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Adaptation of plants and arbuscular mycorrhizal fungi to coal tailings in Indiana

Wendy I. Taheri*, James D. Bever[1]

Biology Department, JH 149, Indiana University, 1001 E. 3rd Street, Bloomington, IN 47405-7005, United States

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ABSTRACT

We tested the potential for arbuscular mycorrhizal fungi to mediate plant adaptation to mine soil conditions utilizing a full factorial experiment involving two fungal communities, two ecotypes of plants and two soil types. We found that plants grew larger with fungal communities derived from mine soil regardless of the soil type in which they were grown. There was no evidence that the plants suffered from aluminum toxicity; however, plants grown in coal tailings produced far less biomass than those grown in low-nutrient clay soil. *Andropogon virginicus* L. grown from seeds collected from a coal mine had increased allocation to roots in sterile soil. *Plantago lanceolata* L. grown from seeds collected from a coal mine also showed an increased allocation to roots. We concluded that harsh edaphic conditions may help reinforce the symbiotic relationship between plants and AM fungi, resulting in more beneficial symbionts.

1. Introduction

Numerous studies have demonstrated adaptation of plants to harsh environments, particularly those represented by metal contaminants as results from mining operations and other anthropogenic pollution (Samuels et al., 1997; Rincon and Gonzales, 1992). These studies demonstrate that plant adaptation can occur quickly. For example, Jules and Shaw (1994) found that plants show heritable resistance to zinc, cadmium and lead in areas contaminated by smelters for less than 90 years. While many studies demonstrate plant adaptation to mine waste, fewer studies have evaluated the potential for root symbionts to contribute to plant adaptation. Arbuscular mycorrhizal (AM) fungi, which associate with most plant species and can serve as intermediaries for uptake of soil nutrients, might be particularly important in mine tailings.

AM fungi have been shown to alter uptake of metals by plants, in some cases enhancing uptake (Killham and Firestone, 1983; Davies et al., 2002; Jamal et al., 2002; Dosskey and Adriano, 1993; Vogel-Mikus et al., 2005; Cumming and Ning, 2003) and in other cases reducing uptake (Gildon and Tinker, 1981; Tullio et al., 2003; Toler et al., 2005; Vivas et al., 2005). For example, Cumming and Ning (2003) found that AM fungi conferred Al resistance to *Andropogon virginicus* L. reducing Al uptake and translocation in host plants, whereas Dosskey and Adriano (1993) showed that AM fungi facilitated uptake of zinc in cucumber grown on weathered coal fly ash.

The response of plants to mycorrhizal colonization combined with toxic metal exposure varies with plant species, fungal community, biotic and abiotic conditions, concentration of toxins and pH. This demonstrates complex interactions involving many variables. Yet few studies have examined plant response in terms of adaptation of their symbionts, particularly in comparison to unadapted communities.

We tested the contribution of AM fungal symbionts to plant adaptation under the harsh edaphic conditions found in mine soil. We employed a full factorial experiment with plant populations and fungal communities derived from mine tailings as well as from non-mine sites. Plants, along with their associated microbial communities, were grown in both low-nutrient clay soils and in mine tailings. We repeated this experiment across two plant species using a common grass (*A. virginicus* L.) and a forb (*Plantago lanceolata* L.).

Common garden experiments are a widely used and well-accepted means of evaluating heritable variation within a population (Callaway and Maron, 2006; Schultz et al., 2001; Stahl et al., 1990). Karhu et al. (1996) found the common garden method to be a better predictor for an adaptive trait (bud set) in Scots pine than the standard molecular markers typically used. Notably, this method cannot account for maternal effects.

* Corresponding author. Tel.: +1 812 855 5969; fax: +1 812 855 6705.
E-mail addresses: wtaheri@indiana.edu (W.I. Taheri), jbever@indiana.edu (J.D. Bever).
[1] Tel.: +1 812 855 0771; fax: +1 812 855 6705.

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2. Materials and methods

2.1. Study sites

We chose an open-pit coal mine, designated site 289 by the Indiana Department of Natural Resources, Reclamation Division, as our source site for plant seeds and AM fungi adapted to post-mine conditions. Site 289 is two miles east of Midland, Indiana, in Greene County. It was mined for three years and then abandoned in 1993. It covered an area of 2.5 acres and had a small (approximately 0.5 acres) pond that was dominated by cattails (*Typha*). The pond was heavily polluted. The water was typical of the acid mine runoff known as yellow boy. The mine soil had a pH of 3.83. Aluminum goes into solution when the pH drops below 5.0. Aluminum toxicity inhibits root development at exposure levels as low as 200 μM Al (Cumming and Ning, 2003; Matsumoto, 2000). Low pH and aluminum toxicity are common problems associated with coal mine restoration.

Most of the AM fungi for the mine ecotype (FM) came from plants growing near the banks of the sludge pond. The AM fungal community was dominated by *Paraglomus occultum* but also harbored an undescribed *Entrospospora* species and *Glomus mosseae*.

We harvested seeds from plants that were growing in isolated clumps, so resource transfer along hyphal networks between non-mine soil and coal tailings would not be an issue. Coal tailings were collected and brought from the site to serve as our mine soil (MS).

Our control site was five miles west of Site 289, in Greene County, Indiana, and was near a lake. Although the area had been mined and restored more than ten years ago, the location we selected to collect plants and AM fungi contained an abundance of native plant species, whereas much of the surrounding area was remedial monoculture. We believe this area may have been a small remnant or ridge that was left undisturbed when the area was originally mined.

Control site inoculum showed higher diversity but also contained a large fraction of *G. mosseae*, *P. occultum*, and a few spores from the genera *Entrospospora* and *Acaulospora*. Spore extractions performed at the end of the greenhouse experiment turned up almost all *P. occultum* in both the FM (Fungi from the Mine) and FN (Fungi from the control site or Non-mine) cultures, with only a few vagrant spores of other genera.

Plant selection was based upon the plants we could harvest seeds from at both sites, and which plants had sufficient germination rates to provide enough seedlings for the experiment. The species that fit these criteria were *A. virginicus* and *P. lanceolata*. For logistic reasons, and to reduce disturbance at the control site, we used soil from a road cut near Bloomington, Indiana, as our non-mine soil for the greenhouse experiment.

A. virginicus, commonly known as broomsedge, is a C4 perennial grass native to the eastern United States. It is a weedy species that is common along roadsides and on abandoned coal mines (Campbell, 1983). *P. lanceolata* is also a weedy species found along roadsides, infesting lawns, and in dry, harsh environments. It is a perennial dicot common in most of the United States (USDA, plants database).

Spores from the mine and control site were extracted using the sucrose-centrifuge method (Bever et al., 1996). While whole inoculum was being mixed on a magnetic stirrer, 25 ml aliquots were pipetted from the beaker and then decanted into each pot. Controls received a filtered rinse that was passed through a number 300 sieve, followed by a Whatman® filter to remove hyphae and spores. Prior to decanting, nematodes and insect eggs were picked out under a Nikon dissection scope (Model SMZ-U) fitted with a zoom 2×–8× objective coupled with 10× oculars. The extracted spores were rinsed in a 400 μm sieve and the rinse water was captured and passed through Whatman® filter paper to remove bits of hyphae. Rinsed water from both the mine and non-mine spores was combined and utilized as the microbial wash for the F0 treatment.

2.2. Experimental design

A greenhouse experiment utilizing a full factorial randomized block design was implemented. It included two soil types, three inocula and two ecotypes of two different species of plants. The entire experiment was replicated ten times for a total of 240 plants, which were seeded directly into Deepots™ (Stewe & Sons). Pots were model D40, type H, which were 25 cm deep, with a diameter of 6.4 cm and a total volume of 656 ml.

The two soil types were mine soil (MS) and non-mine soil (NS). Mine soil was composed of coal tailings from the mine mixed with sand in a 50:50 ratio. Non-mine soil was composed of low-nutrient clay soil mixed with sand in a 50:50 ratio. Both media were passed through a 1-in. wire mesh to remove large clumps and turned in a cement mixer to assure thorough mixing of sand and soil/coal. The media were steam pasteurized for three days in a flatbed steamer. This method had previously been tested and verified by our laboratory as sufficient to kill AM fungi.

Fungal treatments were fungi collected from the mine (FM), fungi collected from the control site or non-mine fungi (FN), and a microbial wash (F0). Plant ecotypes for each species were designated mine plants (PM) and non-mine plants (PN). Five blocks were established, each consisting of two full replicates of the experiment and randomized within each block, for a total of 240 pots and 10 complete replicates of the experiment. Analysis of variance found no block effects.

Plants were grown for four months in a greenhouse, with a sixteen-hour day. Each pot received 50 ml of Peterson's 15-0-15 fertilizer, diluted to 5.5 g/L, on a weekly basis. Plants were watered daily. At harvest, plants were cut even with the soil, then dried for 3 days in a 60 °C oven and weighed for above-ground biomass. The remaining roots and soil were removed intact from the deepots, weighed, and then divided roughly in half lengthwise. Each half was then weighed. Roots were extracted from one half of the divided sample, dried for three days in a 60 °C oven and weighed again. Total root biomass was extrapolated based on the fraction of soil. The other half of the roots and soil were reserved as inoculum for a subsequent experiment. Root samples from every pot were stained and checked for absence or presence of AM fungi. All inoculated plants were found to harbor AM fungi and none of the F0 controls were colonized by AM fungi.

Inoculum potential was evaluated using sorghum that was planted in each inoculum type (FM and FN). Two dilutions were prepared, pure inoculum and inoculum diluted 1:10 by volume with sterile sand. The latter represents the same dilution level used for the experiment. Five replicates of each treatment were planted and harvested after 40 days. Roots were stained in trypan blue and mounted on slides. Arbuscules, vesicles and hyphae were counted and scored per McGonigle et al. (1990). No significant differences were found between the two inocula, based on a two-way analysis of variance.

2.3. Statistical analysis

Plant biomass was analyzed with SAS using analysis of variance (Tables 1 and 2). Total biomass was replaced by ranks in order to achieve homogeneity of variance. Root–shoot ratios were arc-sin transformed for the analysis. Plant population, soil type, inoculum and all interactions were tested. The inoculum main effect and interactions were decomposed into two orthogonal contrasts: one for the average effect of mycorrhizal inoculum and the second for differences between the two inoculum sources.

Table 1
Statistical results for *A. virginicus*. Peco is plant ecotype. Feco is fungal ecotype. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi. Live vs. F0 is the average affect of inoculation (FN and FM combined) vs. no inoculation with AM fungi. PN is the non-mine plant ecotype. PM is the mine plant ecotype.

Total biomass (TBM replaced by ranks)				Ars root–shoot ratios			Percent root		
Source	DF	SS	*p*-Value	DF	SS	Colonization	DF	SS	*p*-Value
Block	4	453	NS	4	0.063	<0.0001	4	0.226	NS
Soil	1	16,463	<0.0001	1	0.288	<0.0001	1	0.463	0.0034
Peco	1	1353	0.0024	1	0.002	NS	1	0.066	NS
Soil × Peco	1	5	NS	1	0.014	NS	1	0.024	NS
Feco	2	88,661	<0.0001	2	2.264	<0.0001	1	0.992	<0.0001
Live vs. F0	1	84,808	<0.0001	1	2.259	<0.0001			
FM vs. FN	1	4444	<0.0001	1	0.002	NS			
Soil × Feco	2	8351	<0.0001	2	0.287	<0.0001	1	0.315	0.0148
Soil × F0	1	8340	<0.0001	1	0.254	<0.0001			
Soil × FM vs. FN	1	23	NS	1	0.029	NS			
Peco × Feco	2	49	NS	2	0.072	0.05	1	<0.000	NS
Peco × F0	1	16	NS	1	0.064	0.02			
Peco × FM vs. FN	1	32	NS	1	0.007	NS			
Soil × Peco × Feco	2	381	NS	2	0.002	NS	1	0.078	NS
Error	100	13,917	<0.0001	100	1.23	<0.0001	64	3.211	0.0003

3. Results

3.1. A. virginicus

Plants from the mine differed in average size and root–shoot ratio compared to the control population. Plants from the control site grew 12% larger than plants from the mine (Fig. 1 and Table 1, *p*-value = 0.002). This difference was not apparent in either ecotype of uninoculated plants, all of which were stunted and had an average total biomass less than 0.1 grams per plant (Fig. 2a). Uninoculated plants had root–shoot ratios 90% greater than inoculated plants, regardless of plant ecotype (Fig. 3 and Table 1, *p*-value = 0.05). However, uninoculated plants from the mine (PM) produced a root–shoot ratio 12% higher than uninoculated plants from the control site (PN). Inoculated plants showed no significant difference in root–shoot ratios between plant ecotypes. Interactions between plant population and soil type were not significant.

Total biomass was more than three times higher in non-mine soil than in mine soil. Plants responded positively to inoculation with mycorrhizal fungi and were particularly responsive to the fungi derived from the mine soil. Plants associated with fungal communities from the mine (FM) produced 69% more biomass than plants associated with fungal communities from the control site (FN) in non-mine soil, and 59% more biomass in mine soil (Fig. 2a and Table 1, *p*-value < 0.0001).

Plant root–shoot ratios were affected by both soil type and inoculation state (inoculated vs. uninoculated). Uninoculated plants in mine soil had root–shoot ratios 44% higher than uninoculated plants in non-mine soil, in spite of their depauperate total biomass (Fig. 4 and Table 1, *p*-value < 0.0001). In mine soil they produced root shoot ratios 113% higher than inoculated plants grown in the same media (Fig. 4 and Table 1, *p*-value < 0.0001). In non-mine soil, uninoculated plants produced root–shoot ratios 64% higher than their inoculated counterparts (Fig. 4 and Table 1, *p*-value < 0.0001). There was no significant difference in root–shoot ratios for inoculated plants, with regards to soil type (Table 1). Three way interactions were not significant (Table 1).

3.2. P. lanceolata

In non-mine soil, fungal communities from the mine (FM) promoted a 36% increase in total biomass over the non-mine fungi (FN), and a 122% increase over uninoculated plants (Fig. 7 and Table 2, *p*-value = 0.06). In mine soil, plant biomass production with either fungal ecotype was approximately equal. Uninoculated plants in non-mine soil produced an average biomass of 4.5 g. In mine soil, their average biomass was 0.18 g.

Plants from the mine (PM) had a root–shoot ratio 11% higher than the non-mine (PN) ecotype (Fig. 5 and Table 2, *p*-value = 0.05). In mine soil, uninoculated *P. lanceolata* more than doubled its

Table 2
Statistical results for *P. lanceolata*. Peco is plant ecotype. Feco is fungal ecotype. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi. Live vs. F0 is the average affect of inoculation (FN and FM combined) vs. no inoculation with AM fungi. PN is the non-mine plant ecotype. PM is the mine plant ecotype.

Total biomass (TBM replaced by ranks)				Ars root–shoot ratios		
Source	DF	SS	*p*-Value	DF	SS	*p*-Value
Block	4	814	NS	4	0.0911	NS
Soil	1	93,197	<0.0001	1	0.7173	<0.0001
Peco	1	48	NS	1	0.0556	0.05
Soil × Peco	1	30	NS	1	0.0004	NS
Feco	2	20,379	<0.0001	2	1.0926	<0.0001
Live vs. F0	1	19,447	<0.001	1	1.0926	<0.0001
FM vs. FN	1	925	0.0054	1	0.0000	NS
Soil × Feco	2	645	0.0646	2	0.6174	<0.0001
Soil × F0	1	102	NS	1	0.5979	<0.0001
Soil × FM vs. FN	1	543	0.0317	1	0.0195	NS
Peco × Feco	2	23	NS	2	0.0538	NS
Peco × F0	1	1	NS	1	0.0345	NS
Peco × FM vs. FN	1	22	NS	1	0.0193	NS
Soil × Peco × Feco	2	211	NS	2	0.0113	NS
Error	100	11,446	<0.0001	100	1.4220	<0.0001

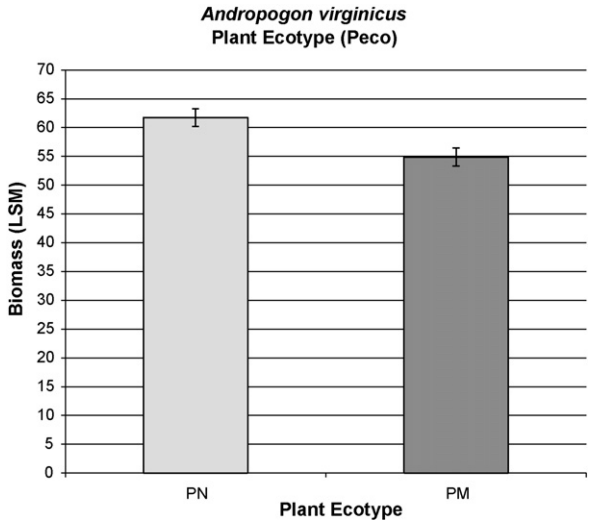

Fig. 1. Plant biomass is reported by SAS as LSM (least squares mean), but since the experiment is balanced, LSM is equal to mean. PN is the plant ecotype from the control site. PM is the plant ecotype from the mine. Reported averages are across all treatments ($p = 0.002$).

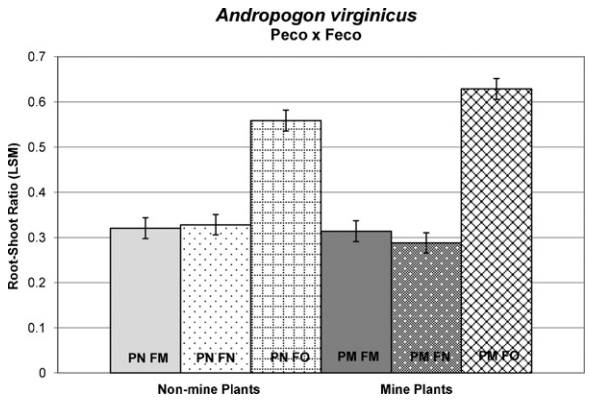

Fig. 3. *A. virginicus* root–shoot ratios by treatment and plant ecotype. PN is the plant ecotype from the control site. PM is the plant ecotype from the mine. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi ($p = 0.05$).

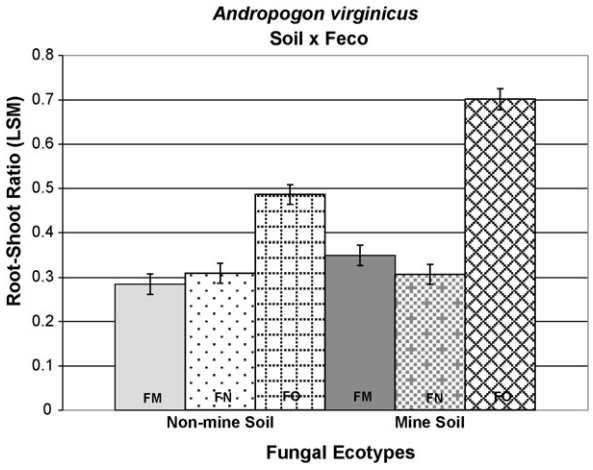

Fig. 4. *A. virginicus* root–shoot ratios by soil type and fungal inocula. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi ($p < 0.0001$).

root–shoot ratios, indicating mine soil conditions induce plants to allocate more resources to their roots (Fig. 6 and Table 2, p-value < 0.0001).

4. Discussion

Growth of both *A. virginicus* and *P. lanceolata* were greatly reduced in mine tailings (Figs. 2 and 7). Although this reduction was expected, we did not observe signs of metal toxicity. At harvest, each plant was carefully examined for evidence of metal toxicity in general, which is typically characterized by necrosis, chlorosis or bronze spotting on leaves. No symptoms of toxicity were observed. Plants grown in coal soil demonstrated stunted growth consistent with nutrient deficiency regardless of species, ecotype or fungal inoculum. Our observation of increased allocation to roots in mine tailings (Figs. 4 and 6) is also consistent with potentially lower availability of nutrients in the mine tailings. Although plants were fertilized weekly, the mine soil was coarser and retained fluids for a shorter period of time than the low-nutrient clay soil. The low aluminum content of the coal tailings may have been caused by leaching and weathering during the years of exposure while the mine was fallow. The mine soil actually had less aluminum in it than the clay soil (16,333 and 20,193 ppm, respectively).

Uninoculated *P. lanceolata* in non-mine soil, while smaller than inoculated plants, looked as if they were robust enough to sur-

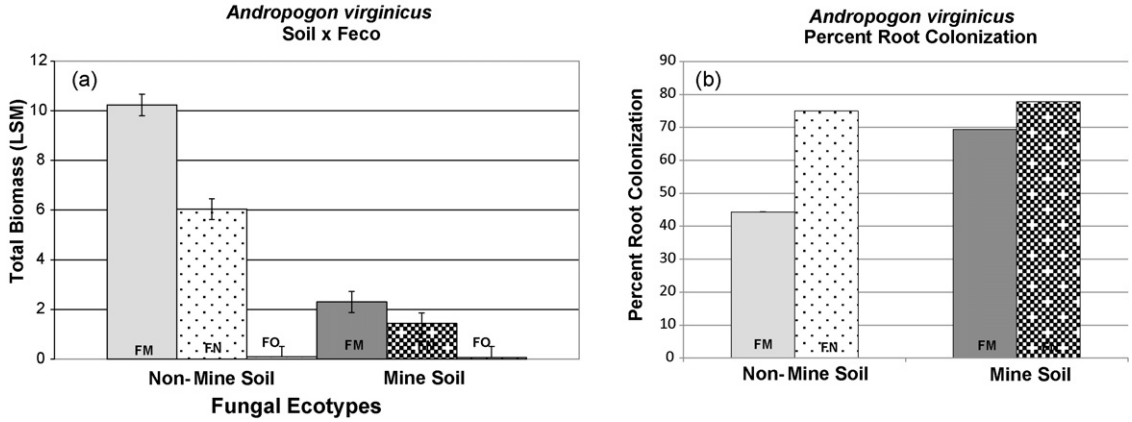

Fig. 2. (a) Total biomass for *A. virginicus* in non-mine soil vs. mine soil, by inoculum type. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi. Biomass includes both plant ecotypes ($p < 0.0001$). (b) Percent root colonization for fungal ecotypes in *Andropogon virginicus*. p-Value for 2-way interactions between soil and fungi is 0.0148, reflecting the mine fungi's much higher root colonization in mine soil than in non-mine soil. The non-mine ecotype did slightly better in mine soil. Error bars are ±0.04 and may not be readily visible due to their small size.

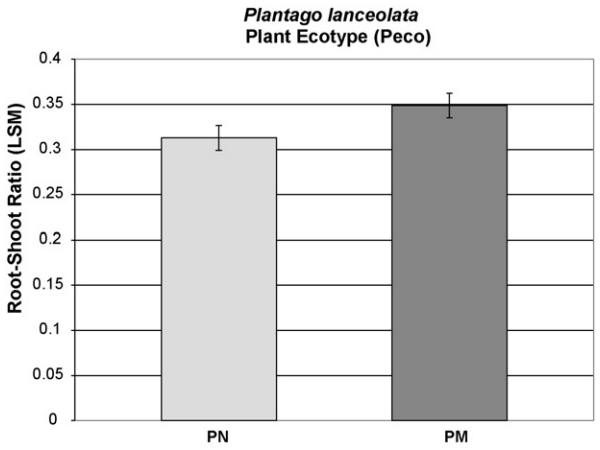

Fig. 5. *P. lanceolata* demonstrates a difference between plant ecotypes in root–shoot ratios ($p = 0.05$). PN is the plant ecotype from the control site. PM is the plant ecotype from the mine.

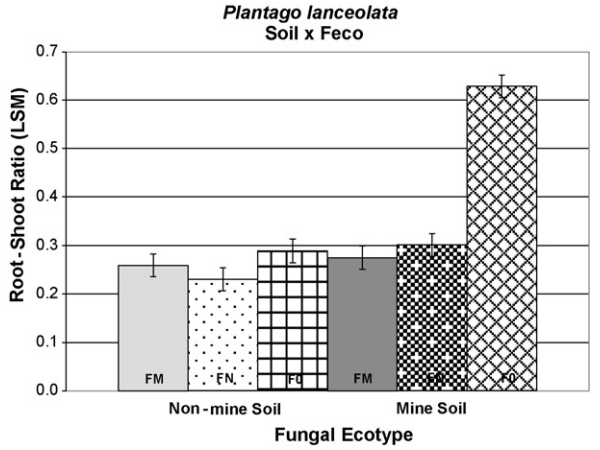

Fig. 6. *P. lanceolata* root–shoot ratios by soil type and fungal inocula. FM is the fungal community derived from mine soil. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi ($p < 0.0001$).

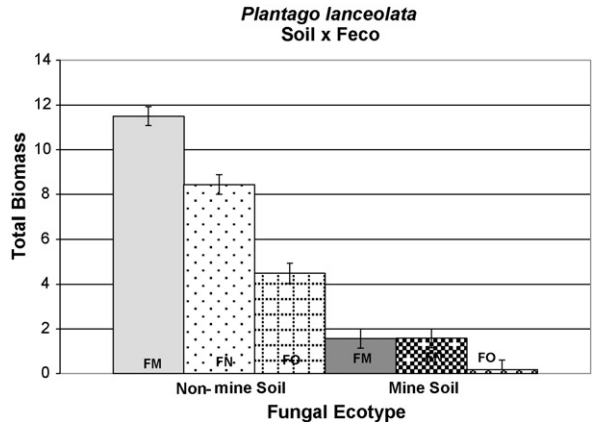

Fig. 7. Total biomass for *P. lanceolata* in non-mine soil vs. mine soil by inoculum type. FM is the fungal community derived from the mine. FN is the fungal community derived from non-mine soil. F0 excludes AM fungi ($p = 0.06$).

vive and reproduce under natural conditions, while uninoculated plants in mine soil appeared too sickly and stunted to survive in nature.

Fungal communities had a large impact on plant biomass (Figs. 2a and 7). In mine soil, *A. virginicus* inoculated with AMF collected from mines grew 60% larger than plants inoculated with AM fungi collected from non-mine soil. *A. virginicus* grown in non-mine soil grew 69% larger when inoculated with the mine fungi compared to plants inoculated with the non-mine fungi. *P. lanceolata* had a similar response to the fungal inoculum. *P. lanceolata* in non-mine soil inoculated with AM fungi from mine soil were 36% larger than plants inoculated with AM fungi from non-mine soil. In mine soil, *P. lanceolata* responded the same to both inocula. This may reflect *P. lanceolata*'s weaker dependence upon AM fungi. We found this pattern to be a powerful and consistent result with both species, across both soil types, suggesting it is not a response to soil, pH, or CEC. Fungi derived from mine soil benefit plants substantially more than fungi adapted to the less stressful edaphic conditions of the control site. The mine group of fungi may be providing more nutrients and essential elements or they may be a weaker sink, withdrawing fewer carbon resources from plants. Johnson (1993) demonstrated that AM fungal communities from fertilized soil were less beneficial to host plants than fungal communities from unfertilized soil. She found that AM fungi from fertilized soil produced fewer arbuscules but still developed the same number of vesicles, giving less in return for what they were receiving from the plant. Stressful environmental conditions may play a role in reinforcing the mutualistic nature of the bond between AM fungi and their hosts.

Both species of plants demonstrated that their two ecotypes had heritable differences from one another. *A. virginicus*' mine ecotype grows more slowly than the ecotype adapted to less stressful conditions (Fig. 1). Its smaller size may also contribute to reduced evapotranspiration. This may reduce water requirements in an environment where the black surface of the coal creates higher temperatures near the ground, resulting in drought-like conditions. Mine soils also suffer compaction, which even further reduces availability of water. Slower growth may also be an adaptation to low nutrient availability.

P. lanceolata's mine ecotype allocated more resources to roots (Fig. 5). Again, this is probably an adaptation to low nutrient availability. More allocation to roots allows a greater area to be explored for mineral resources, and also increases the likelihood of encountering AM fungi. Grasses normally produce a deep and extensive root system, which might explain why we saw a change in root–shoot ratios with *P. lanceolata* but not *A. virginicus*. *P. lanceolata* normally produces coarser, less fibrous, roots, which may be insufficient to the task of acquiring nutrients under poor edaphic conditions and may even fail to anchor them in the coal tailings during periods of heavy rain. The conditions we observed at the mine showed eolian and fluvial processes played an important role in molding an ever-changing landscape where erosion was widespread.

Both ecotypes of *A. virginicus* demonstrated a large increase of root–shoot ratios in the absence of AM fungi. Increased allocation of resources below ground may allow plants to persist longer, and increase their likelihood of encountering an AM fungal symbiont.

These results suggest that plants in the mine are particularly dependent on AM fungi when harsh edaphic conditions exist. We did not find evidence of co-adaptation (i.e., we never observed plant ecotype × AM fungi interactions). However, we did observe a significant difference in plant response to these two fungal communities, with plants growing much better with the fungi derived from mine soil. Given the consistency with other results, this suggests that microbial communities collected from harsh environments might be the best group to draw upon for fungal inoculation of nursery plants destined to be planted in reclaimed areas. Maintenance of these strains might need to be performed in coal tailings, low-pH or low-nutrient media to maintain the properties that make them such strong mutualists.

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