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It Takes an Individual Plant to Raise a Community: TRFLP Analysis of the Rhizosphere Microbial Community of Two Pairs of High- and Low-Metal- Accumulating Plants

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Title: It takes an individual plant to raise a community: TRFLP analysis of the rhizosphere microbial community of two pairs of high- and low-metal-accumulating plants

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Abstract

We used terminal restriction fragment length polymorphism (TRFLP) analysis to look at the microbial community profiles of the rhizosphere surrounding two pairs of high- and low-metal (Cd)-accumulating plants (*Brassica* and *Triticum*). Unexpectedly, the microbial community did not vary with soil type, time, plant type, or metal-accumulating ability of the plant. Instead, when a plant's metal-accumulating ability was well matched to the level of metal contamination in the soil, the microbial populations in the rhizosphere were different than those of the seed endophytes and bulk soil. Unmatched plants had the same microbial community as bulk soil. The plant interaction with the soil, therefore, is essential to forming the bacterial community in the rhizosphere.

Keywords: microbial community, rhizosphere, TRFLP, cadmium

Cadmium occurs naturally in soil but its concentration in agricultural systems may increase after the application of manure and phosphate fertilizers (Alloway *et al.*, 1999; Grant, 2011). Natural variation in the uptake of toxic metals exists among plant species and varieties within a species including durum wheat (Grant *et al.*, 2008). The distribution of Cd in two cultivars (cv) of durum wheat (*Triticum durum* Desf.), Arcola and Kyle, confirmed Kyle as a high-accumulator and Arcola as a low-accumulator (Chan and Hale, 2004). Similarly, *Brassica juncea* is an established metal high-accumulator, while *B. napus* is a low-accumulator (McGrath *et al.*, 2001). The use of these pairs of *Triticum* and *Brassica* offers a good system to study the relationship between Cd tolerance and the microbial community in the rhizosphere. The microbiome is often described as providing a potential mechanism for differential metal accumulation in plants, and can aid in the plant's response to toxic metals. However, to our knowledge there has been no attempt to characterize how the host plant's inherent metal tolerance may shape its rhizosphere community.

Surface-sterilized seeds of high Cd-accumulating *T. durum* cv Kyle and *B. juncea*, as well as low Cd-accumulating *T. durum* cv Arcola and *B. napus* were individually sown in 288 cm³ pots containing one of two soils. Fertilized (high Cd) and unfertilized (low Cd) soil was collected from the top 7.5 cm of an agricultural field near Brandon, Canada. One half of the field had received Cd-contaminated fertilizer from 2002 to 2009. Soil subsamples were sent to A&L Canada Laboratories Inc. (London, Ontario) for analysis of Ca, Mg, NO₃⁻, P, K, Na, B, Cu, Fe, Mn, Zn, Al, % organic matter, pH and cation exchange capacity. The concentrations of total and bioavailable Cd in the soils were determined using a modified United States Environmental Protection Agency test method SW-846 (US EPA, 2000) and Novozamsky *et al.* (1993), respectively, and inductively-coupled plasma atomic emission spectrometry as described in Akhter and Macfie (2012). Among the properties that were measured, only the concentrations of P and Cd varied between the two soils. Before beginning the experiment, 0.172 g/pot P, as Triple Superphosphate (Plant Products Co. Ltd., Brampton, ON), was added to the unfertilized soil to balance the phosphorus concentration. The current Canadian Council of Ministers of the Environment limit for Cd is 1.4 mg/kg soil (CCME, 2013). Both the unfertilized and fertilized soils were above this threshold at 2.38 ± 0.17 and 3.07 ± 0.10 mg/kg (total Cd) and 0.47 ± 0.01 and 0.73 ± 0.02 mg/kg (bioavailable Cd), respectively. Although both soils are Cd-contaminated, we have labeled them as having relatively less or more Cd for the purposes of our experiment. All pots were kept in a growth chamber maintained at 21°C and 60% RH with

a 16:8 hour light:dark cycle and $230 \pm 5.7 \mu\text{mol}/\text{m}^2/\text{s}$ light intensity. One treatment in each soil type was left unplanted to represent bulk soil. Each treatment contained 4 replicates. Plants and soils were harvested after 28 d.

The relative Cd-accumulating ability of each plant species was confirmed following the modified US EPA (2000) method described in Akhter and Macfie (2012). The tissue Cd concentrations were consistent with the relative metal-accumulating abilities of these plants (Chan and Hale, 2004; McGrath *et al.*, 2001). None of the plants showed signs of Cd-stress; therefore, the resulting changes in the rhizosphere communities were not stress-induced.

Bacterial community profiles were generated for unsown seeds, bulk soil, and the rhizospheres. Surface-sterilized seeds for all species were pulverized to obtain the endophytes. Bulk soil samples were collected directly from each unplanted pot. To collect the rhizosphere soil, plant roots were shaken to remove loose soil particles then placed in a 50 mL tube with 25 mL of distilled water and vortexed. Samples were then centrifuged and the soil pellet was collected as the rhizosphere. Total community DNA was extracted from 250 mg of seed tissue, dry bulk soil, or rhizosphere soil using a NORGEN Soil DNA Isolation Kit (Cat # 26500, Biotek Corporation, USA) and diluted to exactly $30 \mu\text{g}/\mu\text{L}$ in filter-sterilized 10 mM Tris, pH 8.0. Bacterial 16s rRNA genes were then PCR-amplified using the fluorophore-labeled primer set 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Marchesi *et al.*, 1998) and PCR parameters outlined in Osborn *et al.* (2000). The amplified DNA was purified using DNA Clean and ConcentratorTM – 5 Kit (Zymo Research Corporation, USA – cat # D4014) before digestion with the restriction enzyme *HhaI* (New England Biolabs Inc.). The samples were sent to the Advanced Analysis Centre Genomic Facility (University of Guelph, Guelph, Ontario) for TRFLP analysis.

The TRFLP fragments were analyzed using GeneMarker[®] AFLP/Genotyping Software version 2.2.0 after undergoing manual alignment. The matrix generated by GeneMarker was turned into a presence/absence matrix, a Bray-Curtis similarity matrix was generated in Primer 5.2.4 and non-metric multidimensional scaling (NMDS) plots were generated using 10 iterations. Analysis of similarity (ANOSIM) was performed for all samples including all factors: time, soil type, genus, plant type, metal accumulating ability, and accumulating ability/soil type match. For this last parameter, the inherent metal-

accumulating ability of the plant and the relative Cd content of the soil were considered: high-accumulating plants in more contaminated soil and low-accumulating plants in less contaminated soil were considered to be “matched” and high-accumulating plants in less contaminated soil or low-accumulating plants in more contaminated soil were considered to be “unmatched”. For all statistical tests a p value of ≤ 0.05 was considered significant.

The following factors were not a source of the differences among bacteria communities: soil type (which includes fertilization regime and therefore Cd concentration and possible differences in P availability; Fig 1A), time (Fig 1 B), plant genus, plant species, and Cd-accumulating ability (data not shown). The lack of difference between the soil types suggests that the concentration of Cd in the fertilized soil was not high enough to select against any bacteria found in the unfertilized soil.

The endophytic bacteria were different between the plant genera and the *Triticum* cultivars (Fig 1 C), which is not surprising (Ahlholm *et al.*, 2002; Manter *et al.*, 2010). If the parent plant were grown in contaminated soil, the endophytes in the seed might be selected to persist with the seedling in a similarly contaminated environment, and *vice versa*. However, the endophyte community profile was markedly different from bacterial communities in both the bulk and rhizosphere soils (Fig 2). Close clustering in an NMDS plot suggests negligible variation among the bacterial communities (one point on the plot represents one community).

The bacteria communities in the rhizosphere of plant types grown in soil that matched their accumulating ability (i.e. Cd-accumulating plant in soil with more Cd or non-accumulating plant in soil with less Cd)) were different than those of plant types grown in unmatched soils (i.e. Cd-accumulating plant in soil with less Cd or non-accumulating plant in soil with more Cd)) (Fig 1 D). This suggests that the plant’s interaction with the soil is essential to forming the rhizosphere bacterial community. The bacterial communities in the rhizosphere of the unmatched plants were the same as the communities in the bulk soil, while the bacterial communities in the rhizosphere of matched plants were significantly different (Fig 2) for all plant types. The plants grown in unmatched soil do not appear to influence the soil environment in a way that would allow new or different bacteria to colonize the rhizosphere. The bacterial communities in the rhizosphere of plants grown in matched soil had the greatest variation among the bacteria communities

(Fig 2), which suggests that these plants were less selective when recruiting soil microbes than were the unmatched plants, which clustered together.

Different bacteria communities in the matched plant/soil rhizosphere compared to the endophyte or bulk soil communities suggests that the matched plants impose greater change to their soil environment, which is reflected in the three-fold higher number of unique fragments detected in the matched compared to the unmatched rhizosphere (Fig 3). The bulk soil samples did not contain any unique fragments. Approximately 40% of the fragments were found in all three treatments. Exudation of compounds that influence the composition of the soil microbial community (Doornbos *et al.*, 2012) may have led to the proliferation of certain bacterial species to bring them above the TRFLP threshold and the subsequent increase in the number of fragments that were detected in the matched rhizosphere. The mechanism used by matched plants to alter the microbial community in the rhizosphere is not known and further investigation into the significance of matching plant metal-accumulating ability to the contamination level of the soil is needed, including the testing of additional low- and high- accumulating pairs of plants. Since the seed endophytes were ruled out as a source of this variation, the most important factor is likely related to chemical changes in the rhizosphere.

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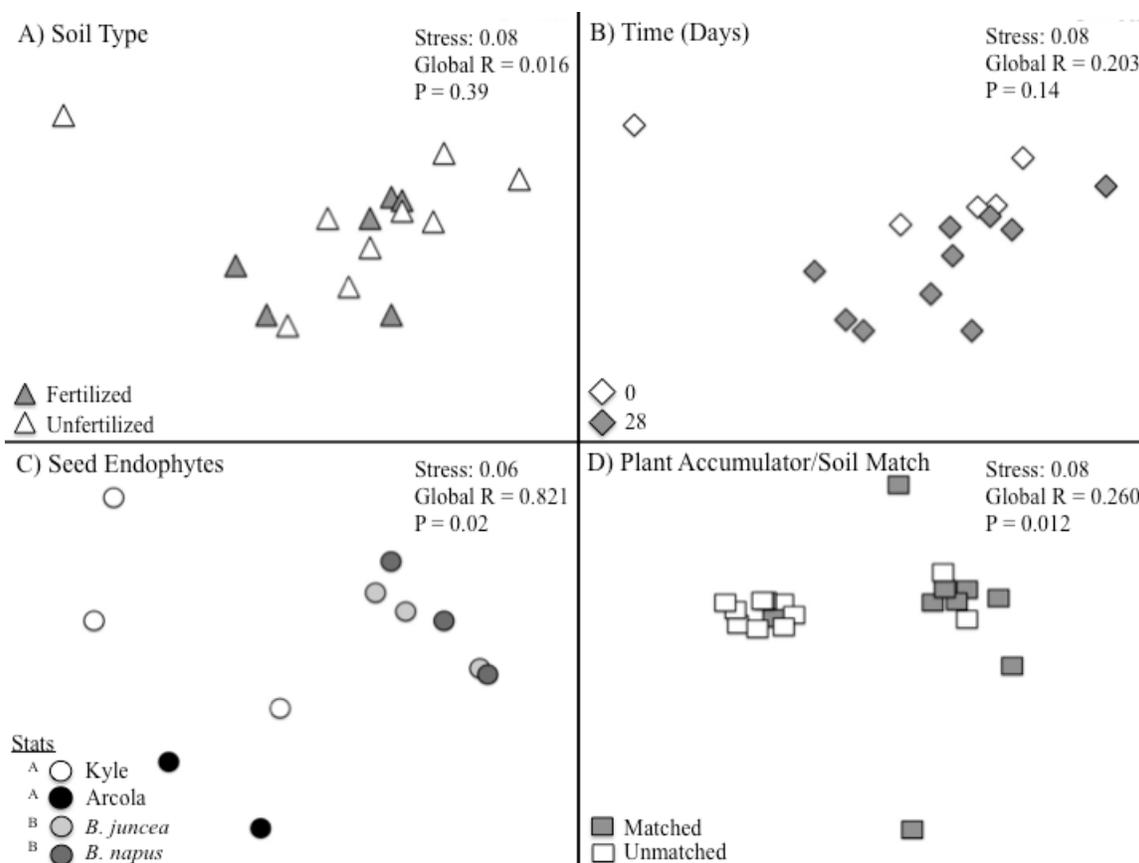


Fig 1. NMDS ordination based on Bray-Curtis similarities of soil bacteria community TRFLP profiles. Dis(similarity) is reflected in the distance between points for A) soil types B) time C) seed endophytes and D) plant accumulating ability/soil type match. Global R and P are based on the overall trend. No significant difference was detected in panel A or B. Seed endophytes not sharing a common letter in the stats column of panel C were significantly different. There was a significant difference between matched and unmatched soils in panel D (ANOSIM followed by a pair-wise post-hoc test, $p < 0.05$, $n = 3$).

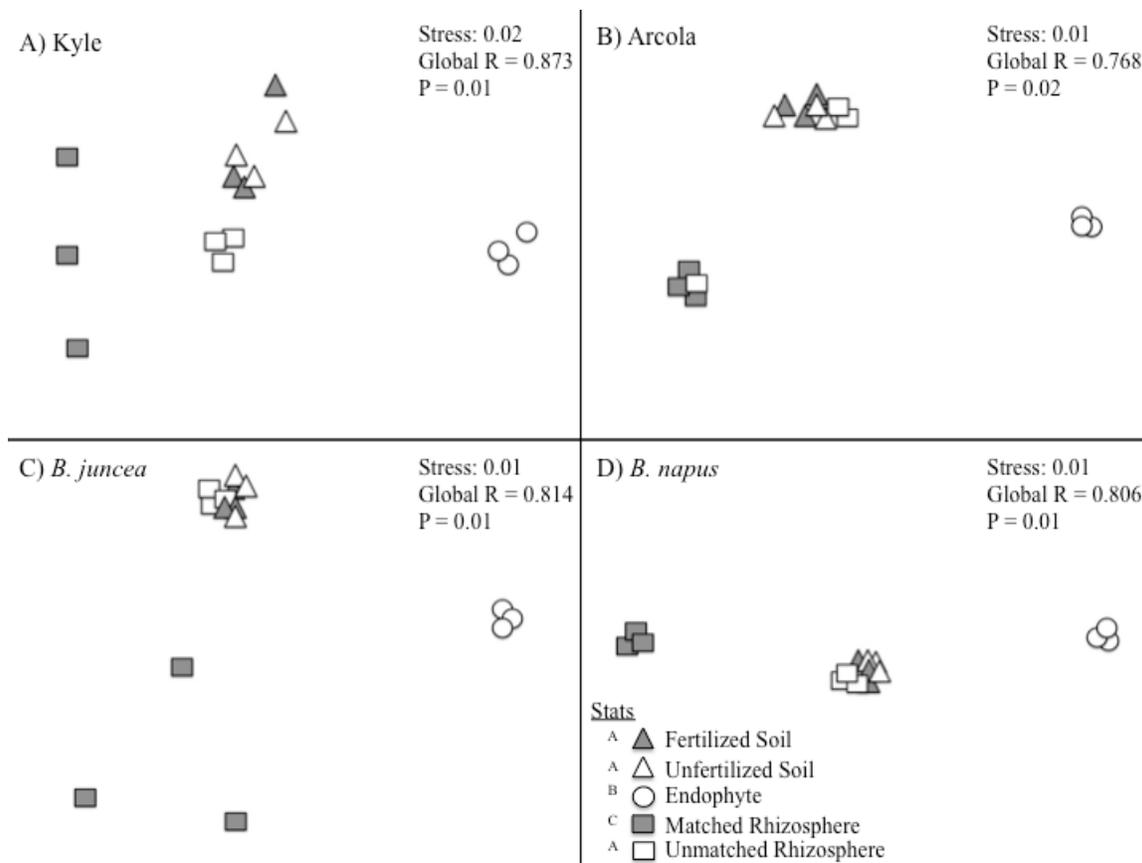


Fig 2. NMS ordination based on Bray-Curtis similarities of bacteria community TRFLP profiles among bulk soil, endophytes, matched, and unmatched soil/plant communities for each plant type. Dis(similarity) is reflected in the distance between points for A) *Triticum durum* var. Kyle B) *T. durum* var. Arcola, C) *Brassica juncea* and D) *B. napus*. Global R and P are based on the overall trend. Statistical results were the same for all 4 panels therefore the stats legend applies to all panels. Treatments not sharing a common letter in the stats column were significantly different (ANOSIM followed by pair-wise post-hoc test, $p < 0.05$, $n = 3$).

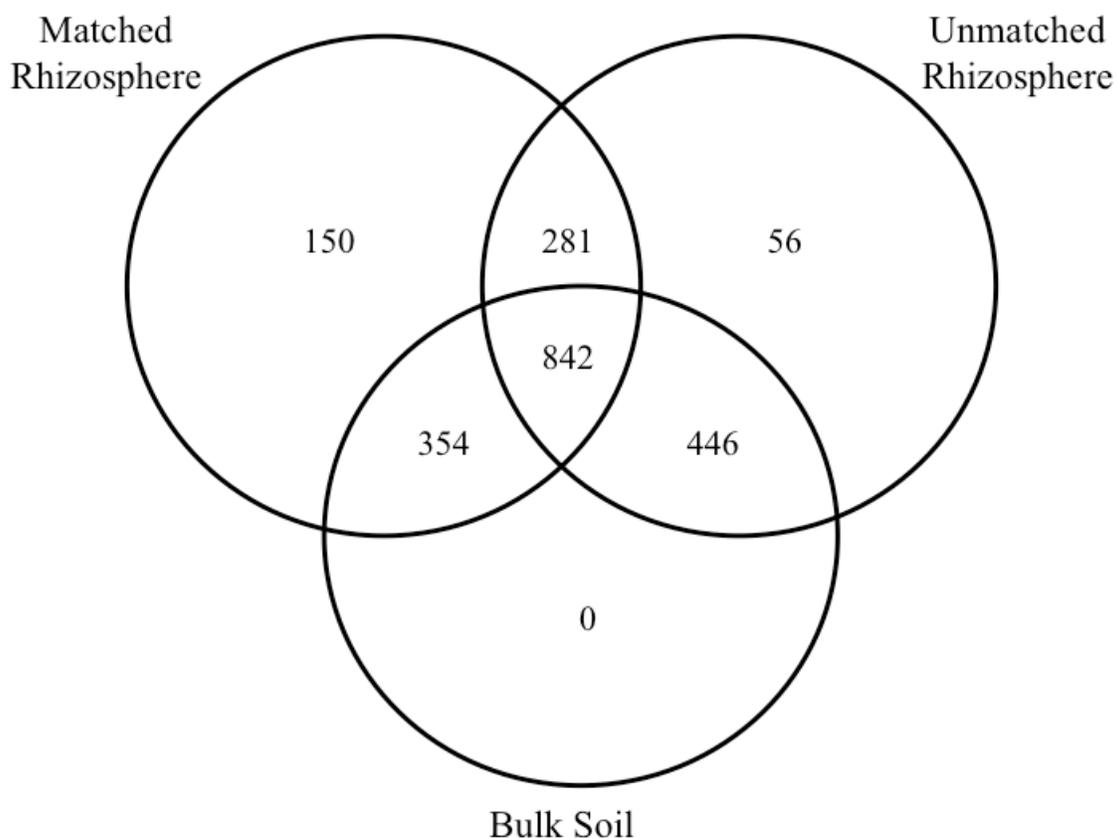


Fig 3. Schematic representation of the number of common fragments among matched rhizosphere, unmatched rhizosphere, and bulk soil samples. The numbers of fragments are based on pooled TRFLP fragment presence/absence data. Within the matched rhizosphere treatment, samples from plants with high accumulating ability in more contaminated soil and those with low accumulating ability in less contaminated soil were pooled. Similarly, within the unmatched rhizosphere treatment, samples from plants with high accumulating ability in less contaminated soil and those with low accumulating ability in more contaminated soil were pooled. For the bulk soil treatment, the two soil types were also pooled. These pools were deemed acceptable since no significant differences were found among the components of each pool (Fig 1 and Fig 2).