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Growth and nutrient concentration of two native forage legumes inoculated with *Rhizobium* and Mycorrhiza in Missouri, USA

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Abstract

The Center for Agroforestry at the University of Missouri has tested numerous native legumes for potential use in agroforestry and selected Illinois bundleflower (*Desmanthus illinoensis* (Michaux) MacMillan ex Robinson and Fern.) and panicled tick clover (*Desmodium paniculatum* (L.) DC.) for further testing. Our objective was to document the effect of arbuscular mycorrhizae (AM) (*Glomus* spp.) and *Rhizobium* on growth and nutrient concentration of these legumes. Seeds were planted in a greenhouse and inoculated with one of two species of AM and/or one of two strains of *Rhizobium*. Plants were harvested after 80 d and data taken on leaf and stem dry weight, root fresh weight, stem height, nodulation, AM colonization, and N, P, K, Ca, and Mg concentration. Inoculation with *Rhizobium* did not affect plant growth in Illinois bundleflower, but colonization by *Glomus intraradices* increased all plant growth variables except stem height. Nutrient concentration was unaffected by the presence of either endophyte. In contrast, inoculation of panicled tick clover with *Rhizobium* str. 41Z10 increased leaf dry weight (32%) compared to the control and root fresh weight (41%) compared to str. 32Z3, and colonization by *G. intraradices* increased leaf dry weight (35%) and stem height (26%). Both species of AM increased P and K concentration (41% and 55%, respectively) in panicled tick clover. Our results suggest that the growth of these legumes can be improved by the use of proper AM species and/or *Rhizobium* strains. However, additional research to identify the best *Rhizobium* and AM inoculates for these plant species is important in developing strategies for their use in agroforestry.

Introduction

In recent years, interest in using native species has steadily increased in the USA, due in large measure to their value as habitat for wildlife. The Center for Agroforestry at the University of Missouri has been evaluating herbaceous native species for incorporation into agroforestry practices. Native legumes adapted to grow under forest canopies can provide wildlife habitat and food, good quality forage production for domestic livestock, and fixed nitrogen for the tree crop. Two native forage legumes currently being evaluated for use as understory species in agroforestry systems are Illinois bundleflower (*Desmanthus illinoensis* (Michaux) MacMillan ex Robinson and Fern.) and panicled tick clover (*Desmodium paniculatum* (L.) DC.).

Colonization by AM has been shown to increase nodulation in legumes (Smith and Daft 1977; Fitter and Garbaye 1994), although this effect may be indirect, i.e., the AM increases phosphate uptake resulting in larger, more vigorous plants that in turn have greater nodulation (Cluett and Boucher 1983). Not surprisingly, *Rhizobium* can also influence AM development and colonization. It was previously believed that AM colonization could be directly linked to inoculation with *Rhizobium* and the resulting increase in nitrogen fixation (El-Kherbawy et al. 1989; Fitter and Garbaye 1994; Hodge 2000). However, Xie et al. (1995) demonstrated that increased AM colonization in the presence of *Rhizobium* is actually linked to the production of plant flavonoids related to host nodulation factors triggered by inoculation with *Rhizobium*.

The relationship between these two endophytes is not static and varies with the strains of *Rhizobium* and species of AM involved. In fact, inoculation with *Rhizobium* can actually decrease spore germination, hyphal length, and colonization by AM in some associations (Hodge 2000). It has been shown that AM fungal hyphae do not invade nodule material, suggesting inhibition via some sort of exclusion mechanism probably related to inter-endophyte competition for root carbohydrates (Bethlenfalvay et al. 1985). Findings by Azcon et al. (1991), working with alfalfa (*Medicago sativa* L.), confirm that there is substantial variation in the synergistic association between *Rhizobium* strains and AM species, and those associations do not always positively affect plant growth and nutrient uptake, suggesting that the mutualism is closely tied to the combination of inocula used.

The effects of AM and *Rhizobium* on the host plant and each other have been well documented for some introduced legume hosts such as alfalfa (Nielsen and Jensen 1983; Azcon and El-Atrash 1997) and soybean [*Glycine max* (L.) Merr.] (Ross 1971). However, no work has been done to investigate the interaction of AM with *Rhizobium* and its impact on native legumes.

The objective of this study was to document the effect of two species of AM fungi and two strains of *Rhizobium* on growth and nutrient concentration of Illinois bundleflower and panicled tick clover. Specifically, we focused on how each species and strain of endophyte affected nodulation, concentration of nutrients in the shoots of the host, and total plant growth (as measured by leaf and stem dry weights and root and shoot fresh weights).

Materials and methods

Seed of Illinois bundleflower and panicled tick clover were surface sterilized by treating for 5 min in 10% bleach, followed by two 5-min rinses in sterile distilled water. Seeds were planted in bleach-sterilized 300-mL pots of a soil: perlite: vermiculite mixture (2: 1: 1; v: v: v) which had been steam sterilized for 2 h per day for 3 consecutive days and then autoclaved for 1 h at 123 ºC and 22 PSI the day of planting. The soil used was a Mexico silt loam topsoil collected at the University of Missouri Agronomy Research Center near Columbia, MO, USA, and possessing the following characteristics: pH = 6; N.A. = 2 meq/100 g; O.M. = 4.8%; P = 35.5 ppm; Ca = 2810 ppm; Mg = 344 ppm; K = 160 ppm; NO_3 = 29.5 ppm; NH_4 = 9.3 ppm. The soil was analyzed by the University of Missouri Soil and Plant Testing Laboratory using procedures described in Brown and Rodriguez (1983).

At planting, seeds of both Illinois bundleflower and panicled tick clover were inoculated singly or in dual combination with one of two common AM species (*Glomus intraradices*, *G. etunicatum*) and/or one of two different strains of *Rhizobium.* For Illinois bundleflower, the *Rhizobium* inocula were strains of *Rhizobium tropici* (522-1, 522-3), supplied by Urbana Labs (Champaign-Urbana, IL, USA). For panicled tick clover, the *Rhizobium* inocula were strains of an unknown species of *Rhizobium* (41Z10, 32Z3) identified specifically for use with panicled tick clover by Liphatech, Inc (Milwaukee, WI, USA). All *Rhizobium* inocula consisted of spores in a peat-based carrier. The AM inocula were provided by Plant Health Care, Inc. (Pittsburgh, PA, USA) and consisted of a homogenous blend of infected root pieces and spores. For inoculated treatments, approximately 10 g of each appropriate *Rhizobium* and AM inocula were applied to each 300-mL pot prior to the first watering. A round depression, 2.5-cm in diameter by 2.5-cm deep, was made in the soil media in each pot to which the AM inoculum, seeds, and *Rhizobium* inoculum were added, respectively. The resulting nine treatments for each species (two AM, two *Rhizobium*, four AM × *Rhizobium*, one non-inoculated control) were replicated five times and the experiment was conducted twice (i.e., replicated in time). All treatments were isolated from each other and inoculated separately, and the pots remained separated until seedling emergence to decrease the chance of cross-contamination. Throughout the assay, plants were watered daily and

Table 1. Effects of inoculation with AM fungi or *Rhizobium* on plant growth, AM colonization, and nodulation of *Desmanthus illinoensis* in Missouri, USA.

Treatments	Stem Ht cm	Leaf Dry Wt g	Stem Dry Wt g	Root Fresh Wt g	AM %	Nodules per Plant #
Rhizobium						
None	44.7A[†]	0.9A	0.5A	5.6A	4B	0B
522-1	48.7A	1.1A	0.6A	6.7A	17A	11A
522-3	49.2A	1.1A	0.5A	7.3A	26A	7A
AM						
None	46.1a	0.8b	0.4b	5.2b	0b	3b
G. intraradices	52.0a	1.1a	0.7a	8.0a	26a	11a
G. etunicatum	44.5a	1.0ab	0.5ab	6.5ab	20a	3b

[†]Means followed by the same letters within a column (uppercase = *Rhizobium*, lowercase = AM) are not significantly different at $\alpha = 0.05$.

received weekly treatments with 10 mL of a N- and P-free Bohnsack's nutrient solution (Bohnsack 1991).

Plants were grown in a greenhouse maintained at 32/22 °C (D: N) and 16:8 light:dark ratio. Day length was increased to 16 h using 400W High Pressure Sodium lamps with an average daytime light intensity of 718 micro Einsteins/m^2/s at the bench surface, compared to an average ambient light intensity of 583 micro Einsteins/m^2/s. Beginning 1 week after seedling emergence, pots were rearranged randomly every week because the light was not evenly distributed across the entire bench surface, and we wanted to reduce the chance for enhanced growth by samples located directly underneath the artificial lights. Seedlings were thinned to one plant per pot after 4 wk, and plants were harvested at 12 wk.

At harvest, primary stem height was recorded and then plants were separated into root, stem and leaf components. Fresh weights were recorded for each of these components at harvest, and then roots were washed, packaged and shipped to Soil Foodweb Inc. (Corvallis, OR, USA) for processing and determination of percent AM infection and total number of nodules. To determine percent AM infection, roots were rinsed in distilled water, cut into 1-cm segments, cleared of pigmentation using 10% KOH, acidified with dilute lactic acid, stained with Trypan Blue, then rinsed and cleared of excess stain with water (Phillips and Hayman 1970). The percent AM colonization was then determined by measuring the percentage of 1-cm root segments infected with hyphae compared to the total number of root segments for each sample. Stems and leaves were dried at 50 ºC for 24 h and then weighed to obtain dry weights. Previous study found that 24h was sufficient to achieve constant weight. Because of the destructive sampling technique used by Soil Foodweb to process the root samples, roots could not be returned and, consequently, no dry weights could be recorded. Dried stems and leaves from each sample were ground in a Udy cyclone sample mill (UDY Corp., Ft. Collins, CO, USA) with a 1-mm screen and combined for N, P, K, Ca and Mg analysis by Custom Laboratories (Golden City, MO, USA). Nitrogen content was determined using the Kjeldal method, phosphorus was determined using the Photometric method, and all other mineral concentrations were determined using the Atomic Absorption Spectrophotometric method (AOAC International, 1995).

Data were analyzed using the Statistical Analysis System (SAS) to perform a one-way ANOVA (SAS, Proc. GLM) in a randomized complete block design. Fisher's Least Significant Difference (LSD) tests were used to identify differences between treatment means ($\alpha = 0.05$) (Steel and Torrie 1960). The ANOVA revealed no significant difference between runs and no direct *Rhizobium* x AM interaction, so runs were pooled and only the main effects of *Rhizobium* and AM are discussed.

Results

Effects of Rhizobium

In Illinois bundleflower, the number of nodules per plant was statistically similar for both 522-1 and 522-3 *Rhizobium* strains (Table 1). Inoculation with *Rhizobium* did not significantly affect any of the plant growth factors measured but did affect AM colonization. Plants inoculated with 522-1 and 522-3 had greater AM colonization than the control. No differ-

Table 2. Effects of inoculation with AM fungi or *Rhizobium* on concentration of shoot nutrients of *Desmanthus illinoensis* in Missouri, USA

Treatments	N %	P %	K %	Ca %	Mg %
Rhizobium					
None	2.91A[†]	0.19A	0.48A	1.08A	0.57A
522-1	2.70A	0.18A	0.60A	1.10A	0.56AB
522-3	2.38B	0.18A	0.51A	1.16A	0.52B
AM					
None	2.93a	0.16a	0.47a	1.15a	0.60a
G. intraradices	2.25b	0.19a	0.54a	1.13a	0.51b
G. etunicatum	2.80a	0.19a	0.58a	1.06a	0.54b

[†]Means followed by the same letters within a column (uppercase = *Rhizobium*, lowercase = AM) are not significantly different at $\alpha = 0.05$.

ences were found among *Rhizobium* treatments for P, K, and Ca concentration (Table 2). Shoots from plants inoculated with 522-3 had lower N concentration than the non-inoculated control and 522-1. Magnesium concentration was also lower in shoots of plants inoculated with 522-3 than in the control, but it was similar to plants inoculated with 522-1.

Nodules were found on the control plants of panicled tick clover. We are not sure of the source of this contamination as the sterilization methods for seed and soil were identical to those used for Illinois bundleflower, which had no contamination. Nonetheless, there were significant differences for nodulation among *Rhizobium* treatments (Table 3). Panicled tick clover plants inoculated with 32Z3 and 41Z10 had similar numbers of nodules and had more nodules than the contaminated control plants. Plants inoculated with 41Z10 had greater leaf dry weight than control plants but were similar to those inoculated with 32Z3. Root fresh weight was greater in plants inoculated with 41Z10 compared to 32Z3 but similar to the control.

Plants inoculated with 41Z10 had greater concentrations of N, P, Ca, and Mg compared to 32Z3 but only differed from the control for Ca concentration (Table 4). Concentration of N, P and Ca between plants inoculated with 32Z3 and the control were similar; however, Mg concentration was less than the control. Potassium concentration in plants inoculated with 32Z3 was greater than the control but similar to those inoculated with 41Z10.

Effects of AM

Both species of AM fungi successfully colonized Illinois bundleflower roots, and percent colonization was similar for the two species (Table 1). Plants colonized by *G. intraradices* had greater leaf dry weight, stem dry weight, and root fresh weight compared to control plants. Illinois bundleflower colonized by *G. intraradices* had more nodules per plant than both the control and plants colonized by *G. etunicatum*. Nitrogen concentration was greater in control plants and plants colonized by *G. etunicatum* compared to those colonized by *G. intraradices* (Table 2). Shoots from plants colonized by either AM species had lower Mg concentration than the control.

Glomus intraradices and *G. etunicatum* both successfully colonized the roots of panicled tick clover (Table 3). Plants colonized by *G. intraradices* had increased stem height and leaf dry weight compared to the control, but plants colonized by *G. etunicatum* did not. Stem dry weights and root fresh weights were similar among AM treatments. Concentration of N, P, and K in shoots of panicled tick clover was affected by AM colonization (Table 4). Shoots of plants colonized by *G. intraradices* had less N than the control but were similar to those colonized by *G. etunicatum*. Phosphorus and K concentrations in shoots were greater when plants were colonized by either *G. intraradices* or *G. etunicatum* compared to the control.

Discussion

Previous research on introduced legume species such as alfalfa (Nielsen and Jensen 1983; Azcon and El-Atrash 1997) and soybean (Ross 1971; Xie et al. 1995) demonstrated that *Rhizobium* and AM can significantly affect plant growth, nutrient uptake, and each other through competitive root colonization (Hodge 2000; Bethlenfalvay et al. 1985). In our study, we also found differences among the *Rhizobium* and AM treatments. Specific strains of *Rhizobium* or species of AM resulted in increased plant growth or nutrient concentration; however, results differed between Illinois bundleflower and panicled tick clover. The two AM species and the *Rhizobium* strains selected for each plant species appeared to be effective endophytes because Illinois bundleflower and panicled tick clover were both successfully colonized and nodulated roots developed (Table 1, Table 3). Despite successful nodulation of both species, *Rhizo-*

Table 3. Effects of inoculation with AM fungi or Rhizobium on plant growth, AM colonization, and nodulation of *Desmodium paniculatum* in Missouri, USA

Treatments	Stem Ht cm	Leaf Dry Wt g	Stem Dry Wt g	Root Fresh Wt g	AM %	Nodules per Plant #
Rhizobium						
None	44.2A†	1.3B	0.8A	4.3AB	17A	11B
41Z10	47.0A	1.9A	0.8A	6.1A	12A	24A
32Z3	46.7A	1.7AB	0.6A	3.6B	16A	26A
AM						
None	37.8b	1.3b	0.7a	5.2a	0b	16a
G. intraradices	51.2a	2.0a	0.8a	5.0a	30a	22a
G. etunicatum	48.9ab	1.6ab	0.7a	3.7a	14a	23a

†Means followed by the same letters within a column (uppercase = *Rhizobium*, lowercase = AM) are not significantly different at $\alpha = 0.05$.

Table 4. Effects of inoculation with AM fungi or *Rhizobium* on concentration of shoot nutrients of *Desmodium paniculatum* in Missouri, USA

Treatments	N %	P %	K %	Ca %	Mg %
Rhizobium					
None	2.67AB†	0.15AB	1.18B	1.13B	0.28A
41Z10	2.82A	0.19A	1.46AB	1.33A	0.30A
32Z3	2.62B	0.15B	1.69A	1.08B	0.25B
AM					
None	2.74a	0.11b	0.80b	1.08a	0.27a
G. intraradices	2.65b	0.18a	1.78a	1.23a	0.27a
G. etunicatum	2.72ab	0.19a	1.75a	1.23a	0.29a

†Means followed by the same letters within a column (uppercase = *Rhizobium*, lowercase = AM) are not significantly different at $\alpha = 0.05$.

bium appeared to have little or no effect on growth of either native legume. We would expect inoculation with *Rhizobium* to improve growth compared to the control. We can only speculate that the soil medium had sufficient N for normal plant growth, supported by our finding that *Rhizobium* did not consistently affect nitrogen concentration in either legume (Table 2).

Both legumes exhibited enhanced growth in plants inoculated with *G. intraradices* compared to the controls. *Glomus etunicatum* also tended to enhance plant growth but differences were not significant. Some of the enhanced growth observed in Illinois bundleflower plants colonized by *G. intraradices* may be due to increased nodulation. Plants colonized by *G. intraradices* averaged 11 nodules per plant, whereas those colonized by *G. etunicatum* and the control only averaged 3 nodules per plant. Interestingly, either species of AM increased shoot P and K concentration in panicled tick clover, but neither species of AM affected nutrient uptake in Illinois bundleflower plants other than reducing Mg concentration.

The results of this study suggest that improved yields from native legumes are possible in agroforestry practices through inoculation with the proper AM species and *Rhizobium* strain. However, additional research is needed to identify the best *Rhizobium* and AM inoculates for Illinois bundleflower and panicled tick clover. Knowing which *Rhizobium*, AM, or combination works best for each of these native legumes is important for developing management strategies for use in agroforestry practices.

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