Furthermore, it was established that bacteria are not able to use the *B. thuringiensis* toxin when the latter is bound to clay (21). The proportion of available toxin might have been in concentrations insufficient to induce changes in nitrogen-fixing bacterial communities.

As for the presence of the *npfIII* and *uidA* genes, which encode a neomycin phosphotransferase and a β -glucuronidase, respectively, we could have suspected some impact since they are not normally found in plants (2, 16, 18). The concern was not for the persistence of the genes themselves in soil, as it has already been demonstrated that they did not persist over 4 months (15), but rather for their products. Indeed, neomycin phosphotransferase is an enzyme able to inactivate different aminoglycoside antibiotics, such as kanamycin, neomycin, G418 (Geneticin), and paromomycin. These antibiotics might affect prokaryotic and/or eukaryotic organisms. Their inactivation might therefore influence the overall microbial community. Nevertheless, no significant difference triggered by the presence of those genes and their product was observed in the present study.

The differences observed between samples in plantation and those in natural stands might be the result of different factors, such as the difference in tree genetic background, which might have triggered variations in root exudates and therefore have affected diazotroph communities (5), or stand age (approx-

mately 4 years old for the plantation and 25 and 65 years old for natural stands). Finally, field preparation prior to planting might have caused a lot of modifications to the soil structure and physicochemical properties, thereby possibly driving changes in nitrogen-fixing communities.

Our study shows that there already are, under natural conditions, great differences in diazotroph communities, driven only by stand sites. However, although no significant impact of Bt white spruce was observed for a particular bacterial functional group, further studies might be required to evaluate, among other things, potential horizontal gene transfer between transgenic white spruce and microbial communities. GM trees are novel organisms with particular characteristics that distinguish them from the more widely studied GM crops. Therefore, specific attention should be given to GM trees in general as they are long lived and might have long-term impacts.

ACKNOWLEDGMENTS

We thank A. Séguin and D. Lachance for the production of transgenic white spruce and for setting up the experimental design, and we thank all field assistants. We also thank N. Feau, F. O. Stefani, and I. Lamarque for the revision of the manuscript. We also thank those who developed the different statistical tools we used; their work is very valuable.

This work was supported by grants from the Canadian Biotechnology Strategic Funds.

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