Title: Low variation in arbuscular mycorrhizal fungal associations and effects on biomass among switchgrass cultivars

Running Head: switchgrass mycorrhizal associations

Authors:

Sarah M. Emery[1]*

Erin R. Kinnetz[1]

Lukas Bell-Dereske[2]

Karen A. Stahlheber[2,3]

Katherine L. Gross[2,4]

Dennis Pennington [5]

Institutions:

[1] Department of Biology, University of Louisville, Louisville KY

[2] W.K. Kellogg Biological Station, Michigan State University, Hickory Corners MI

[3]Department of Natural and Applied Sciences; University of Wisconsin-Green Bay WI

[4]Department of Plant Biology and Graduate Program in Ecology, Evolution, and Behavioral Biology, Michigan State University, East Lansing, MI

[5]Department of Plant, Soil and Microbial Sciences; Michigan State University, East Lansing, MI

***Contact information for corresponding author:**

Department of Biology, University of Louisville

139 Life Sciences Bldg., Louisville, KY 40292

e-mail: sarah.emery@louisville.edu; Office phone: 502.852.5940

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Abstract

Switchgrass (*Panicum virgatum*) is a leading perennial bioenergy feedstock crop candidate in North America, with more than 20 cultivars commercially available. In native prairies, switchgrass is known to be strongly dependent on arbuscular mycorrhizal fungi (AMF), but little is known about AMF in switchgrass bioenergy systems. AMF can alter crop yield and belowground ecosystem functioning, so knowing if AMF vary among switchgrass cultivars may improve development of sustainable bioenergy systems. Using a common garden experiment in Michigan, USA, we examined whether twelve switchgrass cultivars, representing both lowland and upland ecotypes, were associated with shifts in AMF, and if this variation was related to aboveground or belowground crop biomass. Cultivars did not differ in AMF root colonization, extraradical hyphal growth, or soil AMF diversity. However, AMF root colonization was 6% higher in lowland compared to upland ecotypes. While cultivars differed in above- and belowground biomass, only one measure of AMF - root colonization - was significantly correlated with root biomass, and no measures of AMF were correlated with yield. AMF are often more important to plant production in sites that are nutrient or water stressed, and so AMF effects on switchgrass establishment and growth may be more important when grown on marginal lands. Future studies of AMF associations with and effects on production of switchgrass cultivars should be done across a range of conditions reflective of the environments in which they will be grown for bioenergy feedstock.

1. Introduction

Switchgrass (*Panicum virgatum*) is a warm-season grass that is a leading perennial bioenergy feedstock crop candidate in North America due to its tolerance of a wide range of environmental conditions [1], high yield [2], and ability to enhance associated ecosystem services such as soil carbon sequestration [3]. Switchgrass is native to North American prairies, where is known to be strongly dependent on arbuscular mycorrhizal fungi (AMF) [4, 5]. AMF are a major component of soil biodiversity, and are known to form symbiotic associations with 80% of land plants [6]. AMF have been shown to benefit host plants via increased nutrient uptake, pathogen resistance, and drought tolerance [7, 8]. AMF also can enhance soil carbon storage through the production of extraradical hyphal networks [9]. In native systems, AMF colonization is associated with enhanced above- and belowground growth in switchgrass [4].

Recent research in bioenergy systems has shown that switchgrass was associated with increased soil AMF diversity and abundance compared to other bioenergy crops such as corn or miscanthus [10-12]. There have also been documented benefits of various soil microbial inocula for switchgrass growth in bioenergy systems [13-15], though none focused on arbuscular mycorrhizal fungi specifically. Most previous studies characterizing belowground associations with switchgrass have only considered one or two of the most common switchgrass cultivars; almost nothing is known about AMF associations with many switchgrass cultivars being considered for bioenergy production.

The 20+ commercially-available switchgrass cultivars available for bioenergy production [16] are broadly classified into upland and lowland ecotypes based on origin. The upland ecotype is typically better adapted to drier conditions and cold temperatures, while the lowland ecotype thrives in areas with warmer temperatures and wetter soils [1, 17]. These differences in tolerance

of environmental conditions has been shown to involve tradeoffs in productivity and physiology aboveground [18]. There is also considerable variation in belowground traits of switchgrass cultivars, including root biomass, root architecture, and specific root length [19, 20], which may correspond to variation in associations with AMF [21]. Given that AMF diversity and colonization can vary greatly among sites in response to soil fertility and management [e.g., 10], the dependence of switchgrass cultivars on AMF may be important for crop success, especially in low-fertility sites [22]. Variation in AMF associations may affect switchgrass yield directly through improvements in nutrient acquisition, drought tolerance, or pathogen resistance [23-25] and may have added benefits important for sustainability, such as soil erosion control [26] and soil carbon sequestration [27]. In this study we determined whether 12 switchgrass cultivars were associated with shifts in arbuscular mycorrhizal fungi (including root and soil colonization and soil diversity), and if this variation was related to aboveground or belowground crop biomass.

2. Methods

2.1 ***Site description:*** This research was conducted at the Great Lakes Bioenergy Research Center's (GLBRC) Switchgrass Variety Experiment located at the W.K. Kellogg Biological Station of Michigan State University in southwest Michigan USA (KBS, 42°23'47" N, 85°22'26" W). This site averages 810 mm yr^{-1} of precipitation and soils are Kalamazoo series fine-loamy, mixed, mesic Typic Hapludalfs [28]. Twelve switchgrass cultivars, including four lowland and eight upland cultivars were established from seed in spring 2009 or 2010 (see Appendix A for additional details on cultivars). All cultivars were planted at a rate of 9 kg/ha pure live seed in 4.6 m x 12.2 m plots arranged in a randomized block design of 12 adjacent plots in each of four replicate blocks (n=48). For this study, plant and soil samples were taken from

104 the middle of each plot to avoid edge effects. Nine of the planted cultivars were commercially
105 available: Alamo (lowland), Blackwell (upland), Cave-in-rock (upland), Dakota (upland),
106 Kanlow (lowland), Nebraska-28 (upland), Shelter (upland), Southlow (upland), and Trailblazer
107 (upland). Three were proprietary experimental cultivars: Expt-A (improved Alamo), Expt-B
108 (improved Kanlow), and Expt-C (improved Cave-in-Rock). All plots were fertilized with urea
109 annually in the spring at a rate of 78 kg N/ha. Pre-emergence weeds were controlled with
110 Quinclorac (Drive®, 1.1 kg ha-1) and atrazine (0.6 kg ha-1). Glyphosate, 2,4-D or dicamba were
111 applied to control post-emergence weeds as needed. Plots were harvested in November in every
112 year, with crop yield estimated from biomass collected using a Wintersteiger biomass harvester
113 weighing system. Crop yield data from this experiment from 2010-2016 are available on the
114 GLBRC website (https://lter.kbs.msu.edu/datatables/510). We used November aboveground
115 yield rather than July aboveground biomass in our analyses, as this measure of production would
116 be the most relevant to producers.
117
118 ***2.2. AMF root and soil colonization:*** In July 2015, we collected soil cores (2 cm diameter x 15
119 cm deep; 10 per plot) from near the base of haphazardly selected switchgrass plants in each plot.
120 Cores from each plot were pooled, sieved through a 4 mm sieve to remove rocks and large roots,
121 and then stored at 4°C until processed. To determine the extent of AMF colonization in plant
122 roots, we extracted fine roots from 100 ml subsamples taken from the pooled soil cores for each
123 plot using a wet-sieve process [500µm sieve; 29]. Roots were cleared with 10% KOH and
124 stained using a 5% vinegar-ink solution using methods modified after Vierheilig et al. [30].
125 Visual estimation of percent root length colonization was made using 100 fields of view per
126 sample under 200x magnification.

To quantify AMF soil colonization, extra-radical hyphae (ERH) were extracted from 20 ml subsamples taken from the pooled cores for each plot. Soil subsamples were suspended in water, then stained and vacuum filtered through a 45 μm filter, following methods described in Staddon et al. [31]. Hyphal length was estimated using the gridline-intercept method [32] at 100x magnification.

2.3. AMF community composition and diversity: To characterize the diversity and composition of the AMF soil community, we extracted DNA from 0.25 g of fresh soil subsampled from the pooled soil core samples. Methods for sequencing and bioinformatics used are detailed in Emery et al. [10]. Briefly, we used Powersoil DNA Extraction kits to isolate DNA (MOBIO Laboratories, Carlsbad, CA, USA) and the 28S rRNA was targeted using AMF specific fusion primers [FLR3-FLR4, 33]. PCR and MiSeq Illumina paired-end sequencing was conducted by the Research Technology Support Facility Genomics Care at Michigan State University, East Lansing, Michigan. Reads were assembled and quality filtered using USEARCH8 (http://drive5.com/usearch/). Sequences were dereplicated, clustered chimera checked, filtered de novo, and clustered into unique operational taxonomic units (OTUs, i.e., DNA sequences or amplicon types) based on 97% identity using the default settings in UPARSE implemented in UEARCH9 [34, 35]. USEARCH quality filtering, chimera checking using UCHIME, and OTU clustering lead to 260 OTUs and 1,902,178 reads. Representative sequences were then classified using the RDP naïve Bayesian classifier against the Fungal LSU training set 11 [36, 37]. Any sequences with bootstrap values below 60% match with Glomeromycota were removed from the dataset. Taxonomic filtering for AMF-specific sequences resulted in 138 OTUs and 1,562,891 reads. We transformed OTU tables using variance stabilizing transformation (VST) in the

DeSeq2 package [38] in R [39] to control for biases in PCR amplification and to avoid biases due to rarefaction [40]. We used the vegan package in R to calculate Shannon Diversity (H') and Chao1 richness based on OTUs.

2.4. Belowground Biomass: In July 2015, root biomass was determined by taking soil cores (2.5 cm x 50 cm) from the base of a switchgrass plant near the center of each plot. Roots were separated from soil by dry sieving through a 1.0 mm sieve, then rinsed and dried at 60°C for 72 h, weighed, and biomass converted to g m^{-2}.

2.5. Data analyses: We compared AMF root and soil colonization, AMF richness (Chao1 index), AMF diversity (Shannon Index; H', based on OTUs), and root biomass and crop yield (g m^{-2}) among cultivars using one-factor analyses of variance (ANOVA) with a block term. We used a planned contrast to evaluate differences in these response variables between ecotypes. We evaluated the associations between AMF and cultivar biomass using ordinary least squares linear regressions with AMF richness (Chao1), AMF diversity (H'), AMF % root colonization, and AMF ERH length as independent variables, and aboveground or belowground biomass as dependent variables. The three AMF variables were not significantly correlated with one another ($r<0.09$; $p>0.99$ for all pairs; data not shown). All analyses were performed using Systat v.12 [41].

We used one-factor blocked PERMANOVA [42] to examine overall differences in soil AMF community composition associated with switchgrass cultivars or ecotypes. To visualize differences in soil AMF community structure we performed Non-metric Multidimensional Scaling (NMS) ordinations [43] with Bray-Curtis dissimilarity measures based on square-root transformed AMF OTU abundance data. OTU singletons were excluded from PERMANOVAs

and ordinations to improve resolution of analyses. PERMANOVA and NMS analyses were performed using Primer v. 6 [44].

3. Results

3.1 AMF: AMF root colonization was high across all cultivars, ranging from 69 -100% and there was no difference among cultivars in AMF root colonization, ERH growth, or AMF richness and diversity (Table 1). While over 150 OTUs associated with 6 taxa within Glomeromycota were identified across all samples (Appendix B), there were no differences in soil AMF community composition between cultivars (PERMANOVA: "cultivar" pseudo-F=1.01, p=0.48; "block" pseudo-F=1.16, p=0.23; NMS not shown). *Rhizophagus* and *Septoglomus* sequences were commonly found in soils associated with all cultivars.

There was some evidence for differences in AMF association between upland and lowland ecotypes. AMF root colonization was high in both ecotypes, but was significantly lower in upland than lowland ecotypes (88.9% vs. 94.5%; Table 1). However, there were no differences in ERH length, AMF richness or diversity between ecotypes (Table 1). Also, there was no significant difference in soil AMF community composition between ecotypes (PERMANOVA: "ecotype" pseudo-F=0.93, p=0.54; "block" pseudo-F=1.27, p=0.09; Fig. 1).

3.2 Aboveground and Belowground Biomass: Aboveground biomass (yield) varied 4-fold among cultivars (502-2051 g m^{-2}) and differed significantly among cultivars (Table 1). This was primarily driven by the low production of Dakota and Trailblazer compared to the other cultivars (Fig. 2a). Overall lowland ecotypes produced more aboveground biomass (17%) than upland

196 ecotypes (1303 vs 1111 g m^{-2}, Table 1, Fig. 2a). No measures of AMF were associated with
197 differences in aboveground biomass among cultivars or between ecotypes (Table 2).

198 Root biomass varied across cultivars and blocks, ranging from 52-534 g m^{-2}. Southlow
199 had more root biomass than any other cultivar (Table 1, Fig. 2b). Despite the high root
200 production of Southlow (upland ecotype), there were no significant differences in root biomass
201 between upland and lowland ecotypes (Table 1; Fig. 2b). AMF root colonization was positively
202 related to root biomass across all cultivars (Table 2, Fig. 3).

203

204 **4. Discussion**

205 ***4.1 Were switchgrass cultivars associated with shifts in arbuscular mycorrhizal fungi?***

206 Although we found significant differences among these 12 cultivars in aboveground production,
207 we did not find any difference among cultivars in AMF root and soil colonization and soil
208 diversity. *Rhizophagus* and *Septoglomus* sequences were commonly found in soils associated
209 with all 12 cultivars. Both of these AMF taxa are characterized as ruderal genera with fast
210 growth and hyphal turnover rates [45]. While differences in methodologies make it difficult to
211 compare AMF diversity across studies, the taxon diversity and composition in this study are
212 similar to that reported in other studies in no-till agricultural systems in this region [e.g., 46].
213 This suggests that even after being used for switchgrass production for eight years, the AMF
214 community of this site still reflected the conventional row-crop agricultural land use history of
215 the site [47]. We had expected that there might be stronger relationships with AMF in lowland
216 compared to upland cultivars as lowland cultivars are more sensitive to drought and so may
217 depend more on AMF for water acquisition in drier upland conditions [48, 49]. We found that
218 AMF root colonization was slightly higher for the lowland compared to the upland cultivars,

however, we did not detect any other differences in AMF communities. Reliance of switchgrass on AMF has been shown to vary across environments, and the beneficial effects may be more pronounced in low fertility or drought-stressed environments [50]. The lack of differentiation in AMF among the 12 cultivars in our study may reflect that the common garden was located on relatively fertile soils at KBS (total N 1.25 ± 0.09 g kg−1 soil; http://data.sustainability.glbrc.org/), which may have suppressed the benefits of AMF [22, 51]. Also the year we sampled (2015) was not a particularly dry year in this region. While AMF associations have not been widely studied in switchgrass, cultivars of other agricultural grasses, including wheat and corn, are known to differ in AMF colonization rates, even under higher soil nutrient conditions which are often suppressive to AMF [52, 53]. It may be that intraspecific variation in AMF associations will become more apparent under stressful conditions, such as extreme herbivory [54], salinity [55], or drought [56]. It is also possible that the AMF communities occupying soils vs. plant roots may differ and so shifts in soil AMF communities may not indicate functional shifts for plants [e.g., 57, 58]. However, other studies have found that AMF communities in plant roots reflect available AMF soil communities [52, 59] , and are more stable seasonally than root AMF communities [60], indicating soil inoculation potentials. It is also important to note that plant-AMF associations are dynamic and may vary throughout the plant's life history [61].

4.2 Was variation in AMF related to aboveground or belowground crop biomass? While we found differences in shoot and root production among these 12 cultivars of switchgrass, these differences were mostly due to a low performance of a few cultivars. The Dakota and Trailblazer cultivars both had very low yield compared to most other cultivars. The Dakota cultivar

originated from a native stand in North Dakota, and offspring were selected for winter hardiness and high seed yields. Dakota also flowers earlier than other cultivars which may limit vegetative growth [16]. Trailblazer originated as a high forage quality cultivar, rather than a forage quantity/biomass cultivar [16], which may also explain its low yield. In contrast, Southlow, an upland ecotype, was developed from seeds collected from 11 native stands of *P. vigatum* growing in southwest Michigan. Belowground biomass was highest in Southlow than any other cultivar in this study. There has been no intentional selection on this cultivar as a bioenergy feedstock, and it is primarily used for restoration of native grasslands [62]. Given documented differences in aboveground biomass production among switchgrass cultivars [16], we expected some tradeoffs in belowground production [e.g., 63]. However, we found no strong evidence for this, possibly because perennial crops can be more resistant to allocation tradeoffs during crop selection [64]. Prior research comparing switchgrass cultivars has found a similar lack of differences in belowground biomass [e.g., 65, 66, 67], though this is not always the case [e.g., 20, 65, 68, 69].

Despite research from other systems showing a positive relationship between AMF root colonization, AMF diversity, and crop aboveground biomass, we did not find evidence of this in our study. There was a significant relationship between AMF % root colonization and belowground biomass, though this effect explained less than 10% of the variation in belowground biomass among plots and was mostly driven by the high root biomass of the Southlow cultivar. While not as widely evaluated as aboveground biomass responses, positive associations between AMF and belowground biomass have been demonstrated in other grass species, especially under stressful soil conditions [70-72]. While AMF colonization has been found to generally increase plant aboveground biomass in greenhouse and field experiments [4,

73-75], the relationships between AMF root colonization and plant biomass is not always straightforward, and the benefits of AMF are often strongest in low-nutrient or droughty soils [50]. Concerns that land conversion to biofuel crops may increase food costs have led to growing interest in using marginal lands for biomass production, and so AMF may have stronger associations with crop yields in these marginal lands [76]. For example, switchgrass grown on acid soils of mine restoration sites were particularly responsive to AMF colonization [77]. Additionally, AMF associations can benefit plants in ways that are not associated with changes in biomass, for example by enhancing reproduction [7]. As switchgrass breeding and cultivar development continue to advance, and as focus moves to use of marginal lands for biomass production, associations with soil organisms such as AMF may change. Future studies of AMF associated with switchgrass cultivars across a wider range of conditions are warranted.

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Table 1. Summary of results from blocked ANOVAs examining effects of switchgrass cultivar on aboveground yield, root biomass, AMF root colonization, AMF extra-radical hyphal (ERH) length, and AMF operational taxonomic unit (OTU) diversity. Significant associations are in bold with significance levels indicated: p< 0.05 (*), p<0.01 (**), p<0.001 (***).

		Aboveground Yield	Root biomass (0-50 cm)	AMF root colonization	AMF ERH length	AMF OTU Chao1 Richness	AMF OTU Diversity (H')
Factor	**df**	**F-ratio**	**F-ratio**	**F-ratio**	**F-ratio**	**F-ratio**	**F-ratio**
Cultivar	11	**4.94*** **	**2.97** **	0.94	0.63	1.76	1.22
Block	3	1.47	2.20	1.49	2.70	0.64	0.81
Post-Hoc Contrast							
Upland vs. Lowland Ecotype	1	**7.92*** **	0.02	**6.55***	0.08	2.90	1.89

502 **Table 2.** Summary of ordinary least squares linear regression analyses examining associations
503 between AMF and aboveground yield and belowground biomass. Significant predictor variables
504 are in bold with significance levels indicated: $p < 0.05$ (*).

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	Aboveground Yield		**Belowground Biomass**	
Predictor Variable	**t-value**	**R^2**	**t-value**	**R^2**
% AMF root colonization	0.63	0.0089	**2.04**	**0.083**
AMF ERH length	-0.343	0.003	-0.113	<0.001
AMF OTU Chao1 Richness	-0.922	0.018	0.456	0.004
AMF Shannon Diversity	-1.097	0.025	1.029	0.023

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509 **Figure 1.** Non-metric multidimensional scaling (NMS) ordination of AMF communities based
510 on OTUs associated with the 12 cultivars of *Panicum virgatum* grown in a common garden
511 experiment. Filled shapes indicate the lowland ecotype.

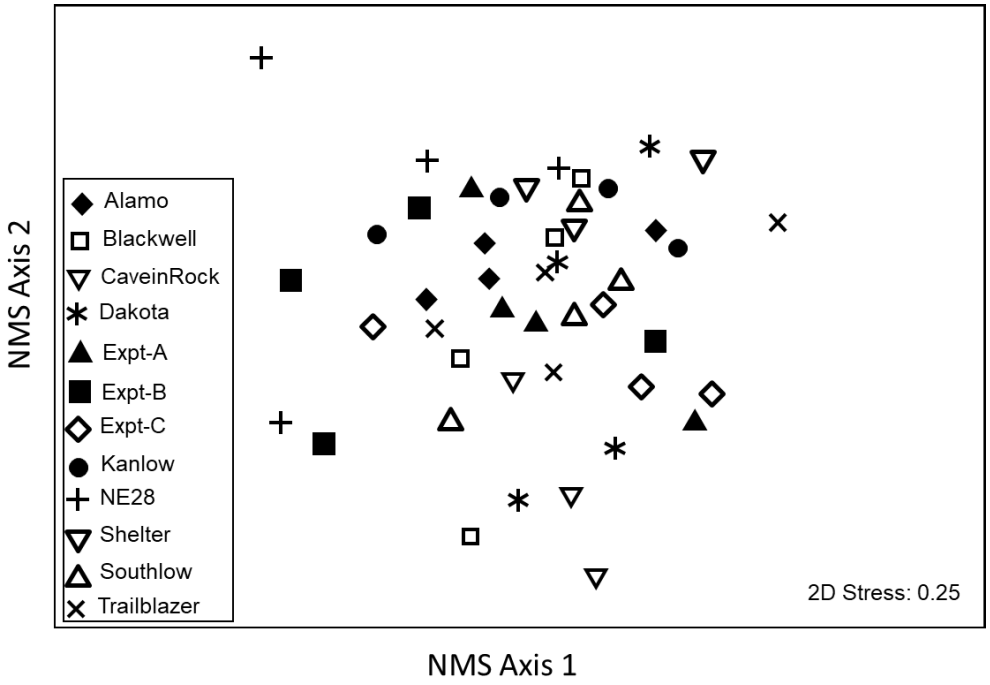

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Figure 2. Biomass production in 2015 for 12 cultivars of *Panicum virgatum* grown in a common garden: a) Aboveground Yield (November), and b) Root biomass (0-50cm; July). Bars are means ±1SE (n=4); light shading indicates the lowland ecotype, dark shading the upland ecotype. Letters on graphs indicate significant Tukey-corrected pairwise comparisons ($p<0.05$).

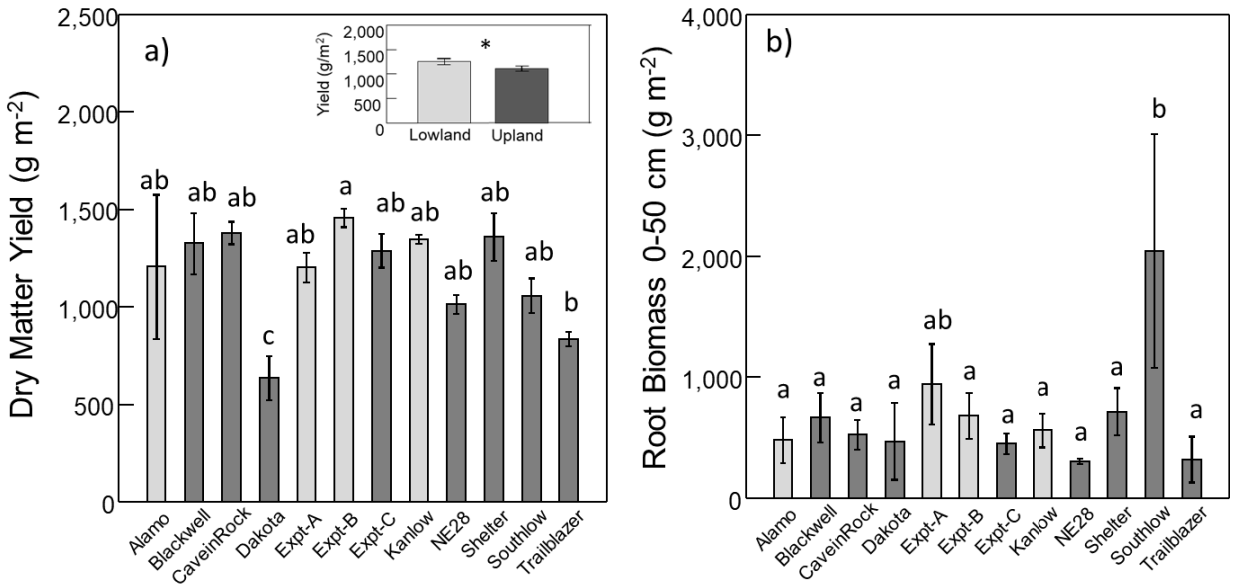

Figure 3. Relationship between AMF root colonization and root biomass across 12 cultivars of *Panicum virgatum* grown in a common garden. Regression model $R^2 = 0.08$, p=0.047.