Journal of Applied Biosciences 89:8263– 8271

ISSN 1997–5902

Mycorrhizal Enhancement of Biomass Productivity of Big Bluestem and Switchgrass in Neutral and Acidic Substrate

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Original submitted in on 24th March 2015. Published online at www.m.elewa.org on 30th May 2015 http://dx.doi.org/10.4314/jab.v89i1.2

ABSTRACT

Objectives: Greenhouse pot studies were conducted to assess the abilities of two arbuscular mycorrhizal fungi (AMF) namely, *Rhizophagus clarus (Rc) and R. intraradices (Ri)* to enhance biomass productivity of big bluestem (*Andropogon gerardii*), as a complementary bioenergy feedstock to and switchgrass (*Panicum virgatum*).

Methodology and results: Big bluestem (BB) and switchgrass (SG) were grown in a soilless substrate adjusted to pH=6.5 or 4.5 and inoculated separately with *Rc* and *Ri*. Plants were grown in the greenhouse for 12 weeks. Results show that AMF significantly enhanced biomass productivity of the grasses over corresponding controls, regardless of pH. Substrate inoculation with *Rc* produced the highest and similar total BB biomass at pH=6.5 and 4.5. However, biomass partitioning into shoot and root differed with pH. Inoculation with *Ri* produced the highest and similar total SG biomass at pH=6.5 and 4.5. SG biomass was more equally distributed at both pHs.

Conclusion and application of findings: Differences in substrate partitioning into shoot and root biomass shown by *Rc*-inoculated BB at 4.5, appeared to be consistent with *Rc* endowing BB the capacity to maintain both relatively high shoot as well as root biomass at pH=4.5. This pattern of substrate partitioning was not shown by *Rc*- or *Ri*-inoculated BB grown at pH =6.5, or *Ri*-inoculated BB grown at pH=4.5. Neither was the pattern shown by *Rc*- or *Ri*-inoculated SG, which maintained relatively similar R/S ratios regardless of pH. The usual biomass partitioning by BB at pH=4.5 deserves further investigation. Different patterns of biomass partitioning notwithstanding, results of this study strongly suggest that BB could complement SG, the model biofuel feedstock, especially under acidic substrate conditions.

Key words: Big bluestem; switchgrass; biofuel feedstock; arbuscular mycorrhizae, substrate acidity.

INTRODUCTION

Heightened global concerns about energy security and environmental sustainability have focused attention on renewable fuels as a means for significantly arresting the world's near-total dependence on finite fossil fuels for transportation. In the US, this focus is largely directed onto biomass-to-ethanol conversions. Currently, corn accounts for 94% of ethanol production (USDA ERS, 2012); however, this is untenable as a long-term proposition. Expansion of corn production to levels needed to meet even a small fraction of the nation's future energy requirements will come with prohibitively steep costs of intensive inputs and associated considerable environmental degradation

(Koo-Oshima, 200). Accordingly, attention has been redirected on warm season perennial grasses WSPGs) for biomass-to-ethanol conversions. The WSPG that has received the greatest attention in the US is switchgrass (*Panicum virgatum, L*), which, the US Department of Energy (DOE) selected as the bioenergy model, based on such desirable attributes as perennial growth, abundant biomass production, excellent nutrient use efficiency, wide geographic distribution and tolerance to abiotic stressors (Wright & Turhollow, 2010). With over 30 years of research, a considerable knowledge base exists on the agronomy, management and breeding of SG (Casler *et al.,* 2007). Accordingly, it will continue to play a leading role as feedstock for bioenergy production in the U.S. in the near future. However, in view of the billions of tons biomass that must be produced annually to meet projected biofuel goals (Perlack *et al.,* 2005), it will be necessary not only to greatly enhance the productivity of switchgrass (SG) but also to diversify biomass production by capitalizing on the inherent potentials of other cellulosic biomass crops (Gonzales-Hernandez *et al.,* 2009). Native WSPGs that have been mentioned as complementary feedstock to SG include prairie cordgrass (*Spartina pectinata*, Bosc ex Link), little bluestem (*Schizachyrium scoparium*, (Michx.) Nash) (Gonzales-Hernandez *et al.,* 2009), big bluestem (*Andropogon gerardii*, Vitman) and eastern gamagrass (*Tripsacum dactyloides* L.) (Anderson *et al.,* 2009*;* Ge *et al.,* 2012). A distinguishing characteristic among these grasses is their tolerances of soil acidity. For example, Keene and Skousen (2010) found that switchgrass could be successfully established on acid mine land for biofuel biomass production. The same characteristic makes eastern gamagrass valuable for improving acidic subsoils, allowing lands considered marginal or impaired to be put to productive use (Gilker *et al.,* 2002). Acid tolerance by big bluestem has not been systematically studied. Projections made at the emergence of switchgrass as biofuel feedstock had assumed biomass productivity of the grass on prime land. It is increasingly recognized that those earlier projections did not adequately account for the critical issue of land availability. Already, over 30% of the world's arable land has been affected by acid degradation largely caused by past unsustainable practices (Von Uexkull & Mutert, 1995). Now, drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Wang *et al.,* 2003). Not surprisingly, debates over land use for food versus bioenergy have been gaining increasing attention (Ross & Hinrichs, 2011) as the world's population continues to increase in the face of shrinking prime croplands, increasing energy demands and environmental degradation. Relatively recent reports have suggested that if biofuel feedstocks (low-input high diversity perennials) are planted on abandoned and degraded lands with marginal productivity, an estimated 26 – 55% of the current world fuel consumption could be met without affecting crop and forage production (Cai *et al.,* 2011). This is a welcome development but first, it is necessary to enhance biomass production of such marginal lands. One approach for enhancing biomass productivity of marginal lands capitalizes on mycorrhizal symbioses (Ghimire & Craven, 2011). Mycorrhizal fungi are ubiquitous, obligate soil symbionts that form close associations with plant roots conferring numerous benefits including to nutrient uptake (Smith & Smith, 2012) and increase tolerance to abiotic stresses on the plant partner (Picardo *et al.,* 2012). This paper reports observations on arbuscular mycorrhizae (AM)-assisted enhancement of biomass productivities of big bluestem in neutral and acidic substrate, using SG as the model biofuel feedstock. Results of the investigations can lead to development of strategies for utilization of acid soils for routine production of alternative biofuel feedstocks.

MATERIALS AND METHODS

Plants and growth substrate: '*Alamo*' variety of switchgrass (*Panicum virgatum* L.) and '*Roundtree*' variety of big bluestem (*Andropogon gerardii* Vitman) were obtained from Star Seeds Inc, Osborne, Kansas. Plants were grown on soilless growth substrate composed of peat moss, vermiculite and sand in a 1:1:1 ratio (Al-Agely and Ogram, 2011). Use of soilless medium avoids AMF suppression by high P concentrations characteristic of agricultural soils.

Arbuscular mycorrhizal fungi (AMF): *Rhizophagus clarus* (formerly *Glomus clarum*) WV234 and *Rhizophagus* (formerly *Glomus) intraradices*, UT125 were obtained from the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM), Morgantown, WV. *Rhizophagus clarus* was originally isolated under acidic soil conditions (pH < 4.0); *R. intraradices* was isolated from soil at pH 6.5-7.5 (INVAM). Host seed preparation and germination: Seeds of *Sorghum bicolor* (L.) Moench were surfaced sterilized using a modified Arabidopsis seed sterilization protocol http://www.plantsci.cam.ac.uk/research/jillharrison/protocols/arabidopsis/sterilization-arabidopsis-seed.pdf. Briefly, sorghum seeds were washed thoroughly under running water, rinsed with 70% ethanol for 30 secs, and then treated with 1.0% of commercial bleach (5.25% sodium hypochlorite) containing few drops of Tween 20. After 15 mins, the seeds were rinsed thoroughly with sterile distilled water 3 times. Surface sterilized seeds were germinated on filter in Petri dishes in the dark for up to 5 days after which time they were transplanted into Deepots (Stuewe and Sons: Tree Seedling Nursery Containers, D40) containing acid washed and autoclave-sterilized turface (Pro League® Calcined Clay).

AMF inoculum propagation and harvesting: Protocols for propagation and harvesting of inoculum were adapted from Giovannetti & Mosse 1980. Briefly, Deepots were filled with approximately 400 mL of turface, and then they were overlain by 40ml inoculum of either *Rc* or *Ri*. Each Deepot was covered with additional 60ml of turface. After planting, seedlings were watered with distilled water every other day. One week after planting, seedlings were watered with a nutrient solution (Peters Professional 15-0-15 Peat-Lite® Dark Weather Feed), which was supplemented with 0.6 mM P as KH_2PO_4 (Dr Kimberly Gwinn, University of Tennessee personal communication). After four weeks, sorghum roots were sampled to determine the extent of colonization using a Trypan blue staining procedure (Kumar *et al.,* 2008) Colonization was assessed as counts of propagules (hyphae, vesicles, spores, and arbusculars) using the grid intersection method (Al-Agely & Ogram, 2011). After 12 weeks, plant feeding was terminated, sorghum shoots were allowed to dry out and mixture of turface containing the propagules was collected, mixed thoroughly and counts of propagules were determined using microscopy (Agley and Ogram, 2011). The mixture was stored at 4°C until used for inoculating BB and SG.

Experimental Design*:* The experiment was conducted in the greenhouse pots (1.5 L capacity) using a factorial design of 2WSPGs x 3AMF x 2pH where WSPGs were BB and SG; AMF = inoculum (Uninoculated, *Rhizophagus. clarus*, and *R. intraradices*), and 2 levels of pH = neutral and acidic (6.5 and 4.5). All treatments were replicated four times with four plants per pot. Treatments were arranged in a randomized design on greenhouse benches and they were rotated frequently in order to minimize position effect within the greenhouse.

Adjustment of substrate pH and WSPG seed pre-germination: Substrate pH was adjusted according to modifications of protocols originally described by Islam *et al.,* (Islam *et al.,* 2004). Instead of $CaCO_3$ used in the original protocol, we used $Ca(OH)_2$ to raise pH of soilless substrate to 6.5. The content of peat left the soilless substrate pH at approximately 4.5 and no further adjustment was needed. Increments of $Ca(OH)_2$ were added to the growth substrate and dose-response curves were generated to allow estimation of amounts of the chemicals required to bring a known amount of substrate to pH=6.5 (1:5 w:v of substrate: 10 mM $CaCl_2$). Seeds of both SG and BB were initially started in germination trays. After seedlings reached 3- to 4-leaf stages, they were transplanted into the 1.5 L pots.

Substrate inoculation, plant growth conditions and harvesting: Seedlings (3- to 4-leaf stage) of switchgrass and big bluestem were inoculated in 5-inch pots containing 1.5kg substrate to give propagule concentration 3.9×10^3 and 3.7×10^3 per gram of substrate *Rc* and *Ri* respectively. Plants were grown for 12 weeks. After four weeks, root samples were examined for inoculum colonization as described earlier and the treatments were watered as needed. Once a week, plants were fed with a modified Hoagland's solution (Al-Agley & Ogram, 2011). At harvest, shoots were cut at 0.5 cm above the top of the pots dried at 60°C and weighed to determine aboveground biomass productivity. Roots were washed to remove substrate materials and they were

dried similarly to determine root biomass productivity of SG and BB.

Statistical Analysis: Mean comparisons of biomass data were made by analysis of variance (ANOVA) using SAS (SAS Institute, 2012).

RESULTS AND DISCUSSION

These investigations focused on AMF-mediated enhancement of biomass productivity by BB for diversifying biofuel feedstock production especially under abiotic stress conditions such as those imposed by acidity. This is in recognition of the fact that 30-40% of the world's arable land has been impacted by acid degradation largely caused by past unsustainable practices (Von Uexkull & Mutert, 1995), and that such lands need to be brought into productive uses in the emerging emphasis on bioenergy. BB was selected for these investigations because of a number of desirable characteristics. First, it is endemic to North America, which is an important consideration for expanding its production area in the U.S. (Anderson *et al.,* 2008). In addition, Jung & Vogel (1992) reported that the *in vitro* fermentability of BB was greater than that for other WSPGs, suggesting that BB may have an advantage over SG when it comes to the production of ethanol and other value-added chemicals via consolidated bioprocessing (Weimer and Springer, 2007).

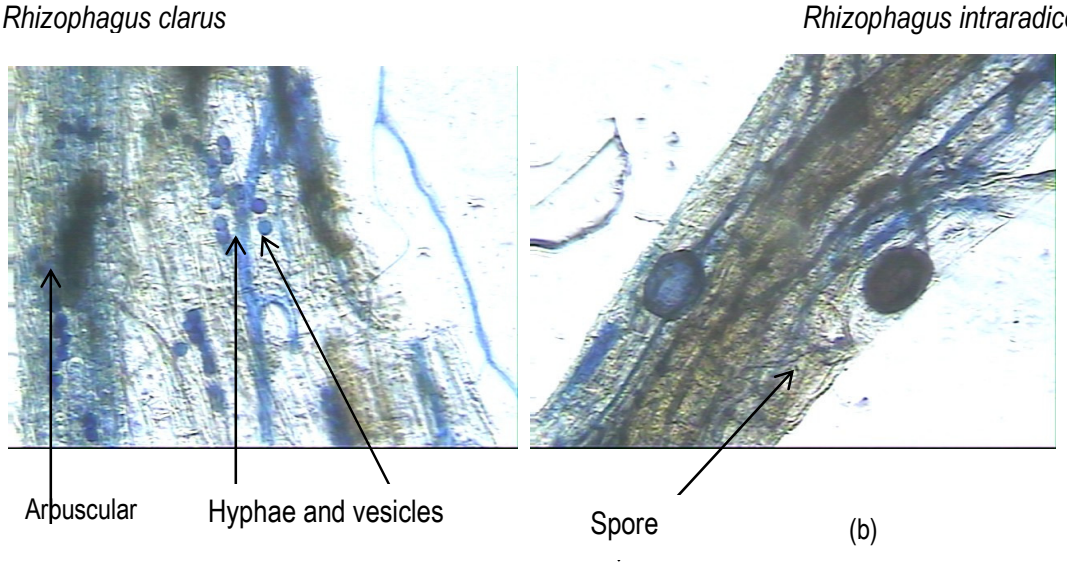

Figure 1: Propagules of (a) *Rc* and (b) *Ri* used for substrate inoculation

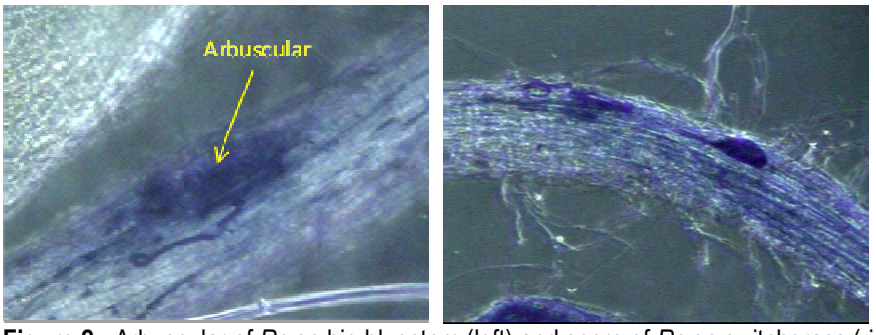

Figure 2: Arbuscular of *Rc* on big bluestem (left) and spore of *Rc* on switchgrass (right).

Propagules (hyphae, arbusculars and spores) of *Rc* and *Ri* for inoculating BB and SG plants: As mentioned in the 'Materials and Methods', *Rhizophagus clarus* was originally isolated under acidic soil conditions

(pH < 4.0); *R. intraradices* was isolated from soil at pH 6.5-7.5 (INVAM). Respective inoculums of Rc and Ri were prepared from a 4-month growth of *Sorghum bicolor* on nutrient solution-fortified turface as described earlier. Figures 1a and 1b are photos of propagules of *Rc* and *Ri* that were used for inoculating the soilless substrate used in these experiments. Figures 2a and 2b are arbuscular of *Rc* on big bluestem and spore of *Rc* on switchgrass during growth of the WSPGs in soilless substrate. AMF-mediated enhancement of biomass productivity by big bluestem: Results presented in Table 1 show that at substrate pH=6.5, inoculation with AM significantly enhanced biomass by BB compared to uninoculated (UN) controls ($p \leq 0.05$). After 12 weeks, *Rc*- and *Ri*- inoculated BB produced 3.5 and 3.0 g dry weight/pot of shoot biomass respectively. This difference was not significant ($p \leq 0.05$) but it was significantly lower than 0.4 g/pot for UN controls (Table 1). Root biomass productivity showed a similar trend to shoot biomass productivity. Thus, substrate inoculation with *Rc* and *Ri* produced similar levels of biomass—7.9 and 7.5 g/pot, respectively compared to only 0.9 g/pot for UN controls (Table1). Thus, substrate inoculation with *Rc* and *Ri* produced similar levels of biomass—7.9 and 7.5 g/pot, respectively compared to only 0.9 g/pot for UN controls (Table1). The ratios of root and shoot biomass productivities (R/S) are shown in Table 1. At pH=6.5, R/S ranged narrowly between 2.25 to 2.50 including that for UN controls. These ratios were useful for the side-by-side comparisons of BB against the model biofuel feedstock, SG. At substrate pH=4.5, the trend of biomass productivity was different from the observation at pH=6.5 (Table 2). At the lower initial pH *Rc*-inoculated BB produced significantly higher ($p \leq 0.05$) shoot biomass (4.2 g /pot) than that produced by *Ri*-inoculated BB (2.8 g/pot). Still, the lower shoot biomass of *Ri*-inoculated BB was significantly higher than the 0.4 g/pot for the control. The contrasting biomass productivity between *Rc*- versus *Ri*-inoculated BB was reflected as significantly higher root biomass of 7.1 /pot compared to 5.0 g/pot for *Ri*-inoculated BB. At the lower pH, R/S ratio for control treatments remained 2.25, similar to the observation at pH=6.5. However, R/S ratio for inoculated treatments declined to 1.69 and 1.79 for *Rc* and *Ri* respectively (Table 2).

Table 1: Effect of AMF Inoculation on Biomass Productivity by Big Bluestem in Neutral Substrate (pH=6.5)[1]

Treatment	Shoot	Root	Total	R/S Ratio
	----------g/pot----------			
Uninoculated	0.4b	0.9b	1.3b	2.25a
Rhizophagus clarus (*Rc*)	3.5a	7.9a	11.4a	2.26a
Rhizophagus intraradices (*Ri*)	3.0a	7.5a	10.5a	2.50a

[1]/Values with the same letters in a column are not significantly different ($p \leq 0.05$).

Table 2: Effect of AMF Inoculation on Biomass Productivity by Big Bluestem in Acidic Substrate (pH=4.5)[1]

Treatment	Shoot	Root	Total	R/S Ratio
	----------g/pot----------			
Uninoculated	0.4c	0.9c	1.3c	2.25a
Rhizophagus clarus (*Rc*)	4.2a	7.1a	11.3a	1.69c
Rhizophagus intraradices (*Ri*)	2.8b	5.0b	7.8b	1.79b

[1/] Values with the same letters in a column are not significantly different ($p \leq 0.05$).

AM-mediated enhancement of biomass productivity by switchgrass: Until recently, WSPG were well known for forage production. Our own research had long focused on their use in phytoremediation of a broad range of organic contaminants (Le *et al.,* 2011; Dzantor *et al.,* 2006; Dzantor & Woolston, 2001; Dzantor *et al.,* 2000). Appropriate evaluation of BB as bioenergy feedstock necessitates side-by-side comparison of feedstock attributes against the bioenergy model, SG. Tables 3 and 4 are biomass productivities by SG at substrate pH=6.5 and 4.5 respectively. Similar to observations with BB, substrate inoculation AMF significantly enhanced shoot and root biomass productivities by SG compared to the biomass production by corresponding UN controls ($p \leq 0.05$). The results show that for SG, substrate pH did not significantly affect shoot or root biomass productivities. After 12 weeks at pH=6.5, *Rc*- and *Ri*-inoculated SG produced practically identical shoot biomass (4.7 and 4.9 g/pot, respectively) compared to only 0.6 g/pot for the control treatment. Corresponding

root biomass productivities were 5.6 and 6.1 g/pot for *Rc*- and *Ri*-inoculated SG respectively. Differences in these values were not significant ($p \leq 0.05$). Table 3 shows that at pH=6.5, R/S ratios for control as well as AMF-inoculated SG ranged narrowly from 1.00 to 1.27 (Table 3). At pH=4.5, *Rc* and *Ri*-inoculated SG produced 4.4 and 5.0 g/pot shoot biomass respectively, compared 1.1 g/pot for controls (Table 4). Corresponding root biomass productivities were 5.2 and 6.0 g/pot respectively. At pH =4.5, R/S ratios ranged even more narrowly from 1.2 to 1.28 for the control and AMF treatments.

Table 3: Effect of AMF Inoculation on Biomass Productivity by Switchgrass in Neutral Substrate (pH=6.5)[1]

Treatment	Shoot	Root	Total	R/S Ratio
	----------g/pot----------			
Uninoculated	0.6b	0.6b	1.2b	1.00a
Rhizophagus clarus (*Rc*)	4.7a	5.6a	10.3a	1.16a
Rhizophagus intraradices (*Ri*)	4.8a	6.1a	10.9a	1.27a

[1/] Values with the same letters in a column are not significantly different ($p \leq 0.05$).

Table 4: Effect of AMF Inoculation on Biomass Productivity by Switchgrass in Acidic Substrate (pH=4.5)[1]

Treatment	**Shoot**	**Root**	**Total**	**R/S Ratio**
	----------g/pot----------			
Uninoculated	1.1b	1.4b	2.5b	1.28a
Rhizophagus clarus (*Rc*)	4.4a	5.2a	9.6a	1.19a
Rhizophagus intraradices (*Ri*)	5.0a	6.0a	11.0a	1.20a

[1/] Values with the same letters in a column are not significantly different ($p \leq 0.05$).

Overall, results of this study show that substrate inoculation with AMF greatly enhanced total biomass productivity by BB to levels that are comparable to, or slightly higher than the productivity by SG, the model bioenergy feedstock (Tables 1-4). The results also showed that in general, growth of BB was characterized by greater root biomass productivity than was observed with SG. For example, with the exception of the *Ri*-inoculated treatments (which produced only 5.0 g) root biomass productivity by BB ranged from 7.0 to 7.9 g/pot, independent of substrate pH. In contrast, root biomass of AMF-inoculated SG ranged from 5.2 to 6.1 at both pH levels. Furthermore, *Rc*-mediated enhanced biomass productivity by BB did not appear to occur at the expense of shoot biomass productivity, a crucial consideration under biomass-to-ethanol bioprocessing. As shown in Table 2, at pH=4.5, *Rc*-inoculated BB produced total biomass of 11.3 g/pot, which was partitioned into 4.2 g for shoot biomass and 7.1 g for root biomass. Under the corresponding conditions, *Rc*-inoculated SG produced similar level of shoot biomass (4.4 g/pot) but only 5.2 g/pot root biomass (total biomass =9.7 g/pot). The ratios of root and shoot biomass production (R/S) were useful for the side-by-side comparisons of BB against the model biofuel feedstock, SG. The manner in which plants partition metabolic production into root and shoot biomass is a topic of ongoing debate (Barbosa *et al*, 2012; Bonifas and Lindquist, 2006]. Certainly, more studies of the nature described here are needed to provide definitive understanding of the phenomena. However, these results suggest strongly that at least for the purpose of side-by-side comparison, R/S ratios may provide one more measure for evaluating BB and potentially other WSPGs as complementary or alternative feedstock to bioenergy model, SG. A notable aspect of biomass partitioning observed during these experiments was the relatively constant R/S ratios for SG plants (R/S = 1.00-1.28) regardless of AMF inoculation or substrate pH. At pH=6.5, BB plants also demonstrated a relatively constant R/S ratio (2.25-2.50). However, at pH = 4.5, R/S decreased from 2.25 for control to 1.79 and 1.67 for *Ri*- and *Rc*-inoculated BB respectively. Perhaps more importantly, *Rc*-mediated relatively high root biomass production was accompanied by relatively high shoot biomass production, similar to the productivity by the SG model. The phenomenon deserves investigation in soil to validate potentials of *Rc*-mediated biomass productivity by BB. Previous investigators have reported the use of AMF inoculation to enhance biomass productivity of SG. For example, Clark *et al.,* 2005 studied four AM isolates (*Glomus intraradices WV895*, *G. clarum WV751*, *G. etunicatum WV579A* and *Acaluspora mellee*) in six acidic

soil types. They concluded that soil type strongly influenced extent of AMF-mediated enhancement of biomass productivity. The investigations by Clark *et al.,* (2005) are particularly relevant to our own overall goal of developing biomass production systems for diversification of biofuel feedstock (in this case, BB) and production of such feedstock under acidic conditions. Ghimire *et al.,* 2009 and Ghimire and Craven (2011) also reported enhancement of SG biomass productivity of SG following inoculation with the ectomycorrhizae *Sebacina vermifera*. In the former study, the investigators noted that although *S. vermifera* was originally isolated from roots of Australian orchids, the association that they created was mutually beneficial. They also expressed caution about the possibility of *S. vermifera* spreading to become infective of weed species. In the later study, the investigators demonstrated ability of *S. verimifera* to enhance biomass productivity under drought conditions. Because of the near total focus on SG as bioenergy feedstock, reports on mycorrhizal mediated enhancement of other WSPGs including BB are very limited. However, some older studies promoted the benefits of AM inoculation to BB biomass production. For example, Hetrick *et al.,* (1990) demonstrated that mycorrhizae improved clipping tolerance of BB, although they cautioned that repeated and intense clipping changed R/S ratios and eventually caused the benefits of AMF to be lost. In addition, Bredja *et al.,* (1993) reported that AM inoculation of soils from an eroded site in the Sandhills of Nebraska greatly improved successful reestablishment of WSPGs at this site. In that study, the site was so mycorrhizae-dependent that a 5-fold P fertilization did not stimulate as vigorous of growth of the grasses as did inoculation with AM. The older reports predated the current emphasis on biofuels; however, they highlight the importance of AM to the enhancement of biomass productivity in degraded lands. In summary, results of the experiments presented here suggest strongly that BB deserves greater consideration than currently exists, as complementary and/or alternative bioenergy feedstock. In particular, *Rc*-mediated total biomass production by the grass could be comparable to or slightly higher than production by SG. These experiments were conducted in soilless substrate to avoid depression of AM by levels of P typical of many agricultural soils. Accordingly, this conclusion requires confirmation in soil. Our goal is to enhance biomass productivity in degraded soils, which are typically deficient in P. Side-by-side experiments of the nature described here but in natural soil, are needed to validate AMF-mediated enhancement of biomass productivity by BB and potentially other WSPGs as biofuel feedstock that can be produced profitably under soil acid and potentially other abiotic stressors.

ACKNOWLEDGEMENT

This research was partly supported through a grant from the USDA/NIFA (#2010-38821-21594) awarded to E.K.D.

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