



Separating the effect of crop from herbicide on soil microbial communities in glyphosate-resistant corn

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Summary

Glyphosate-resistant (GR) cropping systems change the soil environment by introducing novel compounds and glyphosate into the soil environment. Over one growing season, we examined the effect of both the transgenic corn and the use of glyphosate on two groups of rhizosphere microbes, denitrifying bacteria and fungi. Using quantitative PCR to measure microbe abundance, and terminal restriction fragment length polymorphism (T-RFLP) to measure community structure, we found neither crop type (transgenic or conventional) nor herbicide (glyphosate or conventional) affected rhizosphere denitrifying or fungal communities. Instead, our results showed that seasonality was a significant determinant of denitrifier and fungal abundance as well as their diversity in this study, suggesting in the short term, some microbial communities are robust to changes in their environment by GR crops. Crown Copyright © 2008 Published by Elsevier GmbH. All rights reserved.

Introduction

While much has been written about the potential risk of herbicide-tolerant crops, there is little evidence to contraindicate their use. Intuitively, the fact that glyphosate is toxic to some bacteria

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and fungi (Busse et al., 2001) suggests its use could lead to changes in soil microbial communities. Soil microbial communities, in particular rhizosphere microbes, may be particularly sensitive to the effects of genetically modified crops because of their intimate proximity.

Tolerance for glyphosate (or RoundUp) is a common modification to corn, soybean and canola (Widmer, 2007). These plants contain a gene insert that enables them to tolerate glyphosate, which inhibits the synthesis of aromatic amino acids in plants, some microbes (Zablotowicz and Reddy, 2004) and fungi (Feng et al., 2005). Glyphosate-resistant (GR) crops can be treated post-emergence with glyphosate without killing the crop. Thus, these systems are often subjected to repeated use of glyphosate (Gimsing et al., 2004; Montavalli et al., 2004). This changes the soil environment in two ways: first, the influx of carbon, phosphorus and nitrogen in the form of glyphosate, and second, by the introduction of more vegetative material as a result of post-emergent treatment by glyphosate.

While some studies have shown that GR cropping systems negatively affect bacterial numbers (Siciliano and Germida, 1999; Dunfield and Germida, 2001; Siciliano et al., 1998), many more have documented only minor, transient effects. Liphadzi et al. (2005) found that the use of glyphosate on GR soy and corn did not affect soil respiration nor the community identity of soil bacteria. Weaver et al. (2007) also showed that neither GR soy nor glyphosate significantly changed soil microbial communities in terms of structure, function or activity. Lupwayi et al. (2007) found that changes to microbial communities growing with a wheat corn/wheat pea rotation were relatively insensitive to cropping practices, and GR crops produced only small, and inconsistent changes in microbial communities.

These results have been mirrored by other researchers who have shown that plant growth stage (Sessitsch et al., 2004) and tillage (Griffiths et al., 2007) are bigger determinants of microbe communities in the soil than the identity of the crop (GR or conventional). In the absence of GR

crops, glyphosate itself has been shown to affect microbial communities. It has been shown to stimulate microbial activity, both by increasing carbon and nitrogen mineralization by bacteria (Haney et al., 2000), and also by increasing fungal colonization (Kremer et al., 2005).

Such inconsistent findings may be due to two factors. First, it is unlikely that all microbes will be equally affected or resistant to the effects of the GR cropping system. Because of the overwhelming diversity of soil microbial communities, it is essential to target groups most likely to be affected by changes to the growing environment. In this case, we looked at denitrifying bacteria and soil fungi which may be affected by the carbon influx and changes in N and P status associated with GR cropping systems. Second, it is important to separate the effects of the GR crop from glyphosate effects. This is difficult to do, as glyphosate is lethal to non-GR crops. However, we were able to conduct a fully factorial, field study where we were separated the effects of crop type and herbicide treatment on microbe numbers and diversity. We measured the numbers and community composition of two soil rhizosphere microbes to determine if their communities were affected by (1) glyphosate-resistant corn versus conventional corn and (2) glyphosate versus conventional herbicides (isoxaflutole and atrazine).

Materials and methods

Field site and conditions

The experimental field was located at the Elora Research Station of the University of Guelph (Elora, ON, Canada 43°41' 2N, 80°26' 2W). The soil was a Conostogo silt loam soil (sand, 26.1%; silt, 60.1%; clay, 13.8%; pH, 7.3; OM, 5%; CEC, 27.1 cmol kg⁻¹). Average temperatures and precipitation are given in Table 1. The site had no previous history of crops or herbicide application since it was acquired by the University of Guelph 30 years ago.

Table 1. Environmental conditions at Elora Research Station, Elora, Canada from the period of May 29–October 25.

	May 29	August 15	October 25
Average monthly temperature (°C)	11.4	18.4	5.6
Maximum temperature (°C)	19.0	26.1	7.3
Minimum temperature (°C)	3.8	10.6	3.9
Total precipitation (mm)	244.5	145	

Total precipitation was calculated as total amount of precipitation occurring between harvest dates.

Experimental design

This experiment took place during one growing season. We used a two-way factorial design, with crop (RoundUp Ready or conventional corn) and herbicide treatment (RoundUp or conventional herbicides) as the two factors. Plots (12 m × 14 m) were sown (May 10, 2005) with RR corn line DKC35-51 (Monsanto, St. Louis, MO) or its non-transgenic counterpart, DK355, in 76 cm rows. Herbicides were applied as follows, according to manufacturer's instructions: RoundUp (1.8 kg ha⁻¹ atrazine) on June 9 or conventional (79 g of ai ha⁻¹ isoxaflutone+800 g of ai ha⁻¹ atrazine) on June 17. The field was divided into plots corresponding to four treatments (RRcorn+RoundUp, RRcorn+conventional herbicides, conventional corn+conventional herbicides, conventional corn+RoundUp), which were repeated in four blocks. Because this experiment was part of a larger study that did not examine the effect of RU on conventional crops, we treated a 1-m-wide strip of conventional corn treatment with RoundUp, post-emergence at the 4-leaf stage (June 17, 2005). To minimize cross-contamination, the experimental units were separated by a 3-m winter barley (*Hordeum vulgare* L.) border within replicates and by a 14-m border between replicates. Corn was harvested on October 5, 2005.

On each sampling date (May 29, August 15 and October 25, 2005), root samples from five corn plants were collected from each plot, for a total of 80 samples per harvest date. Roots and surrounding soil were immediately stored on ice for transport to the University of Guelph and stored at 4 °C until further processing.

DNA extraction

Roots were shaken to remove bulk soil. The remaining roots and rhizosphere soil were chopped into 2-cm fragments, and 5 g of roots were selected for DNA extraction using the UltraClean-htp 96-well Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA) following the manufacturer's instructions.

Primers

We looked at two groups of rhizosphere microbes that may be significantly affected by the conditions of this cropping system: denitrifying bacteria and root-associated rhizosphere fungi. For both functional groups, we used previously-published primers in a reaction with SybrGreen for quantifi-

cation. For the denitrifiers, we used nirS, which is involved in the conversion of nitrite into nitric oxide, using a cytochrome-cd₁ enzyme. We used forward primer cd3af (5'GTSACGTSAAGGARAC-SGG3') (Michotey et al., 2000) and reverse primer R3cd (5'GASTTCGGRTGSGTCTTGA3') (Throback et al., 2004). For the fungi, we used general fungal primers for the LSU ribosomal DNA, specifically forward FLR2 (5'GTCGTTTAAAGCCATTACGTC3') (Trouvelot et al., 1999) and reverse LR1 (5'GCATA-TCAATAAGCGGAGGA3') (van Tuinen et al., 1998).

Real-time PCR

The quantity of denitrifiers and fungi were measured using real-time PCR. Real-time PCR amplifications were performed on 1 µL DNA extracts with the iCycler (Bio-Rad) in 96-well plate microtubes containing a final volume of 20 µL PCR mixture. The real-time PCR mixture contained 10 µL iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), iTaq DNA polymerase, 50 units mL⁻¹, 6 mM MgCl₂, SYBR Green I, 20 nM fluorescein, and stabilizers) (BioRad), 500 nM of forward and reverse primers, and 10 ng µL⁻¹ of T4 gene 32 protein (Roche, Laval, QC, Canada). Conditions for real-time PCR amplifications included a 1:30 min step at 95 °C followed by 50 cycles of 10 s at 94 °C, 20 s at 53 °C and 30 s at 72 °C. Fluorescence was monitored during the annealing step (53 °C). The copy numbers of target DNA recovered from soil was determined using the sigmoidal curve fitting method as described by Gulden et al. (2007). For the generation of standard curves, 10¹–10⁸ cloned nirS copies from *Pseudomonas stutzerii* were added to sterile water. Critical threshold values and baseline subtracted PCR raw data for each real-time qPCR were exported to MS Excel and prepared for further analysis. Baseline subtracted fluorescence data of each qPCR from the 10-fold dilution series in the soils was used to create linear functions.

T-RFLP analyses

The composition of denitrifying and fungal communities was evaluated using terminal restriction fragment length polymorphism (T-RFLP). This way, end-labeled PCR products are digested with restriction enzymes. Sequence polymorphism can result in fragment size variation. These differences indicate differences in species diversity at the community level, and have been used to

characterize soil fungal communities (Mummey and Rillig, 2006, 2007).

In the present study, DNA from the initial extraction was amplified with FAM-labeled cd3af (5'-FAM-GTSAACGTSAAGGARACSGG3') (Michotey et al., 2000) and FAM-labeled FLR2 (5'-FAM-GTCGTTTAAAGCCAT-TACGTC3') (Trouvelot et al., 1999). PCRs were comprised of a 20 μ L reaction mix containing final concentration of 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), 50 U mL⁻¹ iTaq DNA polymerase, 6 mM MgCl₂, 500 μ M forward and reverse FAM-labeled primers, and 1 μ L of template DNA. PCR conditions consisted of an initial denaturation step at 93 °C for 3 min followed by 35 cycles (93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) and a final extension step of 10 min at 72 °C.

PCR product sizes were verified by gel electrophoresis with a 1 kb GeneRuler™ DNA ladder (Fermentas, Burlington, ON, Canada) as standard and purified using a QIAquick® cleanup kit (Qiagen Inc.). Products were then digested with restriction enzyme *Msp* I (New England Biolabs). The restriction digestions, comprised of a 20 μ L reaction mix containing 15 μ L purified PCR product, 1.5 μ L buffer and 2 U of enzyme. The reactions were incubated for 4 h at 37 °C. T-RF sizes in each sample were determined using an ABI 3730 DNA Analyzer (Applied Biosystems) with LIZ-500 (Applied Biosystems) as the size standard.

Analysis of community structure consisted of determining the profile (sizes and peak heights) of T-RFs in each sample using GeneMapper® software v. 3.5 (Applied Biosystems). The Microsoft-Excel macro Treeflap (Rees et al., 2004; <http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls>) was used to convert fragment sizes to the nearest integer, aligning them with their respective peak heights side by side in two columns. A script (available upon request) was written for use with R (R Development Core Team, 2007) that standardizes the amount of total fluorescence among sample profiles according to the methods described by Dunbar et al. (2001).

Data analysis

All data collected in May prior to herbicide application constitute pretreatment values, and provide base-line values. They were not used in statistical analyses.

Real-time PCR

Data from real-time PCR were analysed using ANOVA, with crop, herbicide and harvest date as

the fixed factors and block as a random factor (SPSS, Inc.). First, five data points from each experimental unit were averaged, and the mean values were used for further calculations. Subsequently, data for both fungi and denitrifiers were then log transformed to satisfy assumptions of normality and homogeneity of variance.

T-RFLP

Community data were analysed by two methods. In the first, each T-RF was considered an operational taxonomic unit (OUT) and total number of T-RFs were calculated per treatment, and the values analysed using ANOVA, with crop, herbicide and harvest dates as fixed factors (as above). DNA were pooled according to treatment and harvest. By pooling the samples, we were able to remove block effects. Therefore, data was analysed as a completely randomized design to improve the power of the analysis. For each of the 4 treatments there were 8 replicates and three harvest dates, for a total of 96 samples.

In the second approach, relationships among samples with respect to T-RFLP profile were evaluated using canonical correspondence analysis (CCA), using the Canoco software for windows version 4.51 (Biometris, Wageningen, The Netherlands). CCA analysis is a community ordination method which uses environmental variables to structure data about species abundance. Detrended correspondence analysis (DCA) was conducted first and showed a strong unimodal response of the species (T-RF) data (i.e., maximum gradient length > 4 SD). CCA analysis was then conducted with forward selection to rank the importance of environmental variables in determining the species data. The statistical significance of each environmental variable (i.e., its contribution to the model of already included variables) was evaluated by a Monte-Carlo permutation test.

Results

nirS

We detected nirS presence in the soil at all harvest dates in all treatments (Figures 1 and 2). Values ranged from undetectable for pretreatment measurements in May to over 60 pg DNA g⁻¹ soil, for the August harvest. Overall, there was a significant harvest effect on the number of NirS copies detected. Regardless of crop type, more copies of nirS were found in plots during August than during

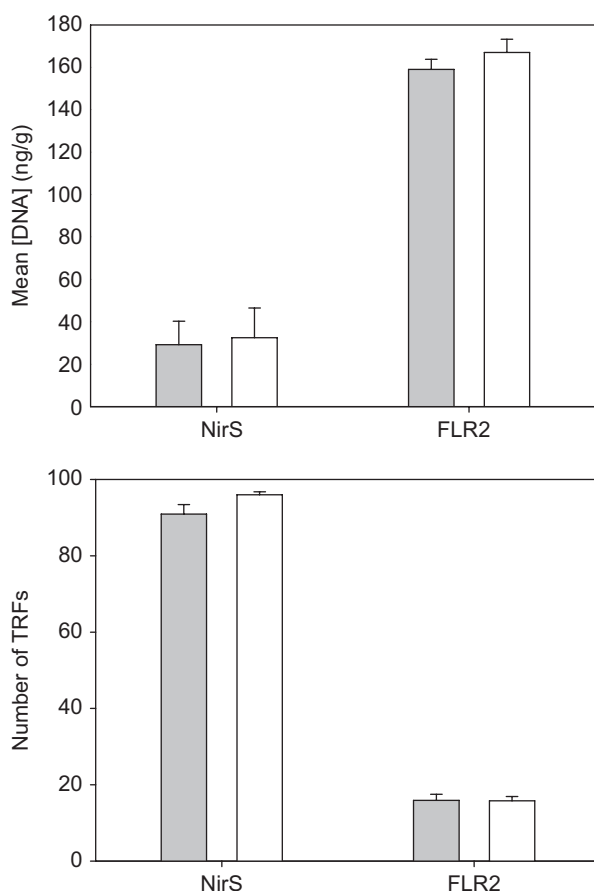


Figure 1. Pretreatment values for (a) DNA concentration ($\text{pg } 5 \text{ g}^{-1}$ wet weight corn roots) and (b) number of T-RFs for denitrifying bacteria (*nirS*) and rhizosphere fungi (*flr2*) at the University of Guelph Research Station, Elora, Canada. Values given are mean $\pm$ S.E.

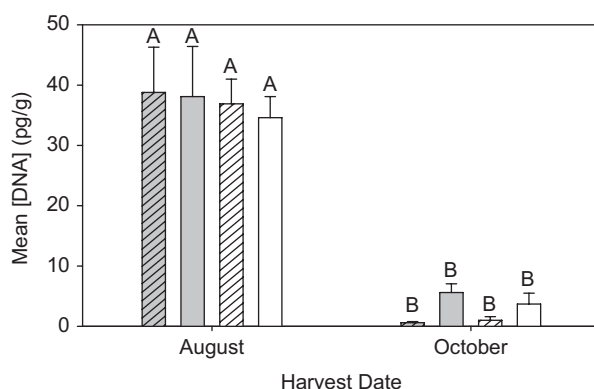


Figure 2. Mean concentration of *nirS* copy number in the rhizosphere of 5 g (wet weight) corn roots harvested from the University of Guelph Research Station, Elora, Canada on two dates. Values are given in DNA $\pm$ S.E. $\text{pg } \text{g}^{-1}$. Black bars and light grey bars represent RoundUp Ready corn grown with RoundUp and with conventional herbicides, respectively. Dark grey and white bars represent conventional corn grown with RoundUp and conventional herbicides, respectively. Different letters are statistically significant at $P < 0.05$.

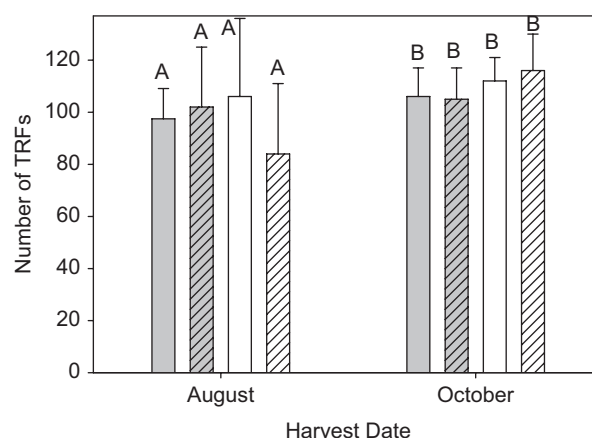


Figure 3. Number of terminal restriction fragments (T-RFs) for *nirS* sequences amplified from root samples at the University of Guelph Research Station, Elora, Canada, on two dates. Values are given as the average number of TRF's $\pm$ S.E. for four treatments: black bars and light grey bars represent RoundUp Ready corn grown with RoundUp and with conventional herbicides, respectively. Dark grey and white bars represent conventional corn grown with RoundUp and conventional herbicides, respectively. Different letters are statistically significant at $P < 0.05$.

October (Figure 2). Average concentration of gene copies was $37.1 \pm 12.5 \text{ pg } \text{g}^{-1}$ soil in August versus $2.8 \pm 3.0 \text{ pg } \text{g}^{-1}$ soil in October.

Conversely, we found more T-RFs in the October harvest than any other (Figure 3). There were an average of 110.2 ± 3.3 T-RFs in October, versus 97.4 ± 3.2 in August. There was no effect of crop or herbicide on the number of T-RFs for *nirS*.

Number of T-Rfs-FLR2

In terms of community analysis, we found that only four out of a total of seven environmental variables were significant ($P < 0.05$) in a preliminary analysis (Figure 4). These were preherbicide harvest (H1) ($P = 0.002$), RoundUp Ready crop ($P = 0.004$), August harvest (H2) ($P = 0.0180$) and RoundUp herbicide ($P = 0.012$). The first two eigenvalues of the CCA were 0.045 and 0.036 compared to 0.146 and 0.065 for an unconstrained ordination (DCA) on the same data set. Thus, while a portion of the variation in community structure was explained by the four environmental variables, other environmental variables not measured in this study accounted for most of the variation. In total, only 4.8% of the total variance was explained by the measured environmental variables.

In the ordination diagram (Figure 4), there was separation of species along axis one, with pretreatment values (H1) occurring far right in the ordination space, the August harvest (H2) near the

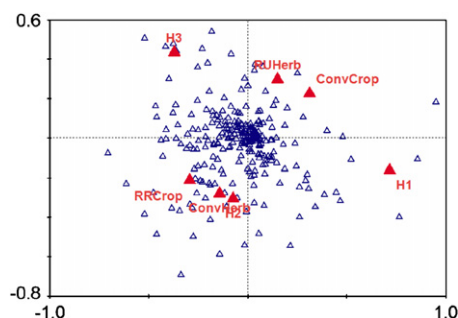


Figure 4. Ordination diagram of denitrifying bacteria (*nirS*) produced by canonical correspondence analysis of species frequencies using the combined data subsets of 95 samples, 335 species, and 7 environmental variables collected from agricultural soil, at the University of Guelph Research Station, Elora, Canada. Plots are of axis 1 ($\lambda = 0.045$) vs. axis 2 ($\lambda = 0.037$). The empty triangles represent species, and the solid triangles represent the environmental variables: H1 = May harvest, H2 = August harvest, H3 = October harvest. RRCrop = RoundUp Ready crop, ConvCrop = Conventional crop, RUHerb = RoundUp herbicide, ConvHerb = Conventional herbicide.

centre, and the October harvest (H3) towards the left of the diagram. Less separation occurred along the second axis with RoundUp herbicide and conventional crop occurring close together, near the top of the ordination space, and conventional herbicide and RoundUp Ready crop occurring together, near the bottom of the ordination space.

FLR2

We detected FLR2 activity in the soil at all harvest dates in all treatments (Figures 1 and 5). Values ranged from undetectable in pretreatment values to over 2300 pg g^{-1} soil for the October harvest. Like *NirS*, there was a significant seasonal effect on the number of FLR2 copies detected, with numbers peaking in August. Mean numbers of FLR2 copies were $111.9 \pm 1.7 \text{ pg g}^{-1}$ soil for August and $70.8 \pm 7.5 \text{ pg g}^{-1}$ soil for October. We found no effect of crop or herbicide on the number of T-RFs for FLR2 (Figure 6).

In terms of community analysis, we found that only two environmental variables were significant out of a total of seven ($P < 0.05$) in a preliminary analysis (Figure 7). These were October harvest (H3) ($P = 0.002$), and RoundUp Ready crop ($P = 0.002$). The first two eigenvalues of the CCA were 0.214 and 0.127 compared to 0.445 and 0.360 for an unconstrained ordination (DCA) on the same data set. Therefore, only a portion of the variation in community structure was explained by the two environmental variables with other, non-measured

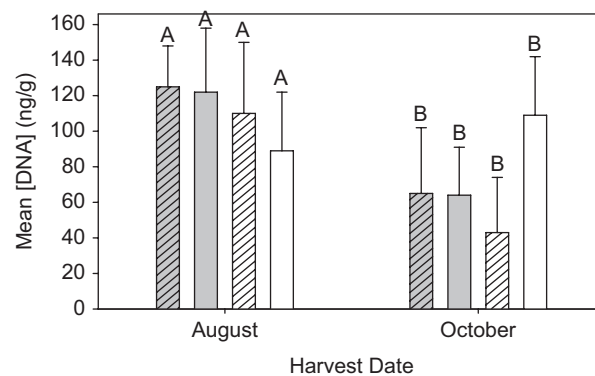


Figure 5. Mean concentration of *flr2* in the rhizosphere of 5g (wet weight) corn roots harvested from the University of Guelph Research Station, Elora, Canada on two dates. Values are given in $\text{pg g}^{-1} \text{ DNA} \pm \text{S.E.}$ Black bars and light grey bars represent RoundUp Ready corn grown with RoundUp and with conventional herbicides, respectively. Dark grey and white bars represent conventional corn grown with RoundUp and conventional herbicides, respectively. Different letters are statistically significant at $P < 0.05$.

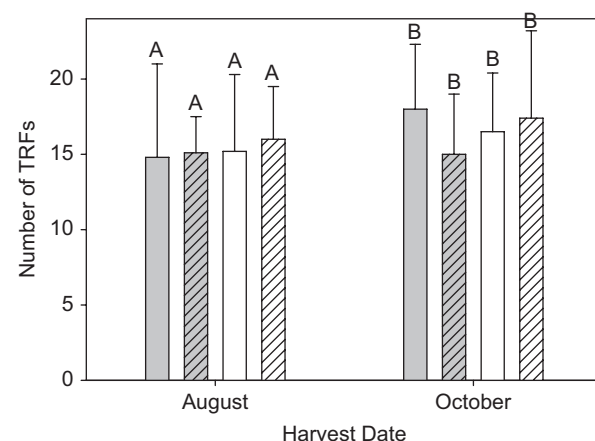


Figure 6. Number of terminal restriction fragments (T-RFs) for *flr2* sequences amplified from root samples from the University of Guelph Research Station, Elora, Canada, on two dates. Values are given as the average number of T-RFs $\pm$ standard deviation for four treatments: black bars and light grey bars represent RoundUp Ready corn grown with RoundUp and with conventional herbicides, respectively. Dark grey and white bars represent conventional corn grown with RoundUp and conventional herbicides, respectively. Different letters are statistically significant at $P < 0.05$.

environmental variables accounted for most of the variation. In total, the environmental variables we measured explained 6.3%.

In the ordination diagram (Figure 7), separation of species was observed along axis one, with the

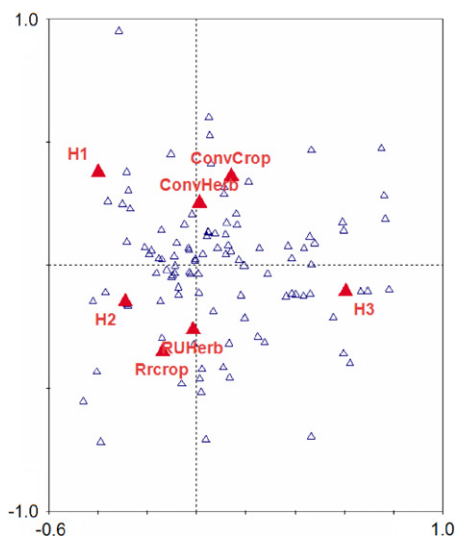


Figure 7. Ordination diagram of soil fungi (flr2) produced by canonical correspondence analysis of species frequencies using the combined data subsets of 95 samples, 144 species, and 7 environmental variables collected from agricultural soil, at the University of Guelph Research Station, Elora, Canada. Plots are of axis 1 ($\lambda = 0.214$) vs. axis 2 ($\lambda = 0.127$). The empty triangles represent species, and the solid triangles represent the environmental variables: H1 = May harvest, H2 = August harvest, H3 = October harvest. RRCrop = RoundUp Ready crop, ConvCrop = Conventional crop, RUHerb = RoundUp herbicide, ConvHerb = Conventional herbicide.

October harvest (H3) occurring far right in the ordination space, the pretreatment (H1) and August harvest (H2) towards the left of the diagram. There was reduced separation along the second axis with RoundUp herbicide and RoundUp crop grouped close together, toward the top of the ordination space, and conventional herbicide and conventional crop occurring together, near the bottom of the ordination space.

Discussion

Crop

Our study found that denitrifiers and rhizosphere fungi responded differently to our GR cropping system. For both groups, crop type made no difference to gene copy number or community structure. It is important to note that C4/EPSPS is a naturally occurring protein in agricultural soils, and was derived for genetic modification from the common soil bacterium, *Agrobacterium tumefaciens* strain CP4 (Zambryski, 1992). Thus it may produce less of an effect on soil microbial populations than modifications that introduce novel or

detrimental proteins into the soil, such as Bt-expressing crops, or cry-proteins. This lack of effect has been shown for many other microbial groups (Liu et al., 2005; Fang et al., 2005; Liphadzi et al., 2005; Weaver et al., 2007).

Still, others have found small, transient effects for microbial populations under GR crops (Dunfield and Germida, 2001; Lupwayi et al., 2007), and some have found distinct changes (Siciliano et al., 1998; Siciliano and Germida, 1999). However, most of these studies looked at all bacterial groups (i.e., 16S) while we looked at only a small portion of these bacteria (NirS). Thus, it is possible that these different groups vary in their sensitivity to C4/EPSPS, and that NirS denitrifiers may be more robust to its effects.

Herbicide

It is more likely that the addition of glyphosate would produce changes in microbial numbers than glyphosate-resistant crops. Glyphosate inhibits the synthesis of aromatic amino acids in plants and some microbes (Zablotowicz and Reddy, 2004), thus is directly detrimental to some soil microbes (Busse et al., 2001). In addition, the use of glyphosate directly changes the chemistry of the soil, most notably by the influx of carbon and nitrogen, both through the herbicide itself, and also through the increased dead fall of vegetation killed by its use (Haney et al., 2000). These inputs could change microbial numbers, either directly, as microbes respond to increased food sources, or indirectly, as they are out-competed by organisms that can use the additional food sources. The stimulating effect of glyphosate on bacteria has been shown in other studies (Haney et al., 2000; Kremer et al., 2005; Ratcliff et al., 2006). This is may be due to the input of resources (carbon). In neither case did herbicide type affect community diversity. In our study, the use of glyphosate did not increase the numbers of denitrifying bacteria or fungi. It may be that moisture levels during the fall of 2005 were too low for organisms to take advantage of the extra resources. One study on soybean showed glyphosate application in conjunction with water stress resulted in lower, not higher numbers of *Fusarium* spp. (Means and Kremer, 2007). Likewise, Zablotowicz and Reddy (2007) showed overall nitrogenase activity decreased in response to glyphosate application and soil moisture stress.

Harvest date

Harvest date had similar effects on denitrifiers and fungi. Because temperature and water

availability are important determinants of microbial growth, it is fitting that harvest date would reveal differences in microbial numbers. In fact, this finding has been shown for diverse groups of microbes, under many different cropping regimes (Busse et al., 2001; Dunfield and Germida, 2003; Griffiths et al., 2003; Schmalenberger and Tebbe, 2002; Sessitsch et al., 2004; Fang et al., 2005).

In our study, DNA concentration peaked during August for both groups. This trend was particularly striking for denitrifiers, whose copy numbers were extremely low in October. This is an unexpected finding, since denitrifiers and fungi are generally most active during the fall, when moisture levels are higher and there is an influx of senescing plant tissue. Because most of the precipitation during the 2005 growing season occurred prior to our August harvest (Table 1), it may be that our site was not more mesic in the fall.

Denitrifier diversity was higher in October than in August (Figure 3). This likely reflects a more mature rhizosphere community, one that represents both the fast growing, early successional species, and those that appear later. Harvest date was also significant in our ordination analysis (Figure 4), with the pretreatment and August harvests accounting for a significant proportion of the variation in NirS diversity. However, it is important to note that total variance explained by the environmental variables was very low (4.8% of total variance). This indicates that other, unmeasured variables were more important in determining the diversity of denitrifiers in our study.

Thus, the diversity of fungi was similar regardless of season. This is surprising since one would expect numbers of fungi to increase during favourable conditions (most notably the fall harvest) with ample resources available to saprotrophs in the form of vegetative litter. However, 2005 was a relatively dry fall, and so numbers of fungal groups detected by this experiment may not reflect the actual capacity of the environment to support more fungal taxa. Again, the total variance accounted for by our environmental variables was very low (6.3%), reflecting similar taxa regardless of treatment, or causal factors beyond the scope of this experiment.

We used T-RFLP as a proxy for community diversity, that is, each T-RF was considered to be an operational taxonomic unit (OTU). This method has been criticized since extra peaks or “species” can be detected due to restriction enzyme inefficiency, polymorphism within individuals (Avis et al., 2006), or shared T-RFLP profiles across species (Dickie and FitzJohn, 2007). However, our measures of diversity were qualitative and not quantitative, so no treatment should have extra peaks. Further,

T-RFLP allows for more replication than cloning and sequencing, so differences between communities can be assessed more easily. Finally, we standardized DNA concentration during our analysis using a method described by Dunbar et al. (2001).

Conclusion

Neither GR corn nor glyphosate had significant impacts on the denitrifying bacteria and fungi in this study. Indeed, crop type, herbicide type and harvest date, overall contributed surprisingly little to the variation observed in gene copy number and community structure for denitrifiers and fungi. This is likely because the crop system in question did not significantly alter the soil environment: the novel protein (C4/EPSPS) is already found in many agricultural soils, and is not biocidal. In contrast, GR crops, which produce novel proteins, or those that require changes to tillage, moisture or herbicide use, may significantly alter soil rhizosphere communities. The use of glyphosate, similarly, did not affect the rhizosphere microbes in this study. Instead, other, unidentified factors appear to buffer the microbial communities in question against any changes resulting from GR crops and increased use of glyphosate. This study looked at effects after only one growing season; future research must assess the long-term consequences of repeated application to determine the full effect of GR cropping systems on microbial communities.

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