Do tree-based intercropping systems increase the diversity and stability of soil microbial communities?

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1. Introduction

The region spanning southern Ontario and Quebec was historically stocked with the most diverse hardwood forests in Canada. Today, this is the country’s most populated area with a scattering of urban centers surrounded by a thriving agricultural industry. The recent expansion of livestock operations and the growing interest for bio-diesel crops are threatening the last remaining enclaves of natural hardwood forests bearing high-value tree species such as red oak (Quercus rubra L.) and black cherry (Prunus serotina Ehrh.) (Cogliastro et al., 2008). The supply of wood from species such as these is critical in maintaining Canada’s furniture and wood cabinet industry, which employs over 100,000 workers and exports goods valued at $14 billion per year. Tree-based intercropping (TBI), a silvoarable approach that combines widely spaced rows of trees and common agricultural field crops, has recently been proposed as a means of reducing the dependence of the Canadian furniture and wood cabinet industries on imports of high-value hardwood timber (Bradley et al., 2008), as well as improving soil fertility and decreasing non-point source pollution (Garrett and McGraw, 2000). Despite the potential economic and environmental benefits expected from TBI systems, their adoption rate in Canada is extremely low compared to European countries and the US. For this reason, our research team...
and collaborators set out to establish pilot study sites in southern Ontario and Quebec, that would provide scientifically vetted support for the implementation of TBI systems in eastern Canada.

One aspect of soil quality that could benefit from TBI, but has not received attention, is the effect of this cropping system on soil microbial diversity. Soil microbial diversity is characterized by several scales of variability, some of which may matter more in terms of their ecological importance. For instance, 1 g of soil may contain over a billion bacterial cells and more than 40,000 bacterial genomes (Torsvik et al., 1990). Therefore, the concept of alpha-diversity, which measures species richness and evenness within a single community, may not be ecologically relevant in the microbial world. However, the composition of independent microbial communities will vary on a larger scale according to local vegetation patterns (Lamarche et al., 2007). Soil microbial community composition at different locations may be, therefore, more diverse in TBI than in conventional monocropping (CM) systems, because higher plant diversity should increase the diversity of carbon-based resources and create more complex spatial patterns of soil properties (Thevathanan and Gordon, 2004).

This higher microbial “beta-diversity” (i.e., landscape level complexity) could play an important ecological role in maintaining microbial functions in the face of environmental stress, as it could provide the soil with a wider array of co-occurring microbial communities adapted to a wider range of environmental conditions (i.e., higher stability). Hence, we hypothesize that TBI systems will increase microbial beta-diversity and tolerance to stress compared to CM systems.

Here, we report on a study where we tested the soil microbial beta-diversity–stability relationship within TBI and adjacent CM systems, in both southern Ontario and Quebec. In each system, changes in soil microbial community composition along a sampling grid were assessed using extractable phospholipid fatty acid (PLFA) profiles. We subsequently mixed random soil samples from each grid, the mixtures were treated with increasing concentrations of a heavy metal (Cu) and microbial biomass was measured. We used Cu as a stress agent because it is a constituent of many fungicides that are applied to crop plants. The ability of the pooled microbial communities to resist Cu stress was used as an index of microbial stability.

2. Materials and methods

2.1. Study sites and sampling

The study compared TBI and adjacent CM fields at two study sites. The first site is located near the Town of St-Rémi (45°16'N, 73°36'W), Québec, Canada. Mean annual temperature is 6 °C and mean annual precipitation is 979 mm of which 22% falls as snow (Environment Canada, 2006). The TBI field was created in 2000 using alternating rows of hybrid poplar clones TD-3230 (Populus trichocarpa Torr. & A. Gray ex Hook. × Populus deltoides Bart. ex Marsh.), NM-3729 (Populus nigra L. × Populus maximowiczi A. Henry), DN-3308 (P. deltoides × P. nigra), and alternating rows of black walnut (Juglans nigra L.) and white ash (Fraxinus americana L.), with 8 m spacings between rows (Rivest et al., 2005). Soybean ( Glycine max L. (Merr.)) was grown between tree rows since 2004. This field was paired with a CM field, situated 300 m southwest, which had been planted with the same crop rotation of maize, soybean, winter wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) as the TBI field. A summary of each field’s characteristics are given in Table 1.

In each field, a rectangular grid of 56 (7 × 8) sampling points was established between tree rows, with 1 m spacings between neighbouring sampling points. In late-August (St-Rémi) and early-September (Guelph) 2006, a 10 cm diameter × 15 cm deep soil core was collected at each sampling point. The soil samples were immediately sieved (2 mm mesh) and transported on ice to coolers in the laboratory where they were kept at −20 °C until analyzed.

2.2. Soil physico-chemical properties and PLFA profiles

Each sample was analyzed for 11 physico-chemical properties. Soil moisture was determined by weight loss after drying subsamples in an air-draft oven (101 °C) for 24 h. Soil pH was measured in 0.01 N KCl (1:3, v/v) using a standard hydrogen probe. Total C and N were measured with a Macro Elemental CN Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Organic matter content was estimated from total C using a conversion ratio of 1.9 (Nelson and Sommers, 1994). KCl (1.0 N) extractable NO3−–N and NH4+–N concentrations were measured colorimetrically with the Griess-llosvay (i.e., salicylate–nitroprusside) and Nelson (i.e., sulfanilamide–N-(1-naphthyl)-ethylenediamine) methods (Mulvaney, 1996), using a Technicon Auto-analyzer (Pulse Instrumentations, Saskatoon, Canada). Following acid digestion, total base cations (K, Ca, Mg, Na) were measured using an Analyst-100 atomic absorption spectrometer (PerkinElmer Corporation, Norwalk, USA).

PLFAs were extracted from all 224 soil samples within 2 months following the method described by Hamel et al. (2006). Briefly, field-moist soil subsamples (4 g dry wt. equiv.) were first extracted with 9.5 mL dichloromethane (DCM); methanol (MeOH); citrate (1:2:0.8, v/v/v) buffer for 2 h, and then with 2.5 mL DCM plus 10 mL saturated NaOH solution for 5 min. The mixture was then centrifuged at 1000 × g for 10 min and the lipid containing phase transferred to a vial. Soil subsamples were extracted a second time with 5 mL DCM:MeOH (1:1, v/v), and the lipid containing phase combined with the first extract. PLFAs were then collected by eluting with 2 mL MeOH through a silica-gel column after discarding other fractions eluted with 2 mL DCM and 2 mL acetone. The fatty acids (FAs) were N2 gas-dried and methylated by adding approximately 0.5 mL of 1:25 (v/v) sulphuric acid:MeOH solution and heating at 80 °C for 10 min. Two mL of hexane were added to the vial and the mixture was vortexted for 30 s, then the aqueous fraction was discarded. The same procedure was repeated with ultra-pure water before a known concentration of methylated FA 19:0 (Sigma–Aldrich) was added to the sample and used as an internal standard to quantify concentrations of the 30 identified and identified indigenous FAs. The extracts were dried down under N2 gas and dissolved in 100 µL hexane, then injected in a HP 6890 gas chromatograph equipped with a flame ionization detector, a 30-m Restek Rtx-1 column, and He as carrier gas. Initial oven temperature was maintained at 140 °C for 5 min, raised to 210 °C at a rate of 2 °C min−1, then raised to 250 °C at a rate of 5 °C min−1. The FID temperature was held at 300 °C.

Microbial community structure at each sampling point was based on the concentrations of 30 identified FAs. Peaks of interest were identified based on retention time using standards (Supelco Bacterial Acid Methyl Ester Mix 47080-U, Sigma–Aldrich). The abundance of these FAs was estimated from the area under each
peak relative to the area below the 19:0 peak, calibrated according to a standard curve made from 19:0 FA standards.

2.3. Microbial stability

To assess microbial stability, we monitored changes in microbial biomass (MB) after treating five bulked soil samples from each field ($N = 20$) to increasing concentrations of a heavy metal (Cu) contaminant. Each bulked sample was comprised of five randomly chosen soil samples within each sampling grid, so as to encompass microbial beta-diversity of each field. Copper was used because it is a component of certain fungicides used in agricultural fields. Each bulked sample was divided into 24 subsamples (24 g dry wt. equiv.), and paired subsamples were then treated with 9 mL of aqueous solutions containing 0, 6, 12, 18, 24, 36, 60, 90, 120, 150, 180 and 240 mg of CuCl$_2$. Each soil sample was mixed with 25 g of sterilized sand to prevent water saturation and retain air-filled pores. The treated soils were left to incubate for 7 days after which microbial biomass was determined by substrate-induced respirometry (SIR) (Anderson and Domsch, 1978). Briefly, 500 mg of a talc:glucose (22:3) mixture was mixed into each soil sample and left to incubate for 100 min. The jar’s headspace was then flushed for 5 min with ambient air, the jar was sealed with a lid equipped with a septum and left to incubate for another 30 min. Headspace air was then sampled with a needle and syringe, injected into a Chrompack Micro GC CP-2002P gas chromatograph (Chrompack, Bergen op Zoom, Netherlands) and analyzed for CO$_2$ concentration. Net CO$_2$ production rate was obtained by subtracting ambient CO$_2$ concentration from sampled CO$_2$ concentration, and converted into an estimate of microbial carbon based on equations given by Anderson and Domsch (1978). For each bulked soil sample, microbial biomass values of all 24 subsamples were plotted as a function of Cu concentration, and this relationship was fitted, using SAS (SAS Institute, Cary, NC, USA) statistical software, to the decreasing exponential function:

$$\text{C}_{\text{mic}} = \left(\text{IC}_{\text{mic}}\right)e^{-\frac{\text{Cu}}{C_{138}}}$$

(1)

where $\text{C}_{\text{mic}}$ is microbial carbon at a copper concentration [Cu], IC$_{\text{mic}}$ is microbial carbon at a zero copper concentration (i.e., $Y$-intercept), e is the base of the natural log, and $k$ is the first-order rate constant. The estimated regression parameters of each bulked soil sample were used to predict the copper concentration ([Cu]$_{0.75}$) at which microbial biomass had fallen to the arbitrary value of 75 ng C$_{\text{mic}}$ g$^{-1}$ soil. The value of [Cu]$_{0.75}$ was then used to denote microbial tolerance (i.e., stability) to heavy metal (Cu) stress in each sample.

2.4. Data analyses

PLFA data from St-Rémi and Guelph were analyzed separately by principal component analysis (PCA). The first two principal component scores for each microbial community within the sampling grid of each field were projected on ordination biplots so as to give a graphic comparison of the spatial heterogeneity of PLFA profiles within each field. Six rare FAs were removed from this analysis because they contributed many zero values to the data matrix. In addition, PLFA data from each site were analyzed for dispersion using PERMDISP (Anderson, 2004a), a non-parametric multivariate permutational extension of Levene’s test for equality of variances. This test was performed on untransformed data using Euclidean distances. Finally, individual FAs within each sampling grid were analyzed for equality of variance and dispersion using both Levene’s test and Moses’ test. The former is more robust than Bartlett’s test for homogeneity of variance in the case of a
departure from normality in the data, and the latter is entirely non-parametric.

The PCA and PERMDISP procedures were repeated using the 11 physico-chemical soil variables that had been measured. These data were first mean-centered normalized because the variables used widely different ranges and units. We then used DISTLM v.5 (Anderson, 2001, 2004b; McArdle and Anderson, 2001), a multivariate multiple regression analysis software, to try to link physico-chemical properties and microbial PLFA profiles. This part of the analysis was limited to the St-Rémi site, where PLFA results concurred with our hypothesis.

The PLFA concentrations of known microbial groupings (Table 2) in the four fields were compared using Tukey’s post hoc comparison tests. Data was log-transformed when necessary to meet assumptions of normality and homoscedasticity. Finally, indices of microbial stability in TBI and CM systems at the St-Rémi site were compared using a Student T-test.

Table 2
Soil PLFAs associated with specific microbial groupings

| Gram positive | i14:0, a15:0, i15:0, i16:0, i17:0, a17:0, br17:0, 10 Me16:0, 10 Me18:0 |
| Gram negative | cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω7 |
| Fungi | 18:2ω6 |
| Arbuscular mycorrhizal fungi (AMF) | 16:1ω5 |

Sources: Fostegaård and Bååth, 1996; Sundh et al., 1997; Zelles, 1999; Hill et al., 2000; Madan et al., 2002; Diaz-Ravina et al., 2006; Balser et al., 2005; Hamel et al., 2006; Rinnan et al., 2007.

3. Results

The average (n = 56) values of 11 soil properties in each field are given in Table 1. In general, soil in the four fields had similar characteristics. The most striking differences were (1) higher pH in the CM than in the TBI field at St-Rémi, (2) a higher pH at Guelph than at St-Rémi, (3) higher organic matter content and higher NO₃⁻ in the TBI than in the CM field at St-Rémi, and (4) higher total base cations in the CM than in the TBI field at St-Rémi.

The average total concentration of identified FAs in the 224 soil samples was 72 ng g⁻¹. Results from PCA based on the PLFA data showed TBI and CM samples from the St-Rémi site to be segregated along the first principal component, with a greater dispersion among TBI samples (Fig. 1A). At the Guelph site, TBI and CM sample scores along both principal components had similar means and standard deviations (Fig. 1B). The first two components at the St-Rémi and Guelph sites, respectively explained 90% and 80% of the total variance in the data set.

Results from PCA based on soil physico-chemical properties showed a clear segregation of samples from TBI and CM fields at both study sites (Fig. 1C and D). The first two components explained 60% and 45% of the total variance at the St-Rémi and Guelph sites, respectively. At the St-Rémi site, biplot scores for the TBI field were more dispersed than for the CM field (Fig. 2C), but PERMDISP analysis revealed the significance of this difference to be only marginal (P = 0.058). At the Guelph site, the dispersion (i.e., standard deviation) of biplot scores for both fields was not significantly different, and PERMDISP confirmed this (P = 0.708). Multivariate multiple regression analyses (DISTLM v.5 software),

![Fig. 1. Ordination biplots resulting from principal component analysis (PCA) of 24 soil fatty acids (frames A and B) or 11 soil physico-chemical variables (frames C and D) found in TBI and CM plots at the St-Rémi and Guelph sites. The proportion of the total variance explained by the first two principal components are shown in parentheses. Bi-directional error bars pinpoint the mean ± S.D. for each field.](image-url)
performed on data from St-Rémi, failed to produce a single statistically significant model linking PLFA profiles to any one of the 11 measured soil physico-chemical properties.

Tukey’s comparisons revealed higher concentrations of arbuscular mycorrhizal fungi (AMF) FAs in TBI than in CM fields at both sites (Fig. 2A). In addition, the TBI field at St-Rémi had a significantly higher ratio of FAs originating from gram positive than gram negative bacteria (Fig. 2B). The TBI field in St-Rémi contained a higher concentration of all FAs than the other three fields (data not shown).

Microbial stability at the Guelph site was not assessed because data failed to produce a significant fit to a decreasing exponential curve or to any alternative trend. At the St-Rémi site, the data conformed well to the exponential decay function, with an average $R^2 = 0.74$ ($P < 0.0001$) for all regression curves, and microbial stability (i.e., $\mu_{0.75}$ values) was significantly higher in the TBI (0.225 ± 0.058) than in the CM (0.102 ± 0.078) field.

4. Discussion

Compared to CM systems, TBI systems present a more heterogeneous vegetation cover and, by implication, a patchier distribution of leaf litter and rooting patterns that can affect chemical and nutritional soil properties. For example, Thevathasan and Gordon (1997) showed that soil mineral N and total C pools in a TBI system were higher within a 2.5 m margin close to tree rows compared to the middle of the crop alley. Likewise, we found a greater spatial heterogeneity of soil physico-chemical properties in TBI compared to CM plots at the St-Rémi site. Soil properties such as pH and available C are expected to control microbial community structure (Jiang, 1996; Drenovsky et al., 2004), hence a greater spatial heterogeneity of soil physico-chemical properties in TBI compared to CM systems at the Guelph site did not differ as they did at the St-Rémi site. Obvious differences between both sites are the types of crops that were grown previously to and during the summer of 2006, as well as differences in soil textural class. Further research should, perhaps, strive to understand how these two factors interact with field-grown trees to control the spatial heterogeneity of soil microbial communities. For example, soybean is a crop that requires less N fertilizer (30 kg N ha$^{-1}$) than corn (120–170 kg N ha$^{-1}$) (CRAAQ, 2003). The effect of high mineral fertilization rates may perhaps mask the effect of plant diversity on microbial communities. For example, Ruppel et al. (2007) found a higher prokaryotic phylogenetic diversity, and a higher diversity of substrate utilisation, in soils receiving low rates of mineral N fertilizers than in heavily fertilized soils. They hypothesized that fertilization results in the selection of specialized microbial communities, hence in fewer functional groups. If this is so, then the reduced fertilization that can be expected in TBI systems over the long term (Thevathasan and Gordon, 2004) could also be beneficial in increasing microbial beta-diversity. As for the differences in soil textural class between the two sites, there is growing evidence that this also can be an important factor controlling microbial community structure (Yao et al., 2006; Lamarche et al., 2007). For instance, Bossio et al. (1998) found higher concentrations of FAs associated to anaerobic bacteria in soils with high clay content, and higher concentrations of FAs associated to aerobic bacteria and fungi in sandier soils. We assume that a clay loam texture, such as at the St-Rémi site,
provides a better structured soil profile than a sandy loam texture, such as at the Guelph site, resulting in a more diverse array of microsite conditions accommodating a wider range of bacterial niches. The higher clay content at the St-Rémi site can result in a greater retention of soil organic matter (Alvarez and Lavado, 1998), especially in TBI plots where litter inputs are greater (Thevathasan and Gordon, 2004). Higher organic C provides more energy yielding substrates and increases microbial diversity (Degens et al., 2000). Consequently, TBI systems may preferentially enhance microbial beta-diversity on heavier textured soils. Higher concentrations of FAs indicating AMF fungi were observed in TBI systems at both sites. The multiple beneficial effects of these symbionts on plant growth are well known (e.g., Jeffries et al., 2003), and AMF community assembly is the focus of an increasing amount of theoretical work on ecosystem functioning (e.g., Maherali and Klironomos, 2007). It would be worthwhile to apply these same fundamental principles to issues of agroforestry and land management. For example, repeated tillage in CM systems can result in diminished AMF densities in soil over time (Kabir et al., 1998), but this tendency could be countered in TBI systems by tree roots providing sustained AMF inocula for successive intercrops. If shown to be true, such an interaction between annual and perennial plant species would have far reaching ramifications, including higher soil aggregate stability in TBI systems due to higher concentrations of glomalin-related proteins in soil (Rillig and Mummey, 2006). The TBI system at the St-Rémi site also had a significantly higher ratio of gram positive to gram negative bacteria. Gram positive organisms tend to have more capacity to sporulate (Doi, 1989), which gives them an advantage in adverse environmental conditions. They also include actinomycetes, which are prolific decomposers (Srinivasan et al., 1991) and producers of antibiotics (Martin et al., 2003).

5. Conclusion

Our hypothesis regarding soil microbial beta-diversity and stability were confirmed at only one of the two sites, thus our conclusions warrant prudence and discretion. More studies of this type need to be accomplished, therefore, with the proper level of replication. Nevertheless, our study has provided the first evidence that microbial beta-diversity may be higher in TBI systems, perhaps more so in heavier textured soils or soils receiving low fertilizer inputs. Our results also suggest that, where applicable, increased microbial beta-diversity may be related to higher microbial stability to withstand heavy metal stress. Finally, our data point to a more prominent presence of AMF fungi and other beneficial soil microbes in TBI systems and this, we believe, should be addressed by future research projects vying to provide scientific arguments favouring the implementation of TBI systems in Canada’s rural landscape.

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