5-2015

Enhancing the Sustainability of Integrated Biofuel Feedstock Production Systems

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Recommended Citation

Ashworth, Amanda Joy, "Enhancing the Sustainability of Integrated Biofuel Feedstock Production Systems. " PhD diss., University of Tennessee, 2015.
https://trace.tennessee.edu/utk_graddiss/3320

To the Graduate Council:

I am submitting herewith a dissertation written by Amanda Joy Ashworth entitled "Enhancing the Sustainability of Integrated Biofuel Feedstock Production Systems." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant Sciences.

Fred Allen, Major Professor

We have read this dissertation and recommend its acceptance:

Patrick Keyser, Donald Tyler, Adam Taylor

Accepted for the Council:

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(Original signatures are on file with official student records.)

Enhancing the Sustainability of Integrated Biofuel Feedstock Production Systems

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Amanda Joy Ashworth
May 2015

DEDICATION

This thesis is dedicated to my parents, Bill and Dorothy Ashworth, who taught me the importance of hard work, dedication, and perseverance. Only through their altruistic love, guiding support, and encouragement have these and previous investigations been possible.

ACKNOWLEDGMENTS

Many ‘thank yous’ are in order to people without whom, completing this work and obtaining this personal accomplishment would not have been possible. Specifically, it has been only through the guidance of caring and supportive faculty such as Drs. West, Allen, and Keyser that lead me to M.S. and PhD degrees.

Dr. Charles West led me to a M.S. graduate program at the University of Arkansas. Under his supervision, I gained an appreciation for grasses (for use in forage, conservation, and cellulosic biofuel production). It was also because of him that I gained confidence in my aptitude to complete graduate-level research. For his devotion and assurance in my abilities as a young scientist, as well as his friendship, I am forever indebted.

I am also greatly appreciative to Dr. Keyser for encouraging me to pursue the doctorate degree, without his continued urging of me to “get on the bus or get out of the way,” this dissertation would not have been completed. Thank you also Dr. ‘Saint Patrick’ Keyser for giving me the opportunity to learn from you over the past four years. Your humor during trying times has been much appreciated.

I would also like to extend sincere gratitude to Dr. Fred Allen for his continued guidance and personal and professional support. It has been because of his patience, hard work, and confidence in my capabilities that this dissertation and many past accomplishments and opportunities have been realized. Your kindness and diligence defines an exemplary advisor, and I am honored to have worked under your tutelage.

Much gratitude is extended to my outstanding committee members, Drs. Don Tyler and Adam Taylor for lending their expertise, providing guidance, and challenging me to do my best.

Last but not least, sincerest of thanks to my beloved friends and family whom have helped me weather the stressors of a PhD student, and have continued to provide support, every step of the way.

It is because of each of you that this accomplishment is possible, thank you.

ABSTRACT

As use of second-generation biofuel crops increases, so do questions about sustainability, particularly their potential to affect fossil energy consumption and greenhouse gas emissions. Nitrogen (N)-fixing legumes interseeded into switchgrass (*Panicum virgatum* L.) may be an alternative to inorganic fertilizer in forage-feedstock systems. Research herein is divided into four general experiments: I). N replacement and feedstock impacts from legume intercrops and biochar in switchgrass; II). N-fixation rates in intercrop systems; III). impacts of biofuel systems under enhanced climate change; and, IV). projected sustainability of regional switchgrass production. Approaches included: characterization of feedstock/forage quality traits based on legume, biochar and synthetic-N applications, and harvest timing; quantification of nitrogenease activity in legumes via two techniques (^{15}N [isotopic] enrichment and N-difference); and, determine impacts from regional switchgrass production, N-input sensitivities, and legume-intercropping via life cycle assessment (LCA). Results suggest pigeon pea, sun hemp, red clover, and partridge pea intercrops, and in some instances, biochar may supply analogous-N to that of synthetic fertilizers to *Panicum* species. Specifically, selected legume fixation may exceed recommended inorganic-N levels (67 kg [kilogram] N ha^{-1} [hectare]) in both temperate humid and semiarid tropical pasture/feedstock systems. N-difference method may be used to measure biological fixation, as it estimated comparable fixation rates to that of benchmark ^{15}N enrichment values. Furthermore, harvest timing can be manipulated to obtain desired feedstock traits. Specifically, overwintering harvests minimized phosphorus and potassium removal, and maximize ethanol yield, hemicellulose, and in field dry-down [10.84 vs. 24.81% ($P \leq 0.05$)]. However, yield losses were observed (22%). Forage yields were generally more responsive to legumes, and legume intercropping may increase switchgrass forage quality ($P<0.05$). In addition, switchgrass adaptation was ‘moderate’ (5-30% weed coverage) under an intensified climatic scenario. Consequently, switchgrass may be produced in the tropics, due to this species drought tolerance, genetic diversity, and competitive growth after establishment. Finally, when agricultural inputs were compared via LCA, nitrogen fertilization resulted in the greatest environmental impacts. Further, legume-intercropping reduced greenhouse gas emissions and groundwater acidification compared with the 67 kg N ha^{-1} rate. Intercropping selected legumes in switchgrass may enhance forage/feedstock quality and yield while reducing non-renewable inputs and greenhouse gas emissions.

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INTRODUCTION

North American native prairie grasses are perennial, drought-tolerant bunchgrasses with a high yield potential on a wide range of soils. Native grasses are adapted to the soils and climates of the United States and their range may include portions of Canada and Central America (Parrish and Fike, 2005). Native pastures are highly productive and could play a significant role in global carbon (C) sequestration. Also, these grasses have the potential to reduce soil erosion, improve water quality, and provide habitat for wildlife and pollinators. These species are suitable for soils considered marginally economical for annual row crops and can improve soils by protecting them from erosion and increasing their C storage, organic matter, aggregation, infiltration, and water-holding capacity. Native grasses generally grow 1–3 m tall and roots of established plants may reach depths of 3-m (McLaughlin et al., 1999; Vogel et al., 2002; Weaver, 1954). These native perennials, particularly switchgrass (*Panicum virgatum* L.), have high water- and nutrient-use efficiencies, meaning they produce relatively high amounts of biomass per unit of water transpired and per unit of essential-nutrient uptake (Bransby et al., 1998; Heaton et al., 2004). Typical of most native grasses, they fix CO_2 by the C_4 photosynthetic pathway (Moss et al., 1969). This pathway enables the plant to tolerate high summer temperatures and moderate-to-high drought stress. Optimum temperature for C_4 photosynthesis is between 35 to 38ºC (Long, 1999). Generally, warm-season C_4 grasses require about one-third to one-half as much water and nitrogen to produce a unit of dry matter, compared to C_3 grasses (Moser et al., 2004). Therefore, C_4 species outperform C_3 species under water deficit and high temperature conditions.

Warm-season grass-dominated plant communities are among the most important C-sequestering terrestrial ecosystems, considering they produce only 6% of the global biomass but have 15% of the global soil organic C (Jobbagy and Jackson, 2000). The maintenance of living roots and crowns has many benefits including acquisition of nutrients and water from deep in the soil profile, a strong energy storage reserve for rapid spring recovery, stable yields during stress years, and continual soil organic matter formation; considering, more than 95% of the C in C_4 grasses is belowground as soil organic matter (McLaughlin and Walsh, 1998). The active pools of roots from native perennial bunchgrasses are major sources of carbon due to fine root turnover, active populations of soil microorganisms and lower-leaf deposition.

Switchgrass has always been a part of North American prairie communities and thus served as forage for native wildlife, and subsequently for cattle grazing on rangeland (Wolf and Fiske, 1995). In the last 50 years, switchgrass was adopted as a cultivated forage crop to fill summer production gaps left by cool-season forages in the central plains, Midwest, and northeast U.S. (Parrish and Fike, 2005). The majority of what is known about the growth, agronomic practices, genetics and chemical composition of switchgrass is derived from research on it as a forage crop for animal production, for which the goal is to maximize cattle conversion of digestible energy, mineral nutrients, and protein. This normally entails grazing the plant when it is green and leafy (late spring to mid-summer) when digestible energy and minerals are in fairly high concentrations, as opposed to grazing stemmy and senescent forage (late summer to autumn) (Wolf and Fiske, 1995). The optimum traits of switchgrass as a bioenergy feedstock, however, contrast with those for forage, in that the bioenergy feedstock needs to be low in mineral concentrations, and yield of lignocellulosic fiber mass needs to be maximized (Parrish and Fike, 2005).

Switchgrass has a reputation for being difficult or slow to establish (Moser and Vogel, 1995; Parrish and Fike, 2005). This small-seeded species generally germinates in spring, followed by little above-ground growth in Year 1; typically attaining only 33 to 66% of its maximum production capacity during the first and second years, respectively, before reaching its maximum yield potential in year three and thereafter. This yield pattern is a function of the growth strategy of switchgrass, one that allocates energy to developing a deep, strong root system at the expense of above ground growth during establishment (McLaughlin and Kszos, 2005; Casler et al., 2007). Native grasses have a production life span of 15 or more years under proper management (Parrish and Fike, 2005).

During its vegetative phase, the rate of switchgrass development is closely related to accumulated growing-degree days (GDD), while timing of reproductive development is tied to photoperiod (Parrish and Fike, 2005). Switchgrass is a short-day plant, meaning that it flowers when exposed to shortening days of a specific length (Benedict, 1940). The development and flowering of switchgrass is location-dependent; however, genetically broad populations will have some plants at anthesis over a 3-wk period (Vogel, 2004). The ability of switchgrass to undergo dormancy in its perennial plant parts (crown buds and roots) toward the end of the growing season is critical for survival through periods of low winter temperatures (Parrish and Fike, 2005; Casler et al., 2004).

The main nutrient requirement of native warm-season grasses (NWSG) is N (Vogel et al., 2002), which is affected by the frequency and timing of harvests, amount of biomass removed, and soil N-mineralization rates (McLaughlin and Kszos, 2005). Even under optimal cropping management, plants usually take up less than 60% of applied fertilizer and often only 40% or less (Sinclair, 2009). Nitrogen uptake and removal in crops is highly variable within a single year, among years, and among sites, even when N-supplies from both the soil and additional fertilizer inputs are adequate (Gastal and Lemaire, 2002). The variability and rate of N uptake during development and its effects on yield have many impacts on the quality of biomass and the environment, as well as the overall feasibility of integrated biomass and forage production (Adler et al., 2006). Standard practices for switchgrass have called for at least 50 kg ha^{-1} N during the year after switchgrass establishment (Year 2), followed by 80 to 100 kg ha^{-1} thereafter (Wolf and Fiske, 1995; McLaughlin et al., 1999). Perennial NWSG, such as switchgrass, translocate significant amounts of nutrients to lower plant parts during senescence. Vogel et al. (2002) reported that nitrogen (N) and nonstructural carbohydrates from aboveground biomass were translocated to stem bases and roots after anthesis and before a killing frost. Nitrogen exits cropland through the export of harvested crops and crop residues, volatilization of nitrogenous gases to the atmosphere (especially during senescence), and leaching of soil nitrate, potentially to neighboring water systems (Anex et al., 2007).

Leguminous species are known to biologically fix N and have, therefore, been used as intercrops for centuries; such species can be grown in tandem with agricultural crops in lieu of synthetic-N (Peoples, 2009). This process, known as biological nitrogen fixation (BNF) occurs through a symbiotic relationship with rhizobia (soil bacteria), which form nodules; where dinitrogen (N_2) from the atmosphere is converted *in situ* into ammonium (NH_4^+), a labile form of N (Graham, 2005). The plant in turn supplies the bacteria with nutrients that it needs to carry out growth and BNF. Legume cover crops that supply ≥110 kg N ha^{-1} achieve crop yields equivalent to those in conventional fertilizer-systems (Tonitto et al., 2006); thereby reducing or eliminating inorganic-N requirements.

Intercropping has proven to increase soil organic matter (SOM), net C, and N through the addition of high biomass via microbial mineralization and biomass decomposition; however, the full extent of this is largely unknown even in traditional cropping systems, and especially for switchgrass production. Under continued practice, sustainable cropping systems can reach equilibrium thus, providing a continual supply of N that increases yield over time. These organic materials can be used to manage existing soil-N by preventing loss from environmental factors, and potentially replacing N due to the slow release (compared to inorganic-N fertilizer), thereby providing N for neighboring crops (Cline and Silvernail, 2002; Wang et al., 2005; Tonitto et al., 2006). Conventional systems supply inorganic-N fertilizer based on paradigms that assume N can be controlled through direct management by supplying large quantities through a single application for immediate crop uptake. This management concept does not take into account interconnected dynamics of soil-N cycling, carbon, and SOM. Additionally, of the total amount of surplus inorganic-N applied to crops in conventional systems, only 45-55% of applied N is recovered in the crop biomass (Galloway and Cowling, 2002).

Biochar is a carbon-rich, biomass-derived material produced by “thermal decomposition of organic material under limited supply of oxygen (O_2), and at relatively low temperatures (<700 ºC)” (Lehmann and Joseph, 2009). Biochar is highly recalcitrant in soils and may be resistant to decomposition, as well as adsorb ions due to its greater surface area and charge density compared to organic matter (Lehmann, 2007). Additionally, most of the labile nutrients in biochar are released slowly, and the material acts as a liming agent due to the high ash-content and alkaline macronutrients. Furthermore, biochar may provide a habitat for soil organisms involved in N, P, or S conversions (Pietikäinen et al., 2000). Some research has suggested that because of the small pore size, biochar may suppress grazing protozoa and nematodes and promote beneficial bacteria and fungi (Warnock et al., 2007), thereby allowing beneficial microbes to transform nutrients more efficiently. Similarly, the porous medium supports the sorption of hydrophobic organic compounds and reduces phenolic compounds (Bornermann et al., 2007; DeLuca et al., 2002). Biochar may also prevent the proliferation of compounds that inhibit nutrient transformation such as denitrification and immobilization (Schimel et al., 1996). This is of particular interest considering the breakdown of and denitrification of synthetic-N is correlated with N_2O emissions, a gaseous form of N with a global warming potential of 310 ($kgCO_2$-eq) (IPCC, 1996). However, it should be noted that the elemental nutrient composition of the final char is dependent on the heating rate and temperature in the process as well as the initial nutrient feedstock profile. For example, greater electrical conductivity (EC) and higher pH are observed in chars when thermochemically decomposed at greater temperatures (>350°C) (Keech et al., 2005). Also, biochar reportedly has the ability to retain N within soils by enhancing ammonia and ammonium retention; reduce nitrous oxide and nitrate leaching fluxes, and enhance biological fixation and benefit microbial communities (Clough and Condron, 2010).

Rationale and Significance

The Southern U.S. climate and soils make it well-positioned for cellulosic feedstock and forage production that will not directly compete with arable land for food production. Already there have been advances in cellulosic ethanol production of switchgrass, and it is widely anticipated that the sustainable dual-purpose forage-bioenergy paradigm will increasingly be adopted in pasture systems (McLaughlin and Kszos, 2005; Vogel et al., 2002). Evaluating nutrient uptake/removal is important in order to minimize environmental impacts of fertilizer application, maximize energy-use efficiencies and produce a quality feedstock/forage with the lowest possible nutrient inputs; thereby enhancing the economic viability of the system (Bransby et al., 2008).

Numerous factors influence the economics of the agricultural sector, including the price of inputs such as fertilizers and herbicides which have experienced steady increases in past decades. Nitrogen is the principal nutrient required in cropping systems, and is applied at a rate of nearly 11 million tonnes of commercial or synthetic N per year in the USA (GAO 2003; USDA 2004). Among all nutrients, nitrogen is the fourth most abundant found in plant tissue and has the greatest impact on soil fertility and subsequent crop productivity (Taiz and Zeiger, 2006). Specifically, inorganic-N is a major, carbon-positive, revenue-negative input in current production paradigms. Inorganic-N has pricing linked to petroleum markets, requires fossil-energy for its production, and can degrade surface and ground water. Moreover, emissions of N_2O, a byproduct of both nitrification and denitrification in soils, is correlated to the production and breakdown of synthetic-N, and is approximately 300 times more potent as a greenhouse gas than CO_2 (Schlesinger, 1991). Petroleum and natural gas are the primary fuels in the US food system and both are now in high demand, and are being imported from various nations (Pimentel et al., 2008). Additionally, as fossil fuels become scarce, costs for production of inorganic fertilizers will increase, thus forcing farmers to seek alternative sources of fertilizers, or accept reduced profit margins and compromised economic viability. Alternatively, farmers will seek alternative sources of fertilizers or adopt forages/bio-feedstocks that are less input-intensive. These realities create challenges to sustainable feedstock production, and reduction of external inputs has important implications for producer profitability, carbon sequestration, and environmental sustainability.

The vulnerability of the agricultural sector to climate variability is well established in literature and the region under investigation is expected to be among the most affected. For example, temperate regions in the U.S. are expected to experience some beneficial effects from climate change (i.e., lengthening of growing seasons, carbon fertilization effects, and improved conditions for crop growth) (Moser et al., 2004). The consensus is that changes in precipitation and temperature will subsequently result in water regime fluctuations, and ultimately land-use changes, thereby impacting agricultural productivity (Adger et al., 2007). This is specifically true in the mid-South, where plant communities are anticipated to be among the most affected by climate-impacts.

Climate change has been observed and its drivers and ensuing impacts are expected to increase in the coming decades. Frequent and extreme droughts, intense rainfall events, and heat waves have serious impacts on forage/feedstock production across the Southeast. This was demonstrated in 2007 when extreme droughts in the Southeast forced many producers to disperse entire herds (National Drought Mitigation Center, 2007). Climate change impacts are projected to include reduced reliable water sources coupled with greater average temperatures. This projection suggests that C_4 species in feedstock production systems will be an appropriate option for adapting to climate change. An appropriate production paradigm is needed for the region in order to disseminate cultural practices to area farmers, thereby enabling them to adapt forage/biofuel systems to changing climates and in doing so, secure food systems.

Climate change will require innovations that allow for adaptation in forage production systems within the “fescue-belt,” a region that produces >25% of the nation’s beef. This region is forecast to be the center of the emerging cellulosic bio-feedstock industry, a development that will place further demand on the region’s forage production through competition for land. Forages in the Mid-South are overwhelmingly cool-season grasses that produce little warm-season forage. Because switchgrass is the primary focus of current feedstock production paradigms, it is critical to evaluate this species in the context of dual-use production systems. Furthermore, climate change may preclude the use of existing tall fescue cultivars due to their C_3 physiology, resulting in increased reliance on forages that are low-input, have high C-sequestration potential, and are adapted to increased frequency and severity of droughts. Therefore, a novel production model based on low-input, native, C_4 perennial grasses must be developed to enable producers to adapt forage systems to changing climates and thus, ensure national food security.

Recent literature has suggested several indirect effects of a warmer climate with increased atmospheric CO_2, such as the increased incidence of pests (weeds and insects) and disease, and decreased soil fertility and water availability. These trends are expected to decrease revenue in feedstock/forage systems, especially in the humid east (Hillel and Rosenzweig, 2009; Rosenzweig et al., 1996). Reilly et al. (2002) forecast an increase in expenditure on pesticides in southern U.S. under anticipated climatic conditions. In another study, Brumbelow and Georgakakos (2001) found that higher irrigation demands in southern regions will be required during extreme climatic events. Also, warmer temperatures are likely to negatively affect soil nutrients and organic matter through increased microbial decomposition, thereby increasing nutrient input requirements in forage systems. Based on agronomic research in lower latitude countries, losses in the agricultural sector (without adaptations) have been projected on the order of $61.2 billion (Reilly et al., 1994; 1996), and significant losses are expected worldwide (Rosenzweig et al., 1996; 2000).

The production and use of cellulose-derived ethanol can potentially reduce greenhouse gas emissions because the C released as CO_2 during combustion is re-assimilated into the subsequent year's regrowth of the biomass crop, which could offset CO_2 emissions to the atmosphere. In addition, the C released via combustion is cycled to and from the atmosphere rather than being unearthed from a fossil source (Parrish and Fike, 2005).

Contemporary policy and research has mainly focused on measuring and modeling greenhouse gasses and their ensuing long-term impacts. Less work has been done to mitigate emissions in the agricultural sector or to offer practices that can be adopted to adapt to these changes as no uniform procedure for landowners to adapt to climate and mitigate climate impacts. Even moderate changes to the climate will provide impetus to adopt low-input, drought-tolerant systems on marginal land. Adaptation in forage-biomass systems in the Mid-South should include tested and matched species that thrive under extreme climatic conditions.

Switchgrass is a potential feedstock for producing biofuels such as ethanol, synthetic gases (syngas), pyrolysis oil, or heat from direct combustion (McLaughlin and Kszos, 2005). Switchgrass is referred to as a cellulosic feedstock because most of the plant dry mass consists of insoluble structural carbohydrates, cellulose and hemicellulose, which are the primary polymers of the the cell walls. As the primary component of plant structure, cellulose is one of the most abundant materials on Earth (Anex et al., 2007). Cellulose is the most abundant polysaccharide in plants, accounting for 15 to 30% of the dry mass of all primary cell walls, and even more in secondary walls (Buchanan et al., 2000). Furthermore, switchgrass has potential to become a major feedstock in the U.S. as the cellulosic bioenergy industry takes shape (McLaughlin et al., 1998).

Switchgrass is classified as a cellulosic biomass feedstock because the entire plant is used; most of the gross energy is contained in the fibrous (lignin, cellulose and hemicellulose) component of the plant. The cellulose is composed of linear chains of β-1,4-linked polymers of glucose, which are hydrogen bonded to each other to form microfibrils (Taiz and Zeiger, 2006). Cellulose microfibrils are aligned with heterogeneous, branched polysaccharides called hemicellulose, which in turn is covalently bonded to a polyphenolic matrix called lignin (Buchanan et al., 2000). The lignocellulosic concentrations are affected by the plants' morphology and physiology, which in turn are largely determined by temperature, photoperiod, precipitation, and genetically programmed ontogeny (maturity) (Moser and Vogel, 1995). Relative to cellulose and hemicellulose, lignin biodegrades slowly, but is more energy dense, with values of around 25 MJ kg^{-1}, similar to that of bituminous coal (the most common fuel for power plants) (Anex et al., 2007). In general, biofuels for combustion and heating should contain low concentrations of water, N, and ash (Sanderson and Wolf, 1995). The N contained in the biomass is mainly responsible for oxides of nitrogen (NOx) emissions from the combustion process, which can react in the presence of sunlight, forming photochemical smog, a significant form of air pollution. Furthermore, alkali metals (e.g. K, Ca, Si) can cause slagging and clogging in the combustion chambers (Adler et al., 2006).

Recent increased public interest, technological advancements, and government programs have driven interest in new cellulosic biofuel plants (Environmental Protection Agency, 2009). However, efficiency improvements at the pilot scale are still needed before commercial-scale plants are realized. One barrier to the commercialization of cellulosic fuels is that significant amounts of feedstock are required in order to meet plant daily requirements; for example a 190-million liter plant needs approximately 1,814 Mg per day (Bohlmann, 2006). Additionally, it is widely assumed that refineries need to be located within 50 km of farms in order to be economically and environmentally feasible (Bransby et al., 2008); making commercial-scale of switchgrass cellulosic plants nonexistent, to date.

Virgin Island farmers face unique challenges to sustainable agriculture as a result of geographic, environmental and socio-economic conditions; therefore, low-external-input farming is a reality for agricultural systems. The USVI is representative of many tropical countries where farmers have limited access to reliable, readily available, or economically feasible external or commercial sources of inorganic or organic soil amendments (Smithson and Giller, 2002). Therefore, soil fertility management is a multifaceted problem and must be managed through alternative organic soil amendments.

Integrated cellulosic biomass for energy and forage for animal production has the potential to address many environmental and economic issues, including energy independence (and thus national security), rural economic development, and environmental sustainability, while providing multiple ecological services. However, for this to be realized, a protocol utilizing economically viable and sustainable nutrient sources over a range of climates and soils is needed for our region. In addition, more data are needed characterizing feedstock and forage materials and substantiating the feasibility of low-input systems, as well further understanding the mechanisms that drive them.

Despite high efficiency in converting fertilizer nutrients to harvestable biomass and low nutrient removal rates, inadequate data exist for predicting yields, feasibility of utilizing biofuel bi-products in agronomic systems, long-term fertilizer nutrient requirements, and the N-supply rates for leguminous cover crops for the Mid-South (Adler et al., 2006). Basic information is needed on dry matter accumulation and macronutrient uptake patterns during the growing season, and post-frost losses in order to construct useful nutrient balances and develop plant response predictions. These data would be useful for managers to plan their harvest time, predict yields, and prevent soil-nutrient mining. Furthermore, establishing a management option for the replacement of synthetic-N based on N-input scenarios is needed for sustainable integrated switchgrass production.

This research will provide much needed field data on switchgrass response to inorganic-N alternatives compared to synthetic fertilization. In addition, results will help establish an intercrop species that will maximize beneficial soil bacteria responsible for producing labile-N in the soil-sphere, thus producing a high quality feedstock/forage, while reducing fossil fuel-based N-inputs and input costs. Lastly, this research will contribute to the understanding of N-mineralization promotion from organic-N sources as they relate to sustainable bioenergy/forage production based on soil-biochemical processes and plant-nutrient retention.

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CHAPTER I

Enhancing the Sustainability of Lignocellulosic Feedstock Production Systems in the Southeast and Under Intensified Climatic Change

ABSTRACT

Second-generation feedstocks such as switchgrass (*Panicum virgatum* L.) have been proposed as sustainable alternatives to fossil fuels although they still require non-renewable based inputs, notably, inorganic nitrogen (N). Consequently, organic-N alternatives that sustain yields should be developed. Because climate change forecasts suggest the southeastern USA may develop more tropical growing conditions, it is important to understand how switchgrass growth may respond to future environmental stochasticity. Therefore, we evaluated switchgrass production in humid temperate and tropical environments under alternative N-sources. Objectives were to i) determine effects of two biochar rates (1 and 2 Mg ha^{-1}), three intercropped legumes [red clover (*Trifolium pretense* L.), partridge pea (*Chamaecrista fasciculate* L.), and sun hemp (*Crotalaria juncea* L.)] versus inorganic-N [67 kg ha^{-1} and a 0 kg ha^{-1} (control)] on desired feedstock characteristics, yield, and soil characteristics; ii) compare nutrient translocation for post-senescence (November 15th) and overwintering (February 1st) harvest date; and, iii) document switchgrass adaptation to more extreme (tropical) growing conditions as a proxy for climatic change response. The same experiment was carried out in the tropics and included a near relative of switchgrass, guinea grass (*Panicum maximum* L.), but only included sun hemp and pigeon pea (*Cajanus cajan* L.) intercrops. Therefore this experiment was conducted at humid temperate (Knoxville and Greenville, TN) and tropical (St. Croix, USVI) locations in a two-factor randomized complete block design. Post-senescence yields were lowest for 0 kg N ha^{-1} and sun hemp, with the low biochar not differing than 67 kg N ha^{-1}, partridge pea, or high biochar rate ($P \leq 0.05$). Over-wintering harvests increased phosphorus and potassium translocation, ethanol yield, fructan, digestible sugars, and in field dry-down [10.84 vs. 24.81% moisture ($P \leq 0.05$)]. However, yield losses were observed (22% decline compared to post-senescence cuts). Conversely, November harvests had greater tissue-N and total digestible nutrients, leading to greater soil-nutrient removals. Consequently, harvest management could be manipulated to obtain desired feedstock traits, whereas soil amendments had little effect on feedstock characteristics. Results from the tropical site suggest that pigeon pea and sun hemp intercrops, and in some instances, biochar (after several field applications) may supply analogous-N to that of synthetic fertilizers to *Panicum* species. Switchgrass adaptation was moderate under an intensified climate, with weed cover differing across all harvests (5-30%), perhaps due to switchgrass photoperiod sensitivity and lack of biochemical adaptations. Consequently, switchgrass may be produced in tropical growing conditions, due to this species drought tolerance, genetic diversity, and competitive growth after establishment on marginal soils; however, photoperiod-insensitive varieties would be required.

Keywords: switchgrass; feedstock characteristics; legume intercropping; biochar; intensified climate

I. Introduction

Fundamental to producing a sustainable bioenergy feedstock is determining alternative organic-nitrogen (N) sources and levels over a range of soils that are more carbon (C)-neutral. Specifically, inorganic-N has pricing linked to petroleum markets, requires fossil-energy for its production, and can degrade surface and ground water (Pimentel et al., 2008). One likely replacement of synthetic-N is incorporation of legumes into biofuel production systems (Snapp et al., 1998). An additional inorganic-N alternative that has gained much attention is biochar, or the by-product of switchgrass and other lignocellulosic biomass from thermochemical conversion processes such as pyrolysis (Lehmann and Joseph, 2009). Biochar may increase carbon sequestration and nutrient retention, thereby decreasing inputs (Mullen et al., 2010). However, in order for legumes and biochar to be employed for feedstock production, a protocol for the most economical and nutrient efficient system is needed.

Soil fertility and crop yield can be increased by leguminous green manure inter-crops through increases in soil-organic carbon, N, and phosphorus (P) compared with weedy fallows and non-leguminous cover crops (Tonitto et al., 2006). However, the extent of this in the humid tropical and warm temperate humid environment for intercropping systems is unknown, especially for switchgrass production. Smithson and Giller (2002) found that biologically-fixed N from legumes can reach levels up to 450 kg N ha^{-1} $crop^{-1}$. Tonitto et al. (2006) in a meta-analysis concluded that legume-based systems could supply up to 350 kg N ha^{-1} $crop^{-1}$ in a fairly continuous distribution, with 50% of the studies having 50–150 kg N ha^{-1} $crop^{-1}$. At this level of N production, legume-based intercrop systems can meet the N requirements for switchgrass feedstock production. These results demonstrate the potential for N-fixing crops to support crop yields while reducing reactive nitrogen emissions and decreasing nitrate leaching up to 40% (Tonitto et al., 2006). However, most studies determining inter-crop impacts on companion crops have been collected in cooler, temperate environments. Further research is needed in tropical and sub-tropical environments to assess yield impacts.

Biochar is assumed to be highly chemically and biologically recalcitrant and able to store carbon in soil with a long turnover time. Consequently, biochar applications may contribute to greater carbon storage, thereby potentially improving soil quality due to the vital role C plays in nutrient cycling (depending on recalcitrance level). Biochar may, in some cases, improve soil characteristics either directly or indirectly, as biochar is highly adsorbent due to its high surface area and charge density and, therefore, may increase soil water-holding capacity and nutrient retention (Chang and Zhihong, 2009; Clough and Condron 2010; Lehmann and Joseph, 2009). A wide range of N concentrations have been reported for biochar (1.8 g kg^{-1} to 56.4 g kg^{-1}) and some nutrients in chars are not labile (Mullen et al., 2010). In addition, biochar nutrient concentration levels are dependent on the feedstock as well as thermochemical operating conditions; therefore, all chars are not equivalent and some may have no yield impact (Chan and Zhihong, 2009). Furthermore, biochar is low in N relative to more stable C-bonded compounds, and therefore, has a high C:N ratio (Sadaka et al., 2014). Heating feedstocks causes volatilization of some nutrients, whereas some nutrients become sequestered in the remaining activated carbon. Anex et al. (2007) determined that when switchgrass co-products are integrated into production systems, a nitrogen recovery rate of 111 kg ha^{-1} yr^{-1}can occur. This represents *ca*. 78% of the N-fertilizer input required, assuming 142 kg N ha^{-1} yr^{-1} [i.e. 10.5 kg N ha^{-1} Mg^{-1} DM switchgrass removed (Anex et al.. 2007)]. Therefore, enhanced nutrient cycling and favorable energy balances are prospective benefits of coupling agricultural and bioenergy systems.

A considerable knowledge gap still exists in terms of understanding the mechanisms and relative nutrient contributions from both biochar and legume intercrop systems. Peer-reviewed reports on biochar in switchgrass are still sparse (Clough and Condron, 2010), and data are needed to test biochar under dynamic field conditions. Similarly, a data-gap exists on legume-intercropping systems for their relative yield contributions in perennial graminaceous feedstock systems [e.g. switchgrass and a near relative, guinea grass (*Panicum maximum* Jacq.)]. Furthermore, research is needed to reduce or eliminate the conventional reliance upon fossil fuel based-N, which is currently the primary input requirement for cellulosic feedstocks. Information is also lacking on feedstock characterization (i.e., potential ethanol yield, cell wall components, nutrient levels, and ash) as affected by harvest timing and soil amendments. In addition, information on biomass water content at post-senescence harvest periods is needed to maximize in-field drying and minimize economic and energy costs (Lindsey et al., 2013).

The Southern U.S. climate and soils make it well positioned for cellulosic feedstock production that will not directly compete for arable land for food production. This area is characterized by its transition from temperate humid to a sub-tropical climate, and is anticipated to be widely affected by climatic changes and could possibly emulate a more tropical climate in the future (IPCC, 1990; IPCC, 2001). In this region, precipitation from 1970-2008 declined 7.7%, while annual mean temperature increased 0.9ºC (USGCRP, 2009). Within the next two decades, temperature in the Southeast is projected to increase and the majority of this increase to be observed in the summer, as the number of extreme heat days are predicted to increase from 90 to 150 (USGCRP, 2009; Rosenberg, 1993). Already more frequent and extreme droughts, intense rainfall events, and heat waves are having serious impacts on crop production across the region.

Our goal was to develop an economically and ecologically sustainable cellulosic energy production model for reducing inorganic-N across an environmental gradient representative of current and projected future climatic conditions. In addition, harvest date may influence sustainability through its impact on nutrient retention and remobilization in feedstocks post-senescence. The result may be a higher quality feedstock for conversion processes, [i.e., lowest nutrient, moisture, ash, and greater digestible sugars (biological conversion) and lignin (thermal-chemical conversion)] and reduced nutrient requirements in subsequent years. This research will contribute to the understanding of N-mineralization promotion from the two organic-N sources (legumes and biochar) as they relate to sustainable bioenergy production. Specific objectives were to: i) determine effects of two biochar rates, four legume inter-crops versus inorganic-N rate on feedstock characteristics, economic feasibility, yield, and soil nutrient additions across a climatic gradient; ii) compare nutrient translocation between fall post-senescence (November 15th) and winter (mid-February) harvests in humid temperate areas; and, iii) document switchgrass performance and adaptation to more extreme climatic conditions compared to a naturalized *Panicum* species in the tropics.

II. Materials and Methods

2.1. Site descriptions

The experiment was conducted over a range of soils and climates, with two temperate locations in Tennessee (TN) and one located near the intertropical convergence zone in St. Croix, USVI (STX-AES). Sites in TN included the East Tennessee Research and Education Center, located near Knoxville (ETREC; 35.53^o N -83.57^o W) on a soil mapped as Huntington silt loam (fine-silty, mixed, active, mesic Fluventic Hapludolls) and at the Research and Education Center at Greeneville (GREC, 36.10° N -82.84° W) on a Dunmore Silty Clay Loam (Fine, kaolinitic, mesic Typic Paleudults). Mean annual precipitation during the duration of the study (2011-2014) at ETREC was 145-cm, with a mean annual temperature of 15.6^{o}C (NOAA, 2013). This site was under orchardgrass (*Dactylis glomerata* L.) hay production for 4-yrs prior to implementation of this experiment. Mean precipitation at GREC was 119-cm, with mean annual temperature of 14.7^{o}C (NOAA, 2013); the site was previously under fescue (*Schedonorous arundinaceus* [Schreb.]) hay production. Accumulated growing degree days (GDD) averaged 4994 and 4105 during the experiment at ETREC and GREC, respectively.

The St. Croix Agricultural Experiment Station (STX-AES; 17.70^o N -64.81^o W) lies in the Tropical Zone in the Virgin Islands. The highest temperatures occur in August or September, and the lowest are in January or February; however, temperature fluctuations are minimal (difference between mean temperatures of the coolest and warmest months is about 6°C). Rainfall is bimodal with the rainy season occurring Jul-Oct. (concurrent with hurricane season), with the remainder of the year being dry with little rainfall. Open-pan measurements indicate evaporation rates exceed the average annual rainfall (Kemp, 1927). The experimental site was located on the southwestern and south-central coastal plain physiographic region (Kemp, 1927) in the major land resource area (MLRA) 273 Land Resource Region Z (Caribbean Region, Semiarid Mountains and Valleys). Mean temperatures during the experiment (July 2013-July 2014) were 28°C, with precipitation totaling 108-cm and 6100 growing degree-days. Field measurements were taken on a Sion Clay (Coarse-loamy, carbonatic, isohyperthermic Typic Calciustolls), which consists of very deep, well drained, and moderately slowly permeable soils, which are formed from alkaline marine deposits.

2.2. Experimental design

This experiment utilized a factorially arranged randomized complete block design with three blocks per location. The first factor, biofuel harvest treatment, included a single post-senescence harvest (mid-November) and a single winter harvest date (early February) for temperate locations 2012-2014. Tropical location harvests included two dry-season harvest (March and November, 2013) and one during the onset of the rainy season (July, 2014). In addition to these post-treatment harvests, one baseline harvest was conducted at this location (July, 2012).

For all locations, soil amendment comparisons (second factor) of biochar at two application levels (1 Mg ha^{-1} and 2 Mg ha^{-1}), three legume intercrops for the TN locations (sun hemp, (*Crotalaria juncea* L. cv. Tropic Sunn; SH), red clover (*Trifolium pretense* L. cv. Cinnamon Plus; RC), and partridge pea (*Chamaecrista fasciculate* L. cv. Lark; PP), all versus inorganic-N (67 kg ha^{-1}) and a 0-application (control). Recent research conducted by Warwick (2010) in TN found that red clover and partridge pea could be successfully intercropped with ‘Alamo’ switchgrass.

Established switchgrass plots (12 m^2), planted at 9 kg pure live seed (PLS) ha^{-1} with 18 cm row-spacing in the spring of 2007 at ETREC and in spring 2008 at GREC were used in this study. Broadleaf weeds were controlled during the establishment year by 2, 4-dichlorophenoxyacetic acid at 0.9 l product ha^{-1}. Legumes were no-till drilled annually into switchgrass stubble at ETREC using a 5-row Hege™ plot drill (Colwich, KS) and a 5-row Great

Plains™ No-Till plot drill (Salina, KS) at GREC. Red clover was planted on 13, 28 February 2012 and 28 February and 13 March 2012 at ETREC and GREC, respectively, at the rate of 12 kg PLS ha^{-1}. Partridge pea and sun hemp were seeded on 12 April and 6 May 2012 and 5 and 16 May 2013, at the rate of 18 and 24 kg PLS ha^{-1} at ETREC and GREC, respectively. Planting depth ranged from 0.6 to 1.3 cm, depending on seed size. Partridge pea and sun hemp were inoculated with cow pea group inoculant (*Bradyrhizobium* spp.) and clover with clover group inoculum (*Sinorhizobium meliloti*).

At STX-AES, switchgrass and guinea grass plots were planted by hand on 60-cm centers on 1 Nov. 2012 (onset of the rainy season). The latter, a tropical *Panicum* species and near relative of switchgrass, was included in the event that switchgrass establishment failure occurred. Guinea grass is very similar physiologically to switchgrass in temperate Tennessee; however, is adapted and naturalized to the environmental conditions of STX-AES. Plot sizes were 1.8 x 6.1 m and 1.8 x 7.6 m for switchgrass and guinea grass, respectively. For STX-AES-AES, comparisons of guinea grass and switchgrass systems were made for biochar (at the same treatment levels as TN sites), two legume intercrops [sun hemp and pigeon pea, (*Cajanus cajan* L. Mill sp.; PiP)] versus inorganic-N (67 kg ha^{-1}) and a 0 kg N ha^{-1} control. Pigeon pea was substituted for partridge pea due to its adaptation to this environment, and red clover was eliminated from the STX-AES trials due to its cool-season growth habit. Legumes were inoculated with cow pea-group inoculant and then interseeded by hand on 13 July 2013 and again on 31 Nov. 2013 at STX-AES. All biochar and fertilizer treatments were applied concurrent with legume seeding.

Inorganic-N applications in the form of ammonium nitrate (NH_4NO_3) were applied annually in a single application at all locations. Each inorganic-N treatment was applied when switchgrass was ca. 30 cm tall (approx. Apr 15). Biochars were applied during spring and summer at temperate and tropical sites, respectively, mixed in a slurry (20% water by volume) to reduce losses due to wind erosion. No macro-nutrients were applied.

Switchgrass biochar in TN was utilized in both study years and locations; however, the high biochar rate (2 Mg ha^{-1}) was only applied on the post-senescence biofuel harvest (Nov) due to limited availability of biochar. Material applied in yr-1 was carbonized at 400ºC and under residence time of 2 h, as described in our earlier study (Sadaka et al., 2014). For material used in yr-2, a continuous, externally-heated auger system heated at 400°C at constant residence time of 8 min was used to produce the biochar (Sadaka et al., 2014). Amorphous structure, nutrient ranges, and cell wall composition of the materials applied during each experimental year were similar (Ashworth et al., 2014).

2.3. Data collection

2.3.1. *Soil characterization*

Pre-treatment soil tests were conducted on a per plot basis to 0-15 cm depths in 2010 to determine initial pH, cation exchange capacity (CEC, meq 100 g^{-1}), base saturation (BS, %), plant side-dress nitrate (PSNT, ppm), and P, K, Mg, and Ca concentrations. Soil sampling was repeated after the final winter harvest (spring of 2013) at TN locations and during July 2013 and 2014 at STX-AES to track elemental fluxes based on treatment applications. Samples were ground to pass through a 1-mm sieve on a Wiley soil crusher (Thomas Scientific, Swedesboro, N.J.). Samples were then analyzed with a Mehlich-1 extractant by UT Soil, Plant, & Pest Center (Nashville, TN) and with Mehlich-3 at A&L laboratory (Memphis, TN) for TN and STX-AES-

AES locations, respectively. In addition, at the latter laboratory, NO_3^--N was determined via the nitrate method (KCl Extraction / Cd-Reduction Method, AOAC 2.4.11), and organic matter by combustion (weight loss on ignition; Schulte and Hopkins, 1996) for STX-AES soils.

2.3.2. Influence of nitrogen amendments on switchgrass yield

Legume stand densities were estimated late-spring annually following green-up using a 1-m^2 frequency grid (Vogel and Masters, 2001) at the two TN locations. Four, 1-m^2 density counts were taken (n=4) on each legume treatment plot and averaged on a per-plot basis.

Two harvests, fall (post-senescence) and late-winter, were implemented annually in 2012-2013 (yr-1) and 2013-2014 (yr-2) in TN. Plots were harvested at ETREC and GREC using a Carter™ forage harvester (Brookston, IN) with a 91cm cutting width. At STX-AES, the two central rows (out of four rows per plot) were hand clipped totaling 2m^2 per *Panicum* species. For both harvests and all locations, plots were cut to a 20.3cm stubble height. Grab samples of biomass (1-2 kg) were collected from all plots at harvest, weighed, dried at 49^oC in a batch oven (Wisconsin Oven Corporation, East Troy, WI) for 48-72 h, and re-weighed to determine moisture content. Samples were then ground through a 2mm sieve on a Wiley mill (Thomas Scientific, Swedesboro, NJ).

2.3.3. Influence of nitrogen amendments and harvest date on switchgrass bio-feedstock characteristics

Soil amendment (i.e., legume inter-crops, biochar, and inorganic-N) and harvest date effects on feedstock characteristics were based on plant total N, total minerals (P, K, Ca, and Mg), and cell wall constituents. Furthermore, feedstock traits (i.e., predicted ethanol yield, ash content, moisture content, and nitrogen tissue concentration) were characterized for each treatment. Switchgrass uptake and removal of P and K were calculated as biomass yield x nutrient concentration.

Ground switchgrass tissue (separated from legumes) was analyzed with near-infrared spectroscopy (NIRS) using a LabSpec® Pro Spectrometer (Analytical Spectral Devices, Boulder, Colorado). Five scans were taken at a scan range of 1003-2500 nm. Equations for the forage nutritive analysis and biomass quality were standardized and checked for accuracy using the Grass Hay equation developed by the NIRS Forage and Feed Consortium (NIRSC [Hillsboro, WI]). Analysis results included acid detergent fiber (ADF), neutral detergent fiber (NDF), NDF digestibility (NDFD), lignin, cellulose, hemicellulose, sugars, fructans, and ash content.

Rapid simultaneous saccharification and fermentation (SSF) ethanol yields are highly correlative to NIRS forage quality methods, as the same cell wall digestibility and fermentation concepts in rumen and in vitro apply (Lorenz et al., 2009a). Consequently, cell wall digestibility measurements were used to determine convertibility and availability of structural cell wall carbohydrates, including NDF and NDFD. The former includes insoluble composites from the neutral detergent and contains total cell wall concentration (i.e., lignin, hemicellulose, and cellulose). The latter is determined by combining in vitro true digestibility (IVTD) and NDF and represents availability of cell wall carbohydrates and is an important determinant for feedstock convertibility (Lorenz et al., 2009b). Therefore, resultant NIR values (NDFD and NDF) were fit to a regression model [NDFD + NDF-0.114+ 0.00229(NDFD) + 0.00117(NDF)] to determine estimated EtOH yield (L kg^{-1} DM), based on Lorenz et al. (2009b), in that these two digestibility measurements are very strong (R^2=0.93) predictors of actual in vitro ethanol yield

2.3.4. Influence of climate change on switchgrass production

Switchgrass response to an environment representative of potential future climate in the SE USA was tested at STX-AES by conducting an initial variety test of lowland cultivars including cv. Alamo, Kanlow, Blade 1101, and Cimarron prior to experimentation for this site (2012). As a result of the more favorable adaptability of cv. Alamo in STX-AES, (all other accessions yielded <1 Mg ha^{-1}) this cultivar was used in the subsequent experiment. In addition, due to weed pressure and adaptability questions regarding switchgrass under tropical conditions, weed yield (broad leaf and grass weed yield) were collected at STX-AES, as well as *Panicum* species' heights at time of harvest on a per plot basis.

To quantify and compare the effectiveness of switchgrass establishment in the tropics, an index was adapted from Linares et al. (2010), known as the cover crop/weed index (CCWI). This tool was established to determine the efficacy of weed suppression by cover crops (Linares et al., 2010). Consequently, we adapted this concept to evaluate the effectiveness of switchgrass establishment relative to that of the weeds present [switchgrass/weed index (SGWI)] by calculating the ratio of switchgrass biomass accumulation to that of weed biomass. Therefore this index is expressed as switchgrass dry weight (DW) production (SGDW) relative to weed dry weight [WeedDW; (SGWI = SGDW/WeedDW)].

2.3.5. Economic implications of soil amendment alternatives

Higher yielding and lower input systems will lower break-even prices. Therefore we determined break-even prices from soil amendments in switchgrass production for TN. The analysis for legume intercropping was based on forage budgets developed by University of

Tennessee, Department of Agricultural & Resource Economics developed by the University of Tennessee's Center for Native Grasslands Management. Establishment costs were assumed to be the same for all treatments for the economic model and included:

1) Glyphosate applied at fall burndown, spring burndown, and post-emergence at a rate of 0.31, 0.31, and 0.14 L product ha^{-1}, respectively. Glyphosate cost was estimated to be \$1.05 L^{-1}.

2) Switchgrass established with no-till methods at 9 lbs PLS ha^{-1}.

3) No N or lime applied during establishment; P and K applied at 44.8 and 89.6 kg ha^{-1}, respectively.

4) Interest (6%) on operating capital calculated at 6 months.

5) Fixed expenses for establishment included depreciation, interest, equipment housing, and insurance.

6) Labor expenses calculated at \$8.50 hr^{-1}.

7) Establishment costs amortized over 15 years.

8) Yield assumed to be no different than the current recommended inorganic-N rate (i.e. 5.7 Mg ha^{-1} annually).

Once prorated establishment costs were determined, management costs for each treatment were calculated. All treatments included labor, fixed (prorated establishment costs, land costs, depreciation, interest, housing, and insurance), and variable expenses. Variable expenses included machinery costs (fuel, oil, and filters), interest on operating costs, and costs of applying a particular treatment. Assumptions of each model were:

Nitrogen treatment:	N applied at 67 kg ha^{-1} at \$352 Mg^{-1} (April 2013 price)
Legume treatments:	Sun hemp planted at 34 kg PLS ha^{-1} at \$1.11 kg^{-1} PLS
	Partridge pea planted at 20 kg PLS ha^{-1} at \$6.89 kg^{-1} PLS
	Red clover planted at 13 kg PLS ha^{-1} at \$5.66 kg^{-1} PLS

Biochar treatment: High application rate applied at 2 Mg ha^{-1} at \$0.46 kg^{-1}

2.4. Statistical methods

Switchgrass yield, feedstock quality, nutrient uptake and removal (P and K), potential ethanol yield, fluctuations in soil characteristics ([both annual and delta (yr3-yr1)], and legume density was initially analyzed in a global model (combined across harvest treatments, TN locations, and years). Analysis of variance tests were performed using the Mixed procedure [(SAS V9.3; SAS Inst., Cary, NC) with block, location, and year considered random effects (SAS, 2007). Legume species, biochar and inorganic-N levels, and harvest date were considered fixed effects. Mean separations were performed by the SAS macro 'pdmix800' (Saxton, 1998) with Fisher's Least Significant Difference (LSD) with a Type I error rate of 5%. Based on the random effects probability level, further pooled models were compared where appropriate. Temperate and tropical locations were analyzed as separate experiments, due to species, climatic, and harvest timing variation. For all models, the Shapiro-Wilks test was used to test for normally distributed residuals and the Levene's F-test was used to test homogeneity of variances.

III. Results and Discussion

3.1. Soil characterization

Changes in soil chemical characteristics (delta=yr3-yr1) indicated minimal transformations when combined across TN locations. Specifically, neither delta soil pH, P, Ca, Mg, PSNT, CEC, nor BS were impacted by soil amendment treatments ($P \geq 0.05$). Only delta potassium (K) was slightly impacted by soil amendments [P=0.05; data not shown (DNS)], with the low biochar rate, sun hemp, and partridge pea treatments resulting in K increases. Measurable fluctuations often require several years for inherent biochemophysical alterations. Initial macro and micronutrient (P, K, Ca, and Mg) levels at GREC were all considered ‘high’ or ‘medium,’ with the exclusion of a few plots testing ‘low’ for potassium and ‘sufficient’ for magnesium. Conversely, at ETREC, all plots tested ‘low’ in potassium, ‘sufficient’ in magnesium, and a variety of ratings (‘low-high’) for phosphorus (DNS). Over the study period P, Ca, and CEC, increased, while soil pH, magnesium, and base saturation decreased (Table 1.1); however, there were no soil amendment effects ($P \geq 0.05$).

For STX-AES, delta (yr2-yr1) soil characteristics were generally not affected by soil amendment treatments, excluding nitrate-N ($P=0.02$) for the switchgrass experiment. Specifically, all soil amendment treatments resulted in NO_3^- reductions, with the control, high biochar, and pigeon pea having the greatest reductions, whereas sun hemp and inorganic-N treatments resulted in fewer losses (DNS). Generally, organic matter, CEC, pH, K, Ca, and Mg, increased from baseline levels during the experimental period; whereas only NO_3^- and P were reduced. Thereby indicating favorable soil characteristics post-switchgrass establishment, despite non-senesced tissue being harvested at this location (due to lack of ‘overwintering’ from environmental signals, and consequently greater nutrient removal). However, no initial or final soil characteristic differences were observed for either switchgrass or guinea grass as a result of experimental treatments ($P>0.05$; Table 1.2).

For guinea grass, soil nutrients were not removed nor were any chemical characteristics directly altered ($P>0.05$) by soil amendment. In addition, soil organic matter, pH, Mg numerically increased, and NO_3^-, Ca, P, and K were reduced ($P>0.05$), suggesting neither inter-crops nor biochar added or removed/chelated soil macronutrients (Table 1.2).

3.2. Influence of nitrogen amendments on switchgrass yield

Under the global yield model (analyzed across years and locations), harvest treatments differed (*P*=0.02), with the over-wintering harvests yielding 22% less biomass, likely due to greater leaf loss and weathering of plant tissue. Likewise, Adler et al. (2006) reported that switchgrass yield in Pennsylvania decreased by almost 40% during winter (with above-average precipitation) when harvest was delayed until spring; with the water content decreasing from about 35 to 7% of fresh weight. In addition, under our model, neither soil amendments nor harvest x soil amendment interactions impacted yield (*P*>0.05). Furthermore, location (*P*=0.24), year (*P*=0.19), and location x year interactions (*P*=0.07) did not impact switchgrass biomass yield.

Consequently, yield was analyzed by harvest treatment in a simplified model with temperate locations and years pooled. Soil amendments impacted post-senescence biofuel harvest (*P*=0.01) with 0 kg N ha^{-1} and sun hemp inter-crops yielding the lowest biomass, neither of which differed from high biochar, partridge pea, or red clover (Table 1.3). Conversely, low biochar was not different than the 67 kg N ha^{-1}, nor was this inorganic treatment different than partridge pea, or high biochar. Thereby suggesting the lower biochar rate is preferred, perhaps because of the covering of switchgrass stubble during higher biochar applications. Such contrasting benefits of biochar were observed in a meta-analysis in that approximately half of the studies observed crop yield increases, whereas, the other half resulted in none or even negative yield responses (Spokas et al., 2012); however, no studies have been conducted (to the authors knowledge) on switchgrass yield response from biochar. In addition, partridge pea may have more favorable yield impacts than the other two legume inter-crops tested in this study.

The over-wintering harvests (mid-February) were not influenced by soil amendments ($P \geq 0.05$), likely due to biomass losses overriding any yield differences that may have occurred as a result of soil amendments. Furthermore, neither harvest regime had influential location, year, or location x year interactions ($P \geq 0.05$).

Legume density [based on our global model (combined across temperate locations and years)] varied among legumes ($P<0.0001$), however, neither harvest, legume x harvest, year, location, nor location x year affected plant frequency ($P>0.05$). Trends for each harvest regime were similar ($P \geq 0.05$; Fig. 1.1) suggesting harvest date did not impact legume self-re-seeding or persistence. When compared across harvest dates, red clover frequency was greatest (20.1 m^2), followed by partridge pea (11.9 m^2), with sun hemp averaging only 4.8 m^2, even with annual re-seeding. Consequently, sun hemp is not considered compatible with switchgrass growth in temperate environments, likely due to the overlap in these species breaking dormancy, which induces seedling competition with established switchgrass.

3.3. Influence of nitrogen amendments and harvest date on switchgrass feedstock characteristics

3.3.1. *Switchgrass response in temperate locations*

When combined across temperate locations and years, plant-nutrient uptake and removal for both P and K varied based on harvest timing ($P<0.0001$), but not for soil amendment, soil amendment x harvest interactions, location, year, or location x year interactions ($P \leq 0.05$). Phosphorus removal was greater for November than over-wintering harvests (19.1 vs. 11.0 kg ha^{-1}, respectively; Table 1.3). Potassium followed similar trends with more removed by November than over-wintering harvests (50.2 vs. 25.02 kg ha^{-1}). For both nutrients, control plots generally had the lowest P and K removal during the fall harvest, with all other soil amendments being the greater for this harvest (*P*≥0.05; Table 1.3). Removal concentrations (both post-senescence and overwintering) determined in our study were similar to those reported by Kering et al. (2013) for switchgrass harvested in December for P (11.7-16.4 kg ha^{-1}) and K (32.0-42.0 kg ha^{-1}). These results suggest that P and K decline through winter, due to nutrient translocation completion. The higher volume of P and K sequestered in senesced material has implications for feedstock usage in thermal chemical conversion due to the high slagging potential of these nutrients in combustion chambers (Boateng et al., 2007), as well as higher nutrient input requirements long-term.

Potential ethanol yield was affected by harvest timing (*P*<0.0001; Table.1.3), as over-wintering biomass resulted in greater potential per unit ethanol yield (1,210 vs. 1,150 L EtOH kg^{-1} DM). This increased ethanol yield for winter-harvested biomass may have been due to greater presence of digestible fibers and fermentable sugars (5 and 6 carbon), and because of in-field breakdown of waxy cuticles that inhibit sugar availability (Adler et al., 2006). However when harvestable biomass per harvest regime is taken into account, over-wintering ethanol potential is lower (12,700 vs. 15,520 L EtOH ha^{-1}) than that of post-senescence harvest periods. Soil amendments did not impact potential ethanol yield of harvested biomass, nor did any soil amendment x harvest interactions (*P*>0.05); indicating greater influences from the physical state (harvest period) of feedstock than physiological (from soil amendment and soil management) on feedstock attributes.

When combined across temperate locations and years, soil amendment treatments had marginal, albeit not significant impacts on feedstock characteristics, likely due to the vast genetic diversity of this species; consequently, non-homogenous plant cell constituents across populations may override any detectable chemical or physical characteristics. Chemical differences observed for harvest date (Fig 1.2) may have been due to several factors including i) remobilization of nutrient to plant storage organs, ii) leaching of soluble nutrients from the standing biomass; and, iii) weathering of senesced leaves resulting in deposition on soil surface. Specifically, the November harvest had greater nitrogen, sugar, and consequently TDN ($P \leq 0.05$). Conversely, late-winter early-spring harvests had greater NDF, lignin, fructan, and hemicellulose concentrations ($P < 0.0001$). Therefore, the only tested feedstock traits not impacted by harvest timing were cellulose and ADF ($P > 0.05$). Higher NDF and lignin for the second harvest indicates losses in digestibility of plant material, likely due to leaf loss and greater stem constituents that are greater in recalcitrant phenolic chains, rather than digestible sugars. Similar results were reported by Adler et al. (2006) with delayed (spring) harvests resulting in higher ethanol yields due to greater glucose, lignin, hemicellulose, and carbohydrates yields. Consequently, based on our results, harvest management could be manipulated to obtain desired feedstock traits based on conversion process (i.e., greater lignocellulosic and sugar yields favor fall harvests for biological conversion, whereas the reduced mineral concentrations during spring may favor thermochemical conversion).

Switchgrass moisture content (MC) was greatly influenced by harvest date ($P < 0.0001$) with overwintered material having a much lower MC (10.8%) than that of November harvested tissue (24.8%). Switchgrass tissue moisture content for both harvest dates decreased to low enough levels after in-field dry-down for direct storage (≤ 200 g kg^{-1}, wet weight basis). However, the 11% moisture content during the over-wintering harvest would require little if any additional dry-down for biological or thermochemical biofuel conversion. In addition, there was an important harvest x soil amendment interaction (*P*= 0.01), in that the overwintering harvest for the high biochar had comparable MC levels to that of the November harvest (27.1%), suggesting switchgrass amended with biochar inputs may result in continued growth later into the growing season with delayed senescence, and consequently may require greater levels of drying before conversion.

3.3.2. *Switchgrass and a native Panicum response in the tropics*

Switchgrass yield in the tropics was impacted by harvest timing (*P*=0.003) but not soil amendments, nor soil amendment x harvest ($P \geq 0.05$). Specifically, the fourth harvest (July, 2014) yield at this location was greater than that of the previous two [2.96 and 2.69 Mg ha^{-1}, harvest#2 (July 2013) and #3 (March 2014), respectively; Fig.1.3 (a)]. Yields for guinea grass were affected by soil amendment ($P<0.0001$), harvest period ($P<0.0001$), and their interactions (*P*=0.002). Compared across all treatments and harvest periods, yields were greatest for the second harvest (Nov, 2013; 6.4 Mg ha^{-1}), whereas the latter two did not differ (March and July 2014, 2.59 and 3.37 Mg ha^{-1}, respectively), likely due to the biomodal rainfall (drier part of the year) occurring during these harvest periods. Specifically, among all soil amendment treatments and harvest periods, the inorganic fertilizer applications during the 2nd harvest (July, 2013) was the greatest [Fig. 1.3 (b)]. The second highest yielding amendment x harvest period interaction resulted from inorganic-N during the final harvest period (July, 2014), which was not different than the sun hemp intercrop during the 2nd harvest period. In addition, harvests during the dry season (harvest 3 and 4), resulted in the greatest yields for the inorganic treatment, with all others being lower; excluding pigeon pea intercropped stands (harvest 4; July, 2014).

For switchgrass production in the tropics, all feedstock characteristic metrics were impacted by harvest period ($P<0.05$), whereas only P levels were impacted by soil amendments ($P=0.04$), and ADF and P were impacted by harvest period x soil amendment treatments ($P<0.05$). Low biochar applications during the second harvest period resulted in the greatest switchgrass phosphorus-tissue levels, with inorganic-N and pigeon pea levels during the same harvest period not differing ($P\geq0.05$). For ADF, similar results were observed as the low biochar rate and inorganic fertilizer treatments were the greatest, suggesting greater availability of digestible 5 and 6 carbon sugars. Among all harvest periods, harvest #2 (or the first harvest after initial soil amendment treatment applications) resulted in the greatest hemicellulose, N, P, and K tissue levels [Fig. 1.4 (a, b)]. Whereas harvesting after more soil amendment applications (harvest#4) induced greater NDF and ADF, suggesting less digestibility. Results suggest feedstock maturity stage can be manipulated for desired traits, whereas intercropping and amendments impact tissue composition to a lesser extent.

Guinea grass productivity was affected by harvest period, but none by soil amendments [Fig. 1.4 (c, d)]. Similar to switchgrass, all characteristics tested were impacted by harvest timing period [i.e., ADF, NDF, P, K, hemicellulose, and N ($P<0.05$)]. The only important feedstock characteristic impacted by harvest timing and amendment x harvest interaction was NDF ($P=0.02$), with pigeon pea and biochar-high treatments being the greatest (75.9 and 75.6%, respectively) during the second harvest period. The inorganic- N (harvest#4) sun hemp and low rate of biochar (harvest#2) not differing, with all others being lower ($P>0.05$). In addition, potassium removal was greatest for the high biochar rate in this study during the 2nd harvest, which was not different than the lowbiochar rate during the same harvest period (harvest period x amendment; *P*=0.04), with the lowest removal occurring for the control plots during the 2nd cut (2.4 vs 1.9% DM). During harvest#2, similar to switchgrass, guinea grass had the greatest hemicellulose, N, P, and K levels. Whereas final harvests (#4) of guinea grass had the greatest NDF and ADF levels, with the second harvests having intermediate to non-differing levels of the aforementioned characteristics [Fig. 1.4 (c, d)]. Consequently, results suggest, tissue transformational responses to harvest timing for guinea grass and switchgrass are similar.

3.4. Influence of climate change on switchgrass production

Switchgrass adaptation based on the SGWI indicated varying weed competition (5-30%) across all harvest periods, likely due to switchgrass photoperiod sensitivity and lack of biochemical adaptations. In temperate regions, the amount of photosynthetically active radiation or usable light energy on a leaf per unit time [i.e., photosynthetic photon flux density (PPFD)] has greater fluctuations than in the tropics. In addition to PPFD, ultraviolet B (UV-B, 280–320 nm), which is damaging to plant cells is up to ten times higher in the tropics compared to temperate areas (Warren et al., 2003). Because of this high PPFD, tropical species have biochemical adaptations, such as flavonoids to allow for selective-wavelength adsorption and removal of damaging UV-B radiation. Consequently, guinea grass likely has greater flavonoid levels compared to switchgrass, and is photoperiod insensitive due to its heightened adaptability in tropical areas with greater UV-B exposure due to ozone depletion.

Switchgrass' lignocellulosic concentrations are affected by the plant's morphology and physiology, which in turn are largely determined by temperature, precipitation, photoperiod, and genetically programmed ontogeny, as this is a short-day species (Moser and Vogel, 1995). However, to authors' knowledge no researches have attempted to grow switchgrass in the tropics. Variation in temperature and solar radiation are less dramatic in the tropics, however, day length is consistently lower than that at temperate latitudes during summer. Consequently, shorter day length at STX-AES induced flowering of switchgrass within 2 months after each harvest. Such rapid anthesis resulted in reduced vegetative growth and consequently, higher weed yields than that of guinea grass due to lack of canopy cover (0.70 vs. 0.04 Mg ha^{-1}, respectively).

Weed pressure in the tropics is among the greatest challenges to agricultural production (Smithson and Giller, 2002). This was an issue in our study, with weed and grass sward dynamics fluctuating due to grass and weed physiological stage at time of harvest. For instance, for the second harvest period (July, 2013), the SGWI ranged from 2.4-2.9, indicating that switchgrass started prevailing with weeds being predominate in certain niches, indicating 'moderate' weed control (Table 1.4). In part, this could be due to switchgrass obtaining only 33 to 66% of its production capacity during yr-1 at temperate locations due to the slow establishment of this bunchgrass (McLaughlin and Kszos, 2005). This low switchgrass predominance mirrored plant heights, as this harvest period had the shortest plants among all harvest periods [121.1 cm; ($P<0.05$)]. During the third harvest period (November, 2013), the SGWI increased to 18.4-19.2, suggesting switchgrass was dominant with <5% weed cover. During the final harvest period (harvest# 4; July, 2014), the SGWI decreased to 5.4-6.4, with 10-

30% weed presence due to the competitive growth habit of many tropical weeds (e.g. desmodium (*Desmodium intortum* (Mill.) with climbing tendrils). Finally, when SGWI was averaged across all harvest periods, ‘excellent’ weed control was observed in the tropics (Table 1.4; SGWI=9.2). Weed suppression at this location was contingent upon establishment success, as areas with good establishment had minimal weed encroachment. Consequently, switchgrass may be produced in the tropics, and perhaps even under intensified climatic changes due to this species’ drought tolerance and competitive growth habit after establishment on marginal soils; however, more aggressive weed measures and photoperiod insensitive varieties would be required.

3.5. Economic investment potential of soil amendment alternatives

Assuming consistent establishment methods were used for switchgrass, the currently recommended N rate (67 kg N ha^{-1}) resulted in the lowest breakeven price under our assumptions (Table 1.5). The second lowest breakeven price was observed for sun hemp (137.81 \$ Mg^{-1}) and was influenced by low costs associated with annual management compared to other legumes; however, it would not be recommended in temperate environments due to establishment failures and lack of yield effects. Therefore, based on break-even price, red clover was the preferred legume due to lower seed costs, albeit its break-even price was greater (by \$59.63 Mg^{-1}) than the inorganic nitrogen scenario. Further, biochar was the most costly N alternative with breakeven costs >2 fold greater than inorganic nitrogen. Consequently, for inorganic input alternatives to be competitive on a breakeven-cost basis, greater biomass yields would need to be obtained under these management practices, or some secondary benefits would have to be realized (e.g. carbon credits for inorganic-N alternatives).

IV. Conclusions

Given that ETREC was lowest in terminal soil P and K and yield was greatest, and that nutrient removal was not different across temperate locations, suggests that uptake and removal was not limited by nutrient availability, and that mineralization from favorable switchgrass rhizosphere conditions may allow for continued plant uptake in the short-term. For guinea grass and switchgrass in the tropics, there was no notable soil nutrient removal, nor was there any soil characteristics directly altered by amendments, indicating neither inter-crops nor biochar added or removed/chelated soil macro-nutrients, despite non-senesced tissue being harvested at this location. Further monitoring of nutrient removal is needed to determine if nutrient mining may occur given that many elements shifted from adequate to low levels in yr-1 to yr-3 at temperate locations as measurable soil fluctuations are often more attenuated and therefore require more years for inherent biochemical alterations.

Soil amendment treatments impacted yield for November, but not overwintering harvests, due to biomass losses overriding any yield differences. Switchgrass yield from fall harvest was lowest for the 0 kg N ha^{-1} rate and sun hemp intercrops; conversely, the low rate of biochar was not different than the 67 kg N ha^{-1} rate, nor was the inorganic rate different than partridge pea and the high biochar rate. Consequently sun hemp is not considered compatible with switchgrass growth, due to the high competition from switchgrass during germination and seedling growth of the sun hemp. On the other hand, partridge pea and red clover can successfully be interseeded into established temperate switchgrass swards, and may, in some cases, result in comparable post-senescence biomass yields to that of the current recommended inorganic-N rate.

For switchgrass and guinea grass production in the tropics, all feedstock characteristics were impacted by harvest period, whereas only P tissue levels were impacted by soil amendments. The low rate of biochar applications during the second harvest period resulted in the greatest switchgrass and guinea grass P and K-tissue removal levels, respectively, even though tropical soils are notoriously low in these nutrient-levels. Utilization of biochar has the potential to provide a ‘closed-loop’ system, considering the feedstock co-product can be applied to the bioenergy crop the following season, however, further research to determine proper application rates is needed.

Switchgrass adaptation based on the SGWI indicated varying results (5-30% weed coverage) across all harvest periods, due to switchgrass photoperiod sensitivity and lack of biochemical adaptations, compared to a naturalized *Panicum* (guinea grass), which is photoperiod insensitive and has likely greater flavonoid levels due to its heightened adaptability in areas with greater UV-B exposure. Because in the tropics, day and night are nearly equally long, with this pattern being consistent all year and switchgrass’ ontogeny, the shorter day length induced flowering of switchgrass within 2 months after each harvest period. Such rapid anthesis resulted in reduced vegetative growth and consequently higher average weed yields than guinea grass (0.70 vs. 0.04 Mg ha^{-1}, respectively). Consequently, switchgrass may be produced in the tropics, and perhaps even under intensified climatic changes in the southeast due to this species C4 photosynthesis, drought tolerance, genetic diversity, and competitive growth habit after establishment on marginal soils. However, more aggressive weed management and photoperiod insensitive varieties would be advantageous.

A sustainable management practice for temperate growers could be to delay harvests until after maturity during the subsequent winter months to stagger the work load and transport to a refinery, provide out-of-season wildlife habitat, and allow further moisture loss in the standing crop, which confers optimum moisture conditions for safe storage. Nutrient (P and K) translocation, ethanol yield, hemicellulose, and in-field dry down was maximized by delaying harvests in temperate locations where senescence occurs. Such harvest delays may allow for greater presence of digestible fibers and fermentable sugars (5 and 6 carbon), due to in-field breakdown of waxy cuticles that inhibit sugar availability. On the other hand, biomass yield losses were observed (22%) from delaying harvests. Consequently, desired feedstock characteristics can be manipulated by harvest management, however, a trade off with yield reductions must be considered. Therefore, the greater lignocellulosic and sugar yields that occurred during fall harvests favor biological conversion, whereas the reduced mineral concentrations for spring favor thermochemical conversion. Systems tested herein could, in part, close the N loop, reduce inputs, and promote the diversification and long-term sustainability of perennial graminaceous feedstock systems.

Acknowledgments

Authors of this paper would like to express gratitude to the United States Department of Agriculture (USDA), Southeastern Sun Grant for funding for this research, Grant. No. 2010-38502-21854. In addition, authors extend a thank you to the Agricultural Research and Education Centers for their help in collecting data and making this research possible.

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APPENDIX

Table 1.1 Soil characterization of baseline (yr1, 2010) and final (yr3, 2013) results (averaged by treatment), based on soil amendment inputs combined over locations (East Tennessee Research and Education Center and Research and Education Center at Greeneville) and harvest treatments. Soil test results from Mehlich-1 extractant.

Year	**Soil amend-ment**	**pH**	**P**	**K**	**Ca**	**Mg**	**PSNT**[5]	**CEC**	**BS**
				---------------kg ha^{-1}-------------			ppm	Meq 100 g soil^{-1}	%
1	B-High[2]	5.74a	39.3 a	69.5 a[1]	1491.2 a	286.91 a	0.5 b	8.9 a	58.1 a
	B-Low	5.6 a	52.5 a	61.0 a	1569.7 a	289.26 a	1.2 a	9.3 a	57.0 a
	N-0	5.7 a	34.4 a	73.9 a	1465.8 a	290.50 a	0.3 b	8.7 a	56.8 a
	N-67[3]	5.9 a	33.0 a	77.2 a	1527.8 a	304.83 a	0.5 b	8.9a	57.3 a
	PP[4]	5.8 a	33.3 a	71.2 a	1484.5 a	295.33 a	0.4 b	8.8 a	56.2 a
	RC	5.8 a	32.7 a	73.5 a	1502.7 a	305.17 a	0.5 b	9.0 a	57.6 a
	SH	5.7 a	37.3 a	82.6 a	1491.4a	307.66 a	0.6 b	9.0 a	56.5 a
3	B-High	6.5 a	29.5 a	60.1 a	1391.1 a	344.74 a	0.9 a	7.9 a	61.7 a
	B-Low	6.6 a	31.0 a	54.5 a	1390.0 a	311.83 a	0.7 a	7.9 a	60.5 a
	N-0	6.5 a	32.4 a	62.3 a	1369.4 a	336.42 a	0.6 a	7.9 a	60.8 a
	N-67	6.50	28.1 a	72.8 a	1396.9 a	340.92 a	0.7 a	8.0 a	61.3 a
	PP	6.5 a	31.1 a	65.3 a	1459.5 a	354.42 a	0.6 a	8.2 a	61.9 a
	RC	6.6 a	31.4 a	55.9 a	1381.0 a	339.42 a	0.5 a	7.8 a	61.5 a
	SH	6.5 a	32.3 a	63.6 a	1428.9 a	351.31 a	0.5 a	8.2 a	61.1 a

[1] different letters indicate a significant difference by the LSD separation procedure within a given analyte and experimental year at the $P<0.05$ level.

[2] swtichgrass biochar rates: B-high (2 Mg ha^{-1}) and B-low (1 Mg ha^{-1}) applied each spring.

[3] kg N ha^{-1}

[4] legume inter-crops: partridge pea (PP); red clover (RC), and sun hemp (SH).

[5] PSNT: plant side-dress nitrate; CEC: cation exchange capacity; BS: base saturation.

Table 1.2 Soil characterization of baseline [yr1 (2013)] and final year (yr2 (2014)]- results (averaged from on a per treatment basis) for switchgrass and guinea grass, based on soil amendment inputs at the St. Croix, USVI, Agricultural Experiment Station. Soil test results from Mehlich-3 extractant.

Species	**Soil amendment**	**pH**	**P**	**K**	**Ca**	**Mg**	**NO_3^-**	**CEC[5]**	**OM**
				---------------kg ha^{-1}-------------			ppm	Meq 100 g soil^{-1}	%
SG-1[1]	B-High[2]	7.90 a[3]	5.33 a	269.67 a	12646 a	280.33 a	5.67 a	52.93 a	5.23 a
	B-Low	7.90 a	7.67 a	312.67 a	12220 a	302.00 a	5.33 a	51.53 a	4.87 a
	N-0	7.90 a	5.67 a	273.00 a	12522 a	287.67 a	4.33 a	52.47 a	5.10 a
	N-67[4]	7.73 a	24.00 a	254.67 a	10470 a	281.33 a	5.33 a	44.43 a	5.33 a
	PP	7.93 a	11.00 a	313.67 a	12355a	307.33 a	6.00 a	52.17 a	5.17 a
	SH	8.10 a	6.00 a	303.00 a	12497 a	307.67 a	4.00 a	52.63 a	5.10 a
SG-2	B-High	7.80 a	8.00 a	307.67 a	13572 a	345.33 a	2.66 a	57.20 a	5.20 a
	B-Low	7.67 a	8.33 a	327.33 a	12674 a	332.33 a	2.00 a	53.60 a	5.47 a
	N-0	7.80 a	6.67 a	279.00 a	14195 a	322.33 a	2.00 a	59.40 a	5.07 a
	N-67	7.80 a	9.33 a	282.33 a	12581a	337.67 a	3.33 a	53.20 a	4.97 a
	PP[4]	7.53 a	8.33 a	317.00 a	13724 a	343.67 a	2.00 a	57.83 a	4.97 a
	SH	7.77 a	7.33 a	294.00 a	13631 a	336.67a	3.67 a	57.30 a	5.17 a
GG-1	B-High	8.33 a	4.66 a	183.33 a	12313 a	220.67 a	2.00 a	50.93 a	5.33 a
	N-0	8.20 a	4.61 a	182.20 a	13221 a	215.33 a	2.01 a	54.46 a	4.90 a
	N-67	8.30 a	6.33 a	207.67 a	15405 a	242.00 a	4.00 a	63.36 a	4.70 a
	PP	8.20 a	7.33 a	218.67 a	13393 a	214.33 a	2.33 a	55.23 a	4.76 a
	SH	8.23 a	5.33 a	209.33 a	14132 a	228.00 a	2.33 a	58.23 a	4.93 a
GG-2	B-High	7.80 a	6.00 a	187.33 a	18392 a	275.67 a	2.00 a	75.40 a	4.93 a
	N-0	7.56 a	6.03 a	179.82 a	17729 a	263.33 a	2.00 a	72.69 a	5.30 a
	N-67	7.46 a	7.00 a	185.00 a	18973 a	280.33 a	2.00 a	77.76 a	4.90 a
	PP	7.60 a	7.66 a	228.33 a	18472 a	278.33 a	2.00 a	75.83 a	4.90 a
	SH	7.86 a	7.00 a	179.33 a	16899 a	278.67 a	2.00 a	69.56 a	4.80 a

[1] by experiment (i.e. species SG=switchgrass; GG=guinea grass) and sampling period (1=baseline and 2= yr2).

[2] Swtichgrass biochar rates: B-high (2 Mg ha^{-1}) and B-low (1 Mg ha^{-1}) applied each spring.

[3] Different letters indicate a significant difference within a given species (experiment), sampling period, and analyte with the LSD procedure at the $P<0.05$ level.

[4] kg N ha^{-1}

[5] CEC: cation exchange capacity; OM: organic matter.

Table 1.3 Switchgrass biomass and estimated ethanol yield, and P and K removal from fall (mid-November) versus late winter (mid-February-early March) from soil amendment treatments combined across locations (Tennessee Research and Education Centers at Knoxville and Greeneville) and years (2012-2013 and 2013-2014).

Harvest treatment	**Soil amendment**	**Biomass yield**	**Phosphorus removal**	**Potassium removal**	**Estimated EtOH yield**
			--Mg ha^{-1}--		L kg^{-1} DM
Fall	B-High[2]	13.22 abc[1]	54.96 a	20.25 abc	113.05 a
	B-Low	14.95 ab	47.80 a	21.35 ab	116.43 a
	N-0	10.65 c	44.51 ab	16.15 c	115.07 a
	N-67[3]	15.75 a	54.23 a	22.23 a	115.78 a
	PP[4]	13.34 abc	46.50 a	19.14 abc	112.04 a
	RC	12.72 bc	54.08 a	19.53 abc	115.32 a
	SH	12.06 c	49.05 a	17.94 abc	113.85 a
Late Winter	B-High	12.51 a	22.55 b	12.74 ab	121.37 a
	N-0	10.21 a	21.91 bc	10.01 b	120.27 a
	N-67	11.82 a	26.26 a	11.74 b	120.10 a
	PP	9.28 a	19.07 c	7.82 b	120.81 a
	RC	10.48 a	22.33 b	10.92 b	121.00 a
	SH	10.30 a	28.07 a	11.53 b	121.39 a

[1] different letters indicate a significant difference within harvest regime and biomass characterization metric at the *P*<0.05 level using LSD.

[2] swtichgrass biochar rates: B-High (2 Mg ha^{-1}) and B-Low (1 Mg ha^{-1}) applied each spring, excluding low rate for the second experiment due to shortage of material.

[3] kg N ha^{-1}

[4] legume inter-crops: partridge pea (PP); red clover (RC), and sun hemp (SH).

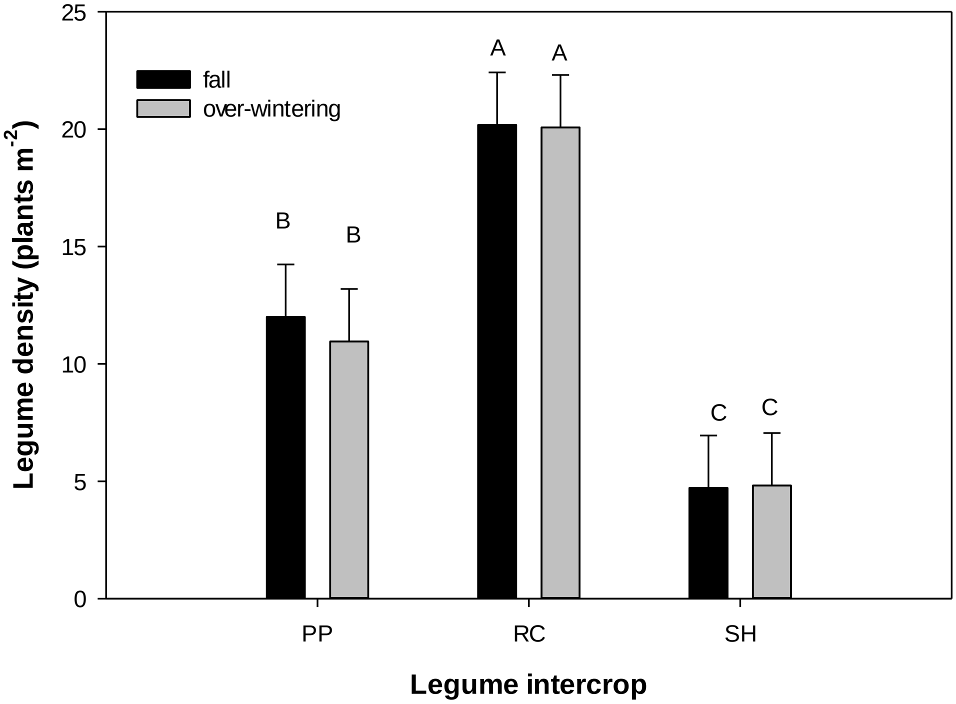

Figure 1.1 Legume density (m^2) combined across locations (Tennessee Research and Education Centers at Knoxville and Greeneville) and years (2012-2013 and 2013-2014). Different letters indicate a significant difference with the LSD procedure across both harvests [fall (post-senescence, mid-November) and over-wintering (mid-February)] at $P \leq 0.05$.

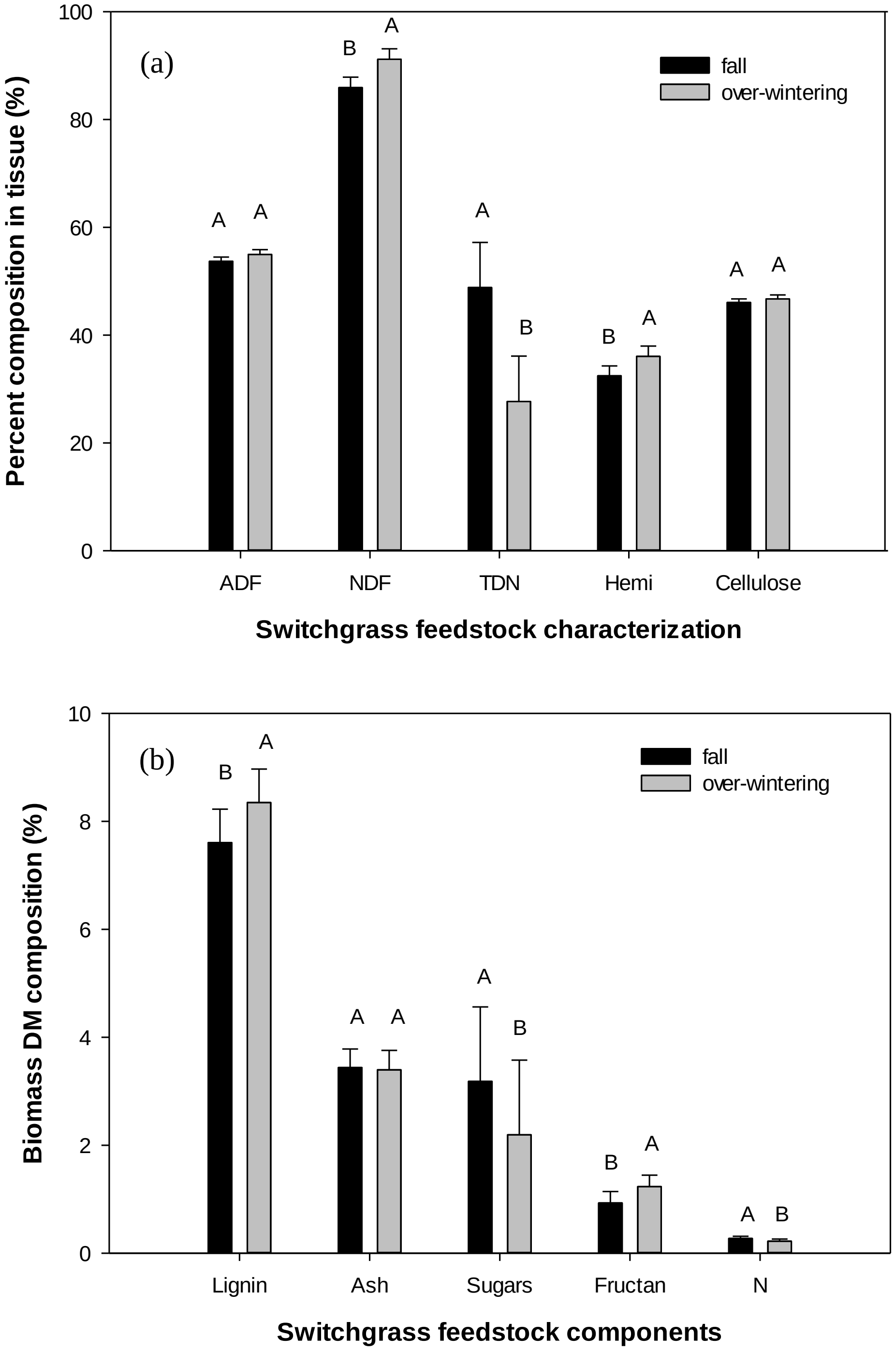

Figure 1.2 Feedstock characterization of switchgrass dry matter (DM) composition (a and b) based on harvest [fall (post-senescence, mid-November) and overwintering (mid-February to early March)] averaged across soil amendment treatments, locations (the East Tennessee Research and Education Center and Research and Education Center at Greeneville) and years (2012-2013 and 2013-2014). Different letters indicate a significant difference with the LSD procedure at the *P*<0.05 level.

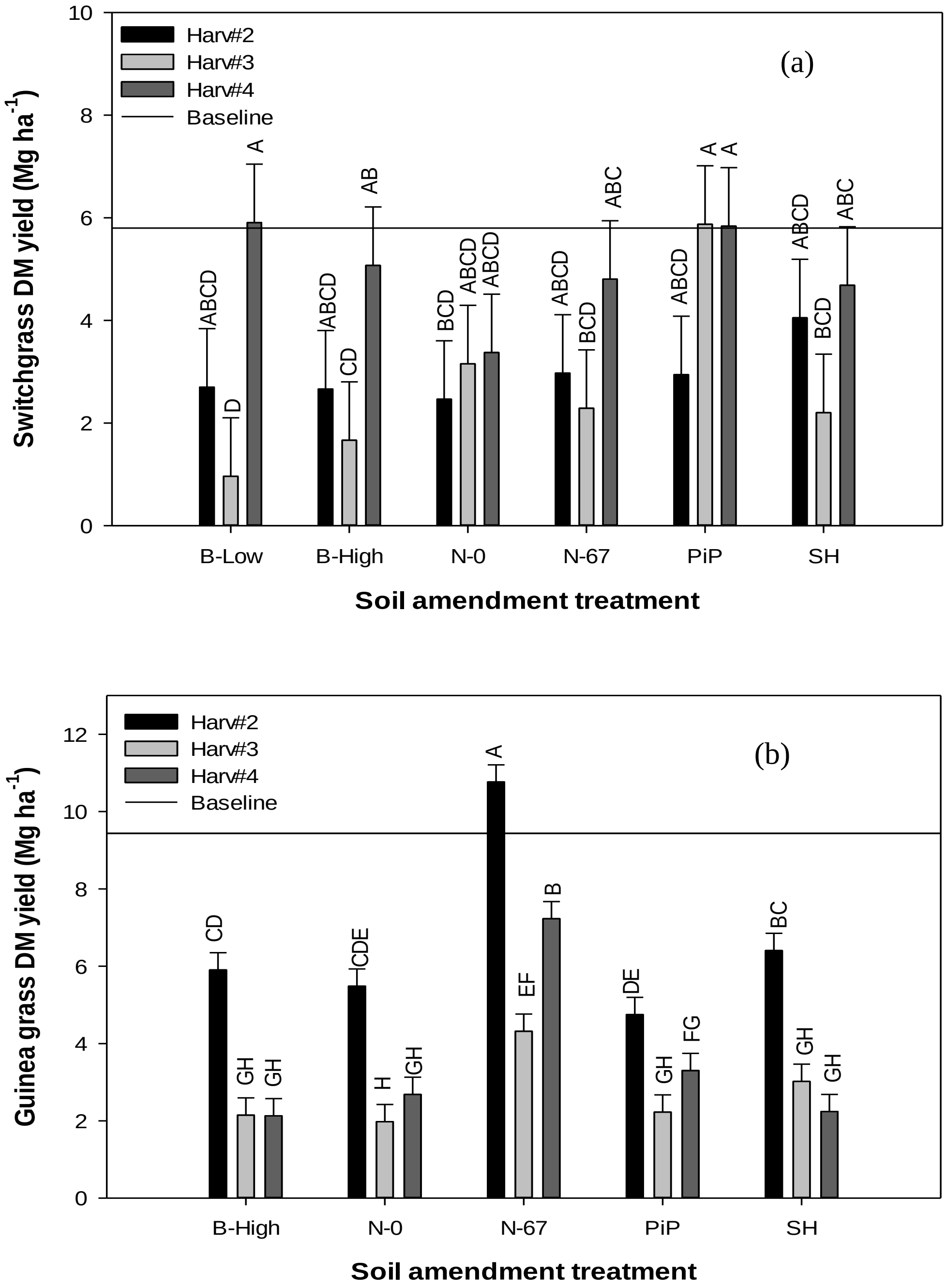

Figure 1.3 Switchgrass (a) and guinea grass (b) dry matter (DM) yield based on harvest timing [baseline (pre-treatment; July, 2013), harvest#2 (Nov., 2013, harvest#3 (March, 2014), and harvest#4 (July, 2014)] per soil amendment treatments at the St. Croix USVI, Agricultural Experiment Station. Different letters indicate a significant difference with the LSD procedure at the $P<0.05$ level. (Soil amendment treatments include: B-high=biochar 2 Mg ha^{-1}; N-0=0 kg N ha^{-1}; N-67= 67 kg N ha^{-1}; PiP=pigeon pea; and, SH=sun hemp.)

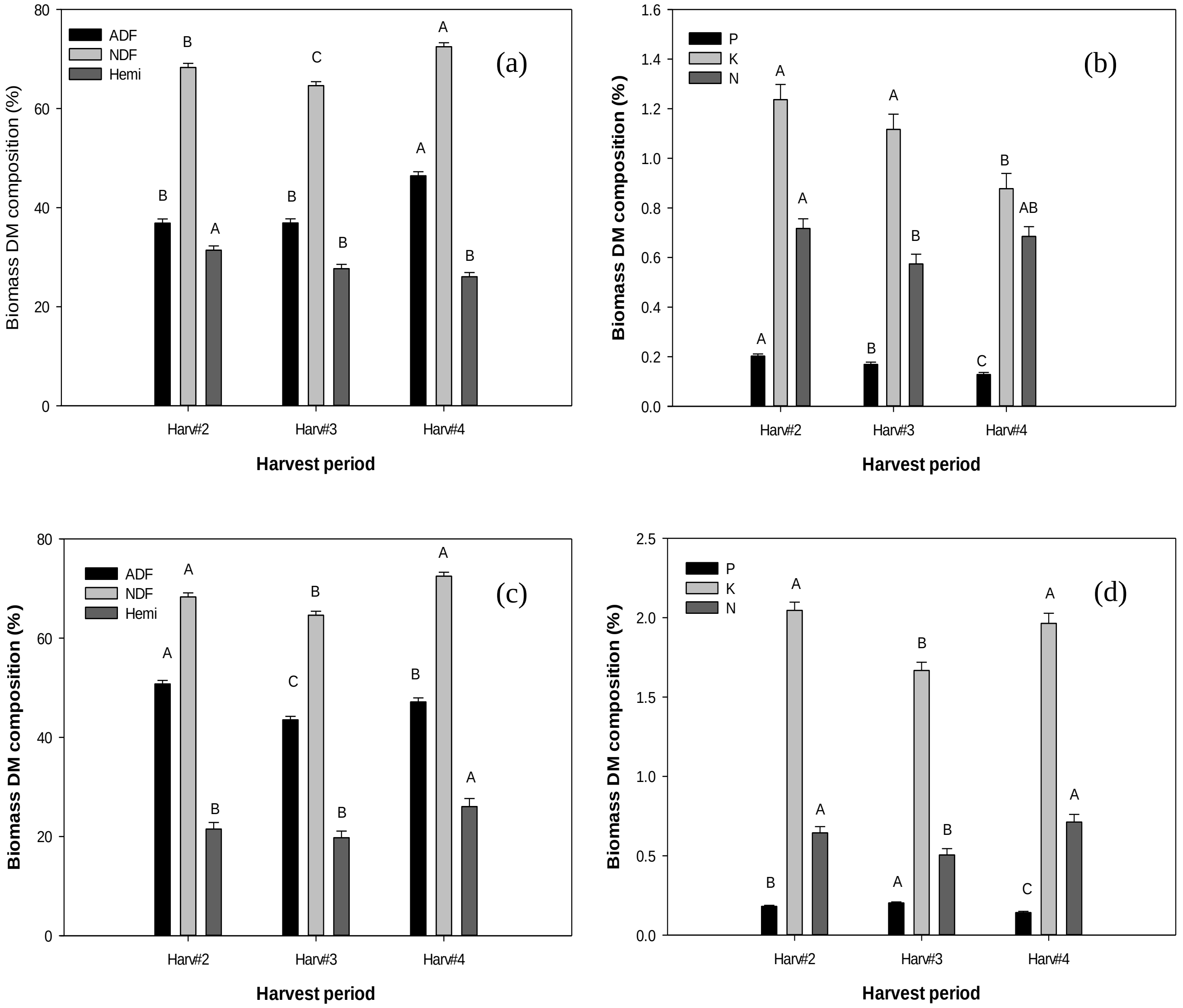

Figure 1.4 Switchgrass (a, b) and guinea grass (c, d) dry matter (DM) feedstock characterization based on harvest timing [baseline (pre-treatment; July, 2013), harvest#2 (Nov., 2013, harvest#3 (March, 2014), and harvest#4 (July, 2014)] averaged across soil amendment treatments at the St. Croix USVI, Agricultural Experiment Station. Different letters indicate a significant difference with the LSD procedure at the *P*<0.05 level within a given component across harvests. (Acid detergent fiber=ADF; neutral detergent fiber=NDF; hemicellulose=hemi.).

Table 1.4 Outline of switchgrass weed index (SGWI) categories to determine adaptability to a tropical environment. [Adapted from Linares et al., 2010].

SGWI value	**Switchgrass adaptability**	**Weed pressure**	**Weed control**
<0.5	SG not competitive	Weeds predominate	Very poor (>70% weeds)
0.5–1	SG coexist	Weeds coexist	Poor
1–3	SG starts prevailing	Weeds prevail in certain niches	Moderate
3–5	SG prevails	Weeds fail to predominate	Adequate
5–15	SG predominate (70% to 90%)	<10% to 30% weeds	Excellent
>15$^{\partial}$	SG completely predominates	<5% weeds	Outstanding

Table 1.5 Total treatment costs (prorated establishment plus annual management costs (ha^{-1}) and the break-even point for switchgrass for each treatment.

Treatment[1]	**Total cost**[2]	**Breakeven cost**[3]
	$ ha^{-1}	$ Mg^{-1}
Nitrogen only	117.31	106.40
Sun hemp	151.94	137.81
Red clover	183.05	166.03
Partridge pea	243.32	212.53
Biochar (2 Mg ha^{-1} rate)	305.63	277.21

[1] Assumes the same establishment practices for all treatments.

[2] Establishment costs amortized over 15 years.

[3] Yield assumed to be equivalent to average yield (i.e. 5.7 Mg ha^{-1}annually) from recommended inorganic-N rate of 67 kg ha^{-1}.

CHAPTER II

Switchgrass yield and stand dynamics from legume intercropping based on seeding rate and harvest management

Abstract: Intercropping legumes may reduce inputs and enhance sustainability of forage and feedstock production, especially on marginal soils. This approach is largely untested for switchgrass (*Panicum virgatum* L.) production, yet producer acceptance should be high given the traditional use of legumes in forage/agricultural systems. Our objectives were to evaluate three cool-season and two warm-season legumes, and their required densities to influence yield and supply nitrogen (N) compared to three inorganic-N levels (0, 33, and 66 kg N ha^{-1}; 0, 30, and 60 lbs ac^{-1}) at three locations in Tennessee (Knoxville [Sequatchie Silt Loam], Crossville [Lilly Loam]; and, Milan [Loring B2 Series]). Fall 2010 seeded, cool-season legumes (red clover [*Trifolium pretense* L.], hairy vetch [*Vicia sativa* L.], ladino clover [*Trifolium repens* L.]) and spring 2011 seeded, warm-season legumes (partridge pea [*Chamaecrista fasciculate* L.], and arrowleaf clover [*Trifolium vesiculosum* L.]) were interseeded into switchgrass at three (high, medium, and low) seeding rates each in two experiments. Harvest treatments were annual single, post-dormancy biofuel (Experiment One) or integrated forage-biofuel (pre-anthesis and post-dormancy; Experiment Two). Year one yield impacts were minimal. During the second harvest year, legumes increased yield versus Yr-1; in general, yields for 33 kg N ha^{-1}did not differ from those for red clover, hairy vetch, ladino clover, or partridge pea ($P<0.05$). Arrowleaf clover yields were not different than 0 kg N ha^{-1}. Forage yields were generally more responsive to legumes ($P<0.05$) than the biomass regime. Legume persistence after 3-yrs was generally greatest for ladino clover and partridge pea. Forage quality (switchgrass only) in some cases was positively influenced by legume treatments, notably hairy vetch and partridge pea ($P<0.05$). Intercropping selected legumes in switchgrass may enhance forage quality and yield while reducing non-renewable inputs, fertilizer costs, and emissions/runoff to air and groundwater.

Key words: biological nitrogen fixation— biomass— forage— legume integration— switchgrass— sustainability

Nitrogen (N) is the principal nutrient required in cropping systems, and is applied at a rate of nearly 11 million tonnes of commercial or synthetic N per year in the USA (GAO 2003; USDA 2004). Among all nutrients, N is the fourth most abundant found in plant tissue and has the greatest impact on soil fertility and subsequent crop productivity (Taiz and Zeiger 2006). Petroleum and natural gas are the primary fuels required for synthetic-N production and therefore exert a strong influence on inorganic-N prices (Pimentel et al. 2008). Thus, increased fossil fuel costs may place pressure on farmers to seek alternative sources of fertilizers, or accept reduced profit margins and compromised economic viability. These realities create challenges to sustainable feedstock production, and reduction of external inputs has important implications for producer profitability (Boyer et al. 2012), carbon balances (Franzluebbers et al. 2000), and environmental sustainability/conservation (McLaughlin and Walsh 1998; Sanderson et al. 2004b).

The main nutrient requirement for switchgrass production is N (Vogel et al. 2002), which is affected by the frequency and timing of harvests, amount of biomass removed, and soil N-mineralization rates (McLaughlin and Kszos 2005; Parrish and Fike 2005). Even under optimal cropping management, plants usually take-up less than 60% of applied fertilizer and often only 40% or less (Sinclair 2006). Nutrient uptake and removal in crops is highly variable within a single year, among years, and among sites, even when N-supplies from both the soil and additional fertilizer inputs are adequate (Gastal and Lemaire 2002). The variability and rate of N uptake during development and its effects on yield have many impacts on the quality of biomass and the environment, as well as the overall feasibility of integrated biomass and forage production (Adler et al. 2007). Standard practices for switchgrass have called for at least 50 kg N ha^{-1} during the year after switchgrass establishment (Year 2), followed by 67 to 100 kg N ha^{-1} thereafter (Wolf and Fiske 1995; McLaughlin and Walsh. 1998; Mooney et al. 2009).

Because legumes biologically fix nitrogen, they have been used as intercrops for centuries and can be grown in tandem with agricultural crops in lieu of synthetic-N (Peoples 2009; Graham 2005). Therefore, integration of legumes into switchgrass forage and feedstock production may provide a viable alternative to inorganic-N, but data on appropriate species and seeding rates are lacking. If a viable model can be developed for switchgrass systems, producer acceptance should be high given the traditional use of legumes in forage-agricultural systems. Soil fertility and yield can be increased by inter-crops because they increase soil-organic carbon (SOC), N, and phosphorus (P) compared with weedy fallows (Tonitto et al. 2006); however, the extent of this in switchgrass systems is unknown. An additional benefit of intercrops is that they suppress weed growth and development by competing for soil-water and nutrients. In addition to direct competition for growth resources, intercrops enhance weed seed decay and increase plant residue (Adler and Chase 2007; Conklin et al. 2002). However, a data-gap exists on legumes species as well as proper seeding rates that can be successfully integrated into forage-biofuel systems in the humid, southeastern USA and contribute to yield; thereby reducing synthetic nitrogen inputs and subsequent greenhouse gas emissions.

Switchgrass can off-set lost forage production by cool-season forages during hot, dry months, and interseeding legumes may increase forage quality by taking advantage of legume growth patterns and improving seasonal distribution of forage production. Other benefits from increasing diversity in pasture systems include greater yield stability under stress or disturbance, ecological facilitation, reduction of weed abundance, and niche-differentiation (Sanderson et al. 2004a;

Sanderson et al. 2005; Finn et al. 2013). In grass-legume swards, legume competiveness is affected by companion species growth habit, photosynthetic pathway, management, and hard seed level (Posler et al. 1993). Because of these potential incompatibilities, botanically stable pasture/feedstock-legume mixtures are often not sustained, and either the legume or grass component declines long-term (Blanchet et al. 1995; George et al. 1995; Warwick 2011). A study by Blanchet et al. (1995) found that hairy vetch interseeded into switchgrass stands persisted into their second year; however, they were not tracked beyond two years. In addition, establishment and persistence of legumes may adversely affect switchgrass growth early in the growing-season if legume growth habits are not considered (George et al. 1995), as Taylor and Jones (1983) found that the red clover component of a switchgrass/red clover mixture overwhelmed switchgrass stands after yr-2. However, research is limited on the persistence of successful legume intercrop species in established switchgrass stands, particularly under various seeding rates.

Harvest management is another factor likely affecting legume persistence and establishment success, as some species may respond to canopy removal whereas others may be unaffected, due to contrasting windows for maximizing legume species photosynthesis and companion crop canopy removal. Such differences have not been investigated in detail, and when to remove grass canopy for effective legume management is still an unresolved issue (Wang et al. 2010). A study by George et al. (1995) tested only forage harvest regimes (June and July), and determined that adequate defoliation in early June is important for minimizing switchgrass competition with legumes (crownvetch [*Coronilla varia* L.], birdsfoot trefoil [*Lotus corniculatus* L.], and red clover). Other studies have looked at the establishment, yield, and persistence of legumes interseeded into switchgrass but did not include harvest treatments (Blanchet et al. 1995; Butler et al. 2013), which is necessary for assessing legume intercropping success under various production systems.

To address this data gap, we conducted research examining switchgrass compatibility with cool- and warm-season legume intercrops compared to synthetic-N fertilization under two harvest systems (two-cut, simulating dual-purpose forage-biomass [pre-anthesis and post-senescence] and one-cut, biofuel harvest [post-senescence]). We evaluated each legume species at three seeding rates to determine densities required to impact switchgrass yield, thereby promoting labile-N in the soil sphere. Specific objectives were to evaluate five (three cool-season and two warm-season) legume species, each seeded at three rates and managed under two harvest systems, and determine their i) influence on switchgrass yield and forage quality compared to three inorganic-N levels; ii) determine persistence of legumes over three years; and, iii) identify legume density thresholds and their associated impact on switchgrass yields.

Materials and Methods

***Site Description*.** Locations included the East Tennessee Research and Education Center, Knoxville (ETREC; 35.53° N -83.57° W) on a soil mapped as Huntington silt loam (fine-silty, mixed, active, mesic Fluventic Hapludolls); Plateau Research and Education Center, Crossville (PREC, Northern Cumberland Plateau; 36.0° N -85.1° W) on a Lilly Loam (Fine-loamy, siliceous, semiactive, mesic Typic Hapludults); and, Research and Education Center at Milan (RECM, East Gulf Coastal Plain, 35.5 ° N -88.4° W) on a soil mapped Collins silt loam (coarse-silty, mixed, active, acid, thermic Aquic Udifluvents). Mean annual precipitation during the duration of the study (2010-2012) at ETREC was 127 m (50 in), with mean annual temperature at 15.3°C (60°F; NOAA 2013). This site had been under orchardgrass (*Dactylis glomerata* L.) hay production for 4-yrs prior to initiation of this experiment. At PREC, precipitation was 153 cm (60 in), mean annual temperature was 13.3°C (56°F; NOAA 2013), and previous management had been tall fescue (*Schedonorous arundinaceus* [Schreb.]) pasture. Mean annual precipitation at MREC was 128 cm (50 in), mean annual temperature was 15.7°C (60°F; NOAA 2013), and had been under row crop production for 4-yrs prior to experiment initiation.

Experimental Design. This experiment tested two-factors arranged factorially under a randomized complete block design. The first factor was harvest system, and included two levels, a single harvest (post-dormancy) and a two-cut system (pre-anthesis stage and post-dormancy). These harvests were chosen to represent a biomass production and an integrated forage-biomass production scenario, respectively. The second factor, N treatments, included five legume species drilled at low, medium, and high seeding rates, and inorganic-N applied at 0 (control), 33, and 67 kg N ha^{-1} (60, 30, and 0 lbs ac^{-1}) in the form of ammonium nitrate (NH_4NO_3) into established 'Alamo' switchgrass. Inorganic-N was applied in a single application when switchgrass was ca. 30-cm tall (12-in) (approximately April, 15) in 2011 and 2012. Legumes and respective seeding rates were: red clover at 9.0, 13.4, and 17.9 kg pure live seed (PLS) ha^{-1} (8, 12, 16 lb ac^{-1}); hairy vetch at 6.7, 10.1, and 13.4 kg PLS ha^{-1} (6, 9, 12 lb ac^{-1}); ladino clover at 3.4, 5.0, and 6.7 kg PLS ha^{-1} (3, 4.5, 6 lb ac^{-1}); partridge pea at 13.4, 20.2, and 26.9 kg PLS ha^{-1} (12, 18, 24 lb ac^{-1}); and, arrowleaf clover at 11.2, 16.8, and 22.4 kg PLS ha^{-1} (10, 15, 20 lb ac^{-1}). Species and seeding rates were selected based on previous work by Warwick (2011) in that species tested herein were deemed most successful in terms of establishment into switchgrass, and medium seeding rates were considered adequate, with high and low levels adjusted accordingly. Therefore, there were 18 N-treatment levels ([3 cool-season + 2 warm-season legumes] x 3 seeding rates + 3 inorganic N levels) and 2 harvest levels, installed in three blocks, creating 108 plots (experimental units) per locale.

Switchgrass was planted at 9 kg ha^{-1} PLS (8 lb ac^{-1}) in spring 2007 at ETREC and PREC and in spring 2004 at RECM. Plots at ETREC and PREC were 7.6 x 1.5 m and 7.6 x 1.8 m (6 x 25 ft and 6 x 30 ft), respectively, with 18-cm (7-in) row-spacing. Plots at RECM were 7.6 x 3.8 m (25 x 12 ft) with 25.4-cm (10-in) row-spacing. Weeds were controlled at ETREC with nicosulfuron {2- ([4,6-dimethoxypyrimidin-2-yl] aminosulfonyl)-N, N-dimethyl-3-pyridinecarboxamide} at 0.98 L ha^{-1} (0.10 gal ac^{-1}) in 2009. Weeds at PREC and RECM were controlled by 2, 4-dichlorophenoxyacetic acid at 0.9 L ha^{-1} (0.09 gal ac^{-1}) in 2009.

Legumes were no-till drilled into switchgrass stubble at ETREC and PREC using a 5-row Hege plot drill (Colwich, KS) and an 10-row ALMACO plot drill (Nevada, IA) at RECM without subsequent reseeding. Cool-season legumes (red clover, hairy vetch, and ladino clover) were planted on 20 October, 28 and 9 September, 2010, and warm-season legumes (partridge pea and arrowleaf clover) were seeded on 24 March, 12 and 13 April, 2011, at ETREC, PREC, and MREC, respectively. Planting depth ranged from 0.6 to 1.3 cm (0.2 to 0.5 in), depending on seed size. All legume seeds were inoculated prior to seeding: partridge pea and hairy vetch with cow pea group inoculum (*Bradyrhizobium* spp.), and clover species with clover group inoculum (*Sinorhizobium meliloti*).

Data Collection. Legume stand densities were estimated late-spring annually following green-up using a 1-m^2 frequency grid (Vogel and Masters 2001). Four 1m^2 (3.3 ft^2) density counts were taken on each experimental unit and averaged. In 2010 (pre-treatment), soil tests were conducted at 15 cm (6 in) depths to determine preliminary levels of pH and soil N, P, K, Mg, and Ca. Samples were ground to pass through a 1-mm sieve on a Wiley mill (Thomas Scientific, Swedesboro, N.J.) and Mehlich-1 extractable nutrients were measured by inductively coupled plasma (ICP) using a 7300 ICP-OES DV (Perkin-Elmer, Waltham, MA). The pH was determined on a 1:1 soil to water ratio using a AS3010D Dual pH Analyzer (Labfit, LLC Burswood, Australia).

Harvest regimes tested included i) a single, end-of-season harvest in November (one-cut system), and ii) an integrated forage and biofuel production paradigm June and November (two-cut system) with each harvest treatment analyzed separately; and, iii) the sum of the two-cut system under an integrated approach. Plots were harvested at ETREC & PREC using a Carter™ forage harvester (Brookston, IN) with a 91 cm (36 in) cutting width, and at RECM with a New Holland 'Crop Cruiser 850' forage chopper with a 2.1 m (6.9 ft) cutting width. For both harvest regimes, switchgrass was cut to a 20.3 cm (8 in) stubble height in 2011 and 2012. Grab samples of switchgrass (1-2 kg; 2.2-4.4 lb) were collected from all plots at harvest, and then weighed, dried at 49^{o}C (120^{o}F) in a batch oven (Wisconsin Oven Corporation, East Troy, WI) for 48-72 h, and weighed again to determine moisture content. Samples were then ground to a 2-mm (0.08-in) particle size on a Wiley mill (Thomas Scientific, Swedesboro, NJ).

Forage quality was analyzed on the first (forage) cut of the two-cut harvest system. The analysis included acid detergent fiber (ADF), neutral detergent fiber (NDF), crude protein (CP), hemicellulose, and ash content. Ground (2-mm; 0.08-in) switchgrass tissue (separated from legumes) was analyzed with near-infrared spectroscopy (NIRS) using a LabSpec® Pro Spectrometer (Analytical Spectral Devices, Boulder, Colorado). Five scans were taken per sample and the scan range was 1003-2500 nm. Samples were compiled across replications per legume seeding level such that only one sample was analyzed per species and N-level per harvest (n =8 per site). In a separate run (n= 3), partridge pea and switchgrass tissue composition was analyzed post-senescence (Mid-Nov) to determine biofuel digestibility affects from intercropping this species with switchgrass. Equations for the forage nutritive analysis and biomass quality were standardized and checked for accuracy using the Grass Hay equation developed by the NIRS Forage and Feed Consortium (NIRSC [Hillsboro, WI]).

Statistical Methods. Three separate models were analyzed to elucidate the relationship between selected legume intercrops and switchgrass. For all models, analysis of variance (ANOVA) assumptions of normally-distributed residuals (Shapiro-Wilk test) and homogeneity of variances (Levene's F-test) were confirmed. When significant differences were found, pairwise post-hoc comparisons of the least squares means were conducted using Least Significant Difference (LSD) at $P \leq 0.05$. Mean separation was performed by the SAS macro 'pdmix800' (Saxton 1998) with Fisher's with a Type-I error rate of 5%. Each model is described in detail below.

Legume Intercropping Impacts on Switchgrass Yield and Forage Quality versus Inorganic Nitrogen Model. Dependent variables, switchgrass yield and forage quality (for forage harvest only; i.e., ADF, NDF, CP, hemicellulose, and ash) were analyzed separately by harvest regime (i.e., one-cut biomass; forage, biomass in an integrated system, and their sum), and included appropriate year and location interactions using ANOVA. In all models, N-treatment was the fixed effect and year, location, and block were entered as random effects using PROC MIXED (SAS V9.3; SAS Inst. Cary, NC).

Intercropped Legume Persistence in Switchgrass Sward Model. Legume persistence (dependent variable; i.e. legume density over years) by species, seeding rate, and harvest treatment (main effects) were analyzed under a repeated measures ANOVA using PROC MIXED (SAS V9.3; SAS Inst. Cary, NC) over the 3-yr sampling period with block entered as a random effect. Legume species, seeding rate, and harvest treatment were all entered as fixed effects and location as a random effect, with year being a repeated measure. For the repeated measure, an autoregressive covariance was used and the denominator degrees of freedom for the Type III F-test were adjusted with the Kenward-Roger method (Gomez et al. 2005). However, the -2 Loglikelihood did not change under the repeated-measure analysis (did not drop by at least 5 per covariance parameter) and the autoregressive correlation value (0.24) indicated a weak correlation among observations, so autoregressive covariance was dropped. Thereafter, an additional model was analyzed using legume density by species and seeding rate within year to assess persistence based on seeding rate impacts by species.

Switchgrass Yield and Legume Density Relationship Model. A multiple-regression analysis was performed to examine the relationship between legume density (independent variable) and biomass yield (dependent variable). One requirement was that adequate legumes existed; therefore, due to poor persistence at PREC in yr-2, this location-yr combination was dropped from this model. Initially, a mixed model analysis of variance (MMAOV) was run separately for legume density per harvest treatment (one-cut biomass; forage, biomass in an integrated system, and their total) with replication, location, and year entered as random effects; legume species, seeding rate, and seeding rate x legume species were fixed effects, with $\alpha=0.05$. Because location and year were not important ($P\geq0.05$) predictive variables for legume density in the MMAOV, these effects were removed in the multiple regression model. Therefore, the simplified regression model (pooled across years and location) accessed legume density per species with the interaction of density and legume species for each of the four harvest treatments. All residuals in the aforementioned models were normally distributed ($P\geq0.05$; Shapiro-Wilk>0.90).

Results and Discussion

Mean soil test results per location indicate moderate-high phosphorus levels in the upper 15-cm, with moderate-low potassium (K), sufficient calcium (Ca) levels, and sufficient-deficient magnesium (Mg) levels (table 2.1). In general, legume persistence and establishment was lowest at PREC, which also corresponds to low-deficient soil-nutrient conditions compared to other experimental sites, suggesting soil fertility was important for successful legume establishment and persistence.

Legume Intercropping Impacts on Switchgrass Yield and Forage Quality versus Inorganic Nitrogen. When combined across all locations and years (2011 and 2012), there were no differences in forage quality results of switchgrass, except for CP ($P=0.013$) with hairy vetch being greatest (7.9%), ladino clover lowest (6.6%), and the remaining treatments not differing ($P<0.05$). This was not found to be the case by Posler et al. (1993), who reported increased CP levels of legume-grass mixtures compared to sole grass crops. However, Posler et al. (1993) as well as others (George et al. 1995) did not separate legume and grass tissue before analysis; consequently, *in situ* forage quality of grass-legume mixtures is likely more positively impacted than what was observed in this study.

For the first study year (2011), RECM was the only location to have forage quality components (CP, NDF, and hemicellulose) impacted by legumes ($P<0.05$; table 2.1). For this location and year combination, CP for switchgrass tissue with hairy vetch and red clover intercropped exceeded all other N-treatments ($P<0.05$). For this location x year combination, hairy vetch treatments resulted in the lowest NDF levels in the switchgrass among all treatments indicating greater digestibility. Similarly, hairy vetch resulted in the lowest hemicellulose levels, with partridge pea and inorganic-N levels being the greatest ($P<0.05$), indicating greater 5 and 6 carbon sugars in switchgrass plant tissue when intercropped with partridge pea and under inorganic-N inputs.

During 2012 forage harvests, forage quality results were impacted by legume species at ETREC. Specifically, CP, ADF, NDF, and ash were impacted by legume intercrops and inorganic-N treatments ($P<0.05$; table 2.1). For CP, legumes did not differ from inorganic-N rates; however, partridge pea treatments had higher levels than that of ladino clover intercrops. Acid detergent fiber and NDF were lowest in switchgrass grown with partridge pea, and was greatest for ladino clover and red clover ($P<0.05$; table. 2.1). Therefore, results suggest greater digestibility and intake by ruminants of switchgrass-forage when intercropped with partridge pea during June. Conversely, greater ash levels were observed in partridge pea intercropped switchgrass tissue compared to inorganic-N treatments (*P*<0.05), suggesting more adverse feedstock characteristics due to slagging in combustion chambers.

Based on post-senescence partridge pea-only tissue composition, if harvested in a switchgrass-biomass mixture, biofuel digestibility may decrease substantially compared to switchgrass-only biomass (average ADF increases of 43% and total digestible nutrient losses of 44%), due to partridge peas' stemmy and fibrous composition (data not shown [DNS]). Such declines from switchgrass-only biomass will likely reduce ethanol conversion efficiency and affect enzyme requirements if harvested in a mixture where partridge pea was a significant component.

When combined across locations and years, the forage and integrated (forage + biomass harvests) harvest treatment yields were impacted by nitrogen and legume treatments, whereas the biomass and biomass-only harvest treatment were not (*P*<0.05). This suggests that the annual removal of the switchgrass canopy may affect yield impacts from intercropping. Under each harvest treatment models, there were no location or year effects (*P*>0.05), but a slight interaction was observed for location x year impacts for the forage harvest (*P*=0.05). Under the forage harvest, the 67 kg N ha^{-1} (60 lbs N ac^{-1}) rate resulted in the greatest yield, followed by 33 kg N ha^{-1} (30 lbs N ac^{-1}), and the 0 kg N ha^{-1} rate being the lowest (*P*<0.05); and, the latter two not differing from any legume species (except for that of arrowleaf clover, which was the lowest). For the integrated harvests, a similar pattern was observed; however, none of the tested legumes were different than the 0 and 33 kg N ha^{-1} (30 lbs N ac^{-1}) rates (*P*>0.05).

For the first year of legume establishment, minimal yield impacts were observed as a result of intercropping. During 2011, the only legume treatment imposed variation in yield occurred at

PREC during the forage ($P=0.029$) and biomass-only harvests ($P=0.005$); all other yields were not impacted by intercrops or inorganic inputs ($P<0.05$; table 2.3). Forage yields at PREC during the first year were greatest for 67 kg N ha^{-1} (60 lbs N ac^{-1}) and did not differ from the 33 kg N ha^{-1} rate (30 lbs N ac^{-1}; $P<0.05$). In addition, the 33 kg N ha^{-1} (30 lbs N ac^{-1}) rate was not different than any legume treatment except red clover, which was not different than the 0 kg N ha^{-1} rate (table 2.3). Similarly, for the biomass-only harvest, the highest N rate resulted in the greatest yields, but was not different than the moderate rate (table 2.3), which was not different than arrowleaf clover, partridge pea, or red clover ($P>0.05$).

During the second harvest year, legumes had more beneficial impacts on yield compared to that of the first legume establishment year. For 2012, the forage yield ($P=0.028$) and biomass-only ($P<0.0001$) at PREC was impacted by treatments. Similarly, at RECM, forage ($P=0.005$), biomass ($P=0.012$), and integrated ($P=0.003$) yields were affected by legume intercrop and nitrogen inputs (table 2.3). Both forage yields at PREC and RECM were greatest at 67 and 33 kg N ha^{-1} (60 and 30 lbs N ac^{-1}). At PREC, the 67 and 33 kg N ha^{-1} (60 and 30 lbs N ac^{-1}) rate produced equivalent forage yields to that of ladino clover and hairy vetch (table 2.3). For this location-year combination, the lowest yielding treatments were red clover, then partridge pea and arrowleaf clover. The biomass-only harvest regime at PREC also had no differences between high and medium synthetic nitrogen rates, which were greater than all other treatments ($P<0.05$); however, partridge pea yields exceeded that of intercropped arrowleaf clover (table 2.3). At RECM, partridge pea mixtures yielded comparable biomass to the inorganic treatments for forage, biomass, and integrated harvests ($P<0.05$). Biomass harvests at RECM revealed that in addition to partridge pea, hairy vetch, and ladino clover, intercropping can supply equivalent N as 67, 33, and 0 kg N ha^{-1} for switchgrass yields. On the other end of the spectrum, for these harvest regimes, arrowleaf clover consistently produced the poorest yields, not different than 0 kg N ha^{-1}.

Intercropped Legume Persistence in Switchgrass Swards. Harvest treatment did not impact legume persistence (P=0.99) and consequently this fixed effect was dropped from the model. In turn, legume species, year, and legume species x year were all important factors (P<0.0001). However, neither seeding rate within legume species (P=0.38), nor seeding rate x year within legume species was significant (P=0.78). The combined model for legume persistence (across locations and seeding rates) illustrated declining legume density trends over years, with the exclusion of arrowleaf clover as it was close to initial low legume levels (4.19 and 4.15 plants m^{-2} [1.28 and 1.26 plants ft^2] in years one and three, respectively [figure 2.1]). Legume species' persistence slightly increased from yr-2 to yr-3 for arrowleaf clover (1.6 plants m^{-2}; 0.49 plants ft^2), ladino clover (2.1 plants m^{-2}; 0.64 plants ft^2), and partridge pea (0.3 plants m^{-2}; 0.09 plants ft^2). This suggests that legume density either leveled off, or could potentially increase over time due to self-re-seeding (partridge pea) or asexual reproduction via lateral stolons (i.e., arrowleaf and ladino clovers).

Among all legume treatments, (across all seeding rates and years) red clover resulted in the highest density (12.6 plants m^{-2}; 3.8 plants ft^2), with partridge pea, ladino clover, and hairy vetch densities not differing from one another (10.3, 9.8, and 9.6 plants m^{-2} [3.1, 3.0, and 2.9 plants ft-^{2}], respectively), and arrowleaf clover being the lowest (3.6 plants m^{-2}; 1.1 plants ft^{-2} [P<0.05]). When combined across all years, red clover at the high and medium seeding rates (17.9 and 13.4 kg PLS ha^{-1}; 16 and 12 lb ac^{-1}) had the greatest densities across all seeding rate legume combinations (27.3 and 25.2 plants m^{-2}; 8.3 and 7.7 plants ft^{-2}, respectively). On the opposite end of the legume density spectrum, arrowleaf clover was the lowest, with seeding rates not affecting density over time ($P<0.05$; figure 2.1).

Within years and across all seeding rates by legume species, persistence differed the greatest during the establishment year (4.2 to 25.5 plants m^{-2}; 1.2 to 7.7 plants ft^{-2}) with red clover being the highest, followed by hairy vetch = partridge pea = ladino clover, > arrowleaf (figure 2.1). During yr-2 (across all legume species) densities dropped approximately 63%, with declines leveling off during yr- 3 (only 6% decline from yr-2). Consequently, arrowleaf clover density during yr-2 was the only species that differed among all legume treatments during 2013 ($P<0.05$).

Yr-1 density (16.0 plants m^{-2}; 4.9 plants ft^{-2}) was greater than yr-2 (6.0 plants m^{-2}; 1.8 plants ft^{-2}) which was not different than that of yr-3 (5.6 plants m^{-2}; 1.7 plants ft^{-2} [$P<0.05$]), when compared across all seeding rates, legume treatments, and locations. ETREC and RECM during yr-1 had the greatest densities (18.6 and 18.8 plants m^{-2}; 5.6 and 5.7 plants ft^{-2}, respectively) compared across all locations and years, followed by PREC in yr-1 (10.6 plants m^{-2}; 3.2 plants ft^{-2}), which was different than the other locations for that year ($P<0.05$), but not different than ETREC and RECM in yr-2 (8.7 and 8.2 plants m^{-2}; 2.7 and 2.5 plants ft^{-2}, respectively). However, the latter two were not different than RECM in yr-3 (7.49 plants m^{-2}; 2.3 plants ft^{-2}). Finally, ETREC densities in yr-3 were different than RECM in yr-3 (3.8 plants m^{-2}; 1.2 plants ft^{-2}); albeit still greater than PREC in yr-2 (1.0 plants m^{-2}; 0.3 plants ft^{-2} [$P<0.05$]).

Seeding rates x legume species did not impact plant density by year ($P<0.05$). Although red clover, partridge pea, and hairy vetch were among the only species that higher seeding rate resulted in greater numeric legume densities for yr-1 (figure 2.2). This trend did not persist in yr-2, with ladino clover and red clover being the only species where greater seeding rates were enumerating in yr-3. Conversely, low and medium seeding rates resulted in numerically greater legume densities for hairy vetch, arrowleaf clover, and partridge pea during yr-3.

Based on legume persistence for all tested legume species, reseeding would be recommended during yr-4, due to companion crop densities likely dropping below an acceptable persistence threshold for target nitrogen fixation (≤67 kg ha^{-1}; 60 lbs N ac^{-1} [Warwick 2011; Peoples et al. 1995]). Reseeding during yr-4 may not be necessary for red clover, ladino clover, and partridge pea, due to exceptionally high persistence and in some cases, out competing switchgrass (in field observations). Consequently, re-seeding would be predicated on site management objectives, soil texture, and physiographic location, and selected legume intercrop.

Among all legume intercrops, partridge pea demonstrated the greatest potential for legume persistence, self-re-seeding in both harvest systems, and for all seeding rates across a diversity of soil textures. However, given the difficulty in seed procurement, potential toxicity for cattle (Liener 1962), need for inoculating seed by-hand, unknown diazotrophic species for partridge pea host, and great potential for dormant and hard seed, this species may not be an ideal candidate. Consequently, ladino and red clover may be more appropriate as a legume intercrop given that persistence was also among the highest after 3-yrs (9.8 and 8.8 plants m^{-2}; 3.0 and 2.7 plants ft^{-2}, respectively), seed come pre-inoculated and with rock phosphate protectant to combat unfavorable conditions, have low dormant-seed level, have high nitrogen fixation rates (Peoples et al. 1995), and are widely distributed. Further, based on the theoretical fixation-transfer of these cool-season species (Peoples and Baldock 2001), and their complementary growth habit from fall through early-spring, these species are likely good intercrop candidates in switchgrass swards.

Switchgrass Yield and Legume Density Relationship. In the regression model (combined across locations and years) for the integrated harvest (forage + biomass) system, seeding rate and seeding rate x legume species interaction were marginally important (*P*=0.05) descriptors of yield, whereas legume species alone was not important for describing combined yields (*P*=0.08; figure 2.3A). Under the integrated model, relationships with legume density were all generally positive (ranging from β=1.63 to 2.56 ±1.22 se) with increasing legume density until approximately10 plants m^{-2} (3 plants ft^{-2}). This trend excluded hairy vetch, suggesting the growth habit of this species (climbing with tendrils), may negatively affect integrated switchgrass yield (*P*=0.04). Overall, across all seeding rates, for all species, there were some marginal trends suggesting positive benefits from legume integration.

Varying yield and legume density relationships were observed for the forage-only and biomass-only harvest regimes. Similar to integrated yield results, forage yields were not impacted by legume species (*P*=0.38), neither by seeding rate (*P*=0.13), nor legume species x seeding rate interactions (*P*=0.16). Conversely, switchgrass biomass-only yields were impacted by legume density (*P*<0.0001) but only marginally by legume seeding rate (*P*=0.07; figure 2.3B), but not by legume species (*P*=0.16), nor legume species x seeding rate interactions (*P*=0.98). Indicating biomass yields may potentially benefit at proper legume seeding rate, or be adversely affected at too high of densities (seeding rate). For this harvest regime, cool-season legumes (red clover, ladino clover, and arrowleaf clover) tended to occur with less frequency at higher legume densities, as biomass was not removed during peak production of these legumes (i.e. forage harvest period).

Summary and Conclusions

There are a multitude of potential benefits from introducing legumes into pasture and monoculture biofuel systems in the humid east, including reduced fertilizer inputs, increased soil carbon additions from green manure, reduced weed pressure due to niche differentiation, and reduced leaching of soil nitrate to groundwater. Switchgrass pastures and biofuel swards can be interseeded successfully with cool-season legumes (ladino and red clovers) and the warm-season legume, partridge pea, without annual re-seeding (≥3 yrs; depending on soil texture, soil fertility, and rainfall). Although annual reseeding may not be required, substantial legume density loss did occur during yr-2 for all species (approximately 63% decreases), likely due to competition from the dense stands of switchgrass used in this study, and canopy closure of this species early in the growing-season. Substantial legume density losses occurred for one of the three locations (PREC), with the lowest persistence occurring by yr-2 (1.0 plants m^{-2}), possibly corresponding to low soil fertility at this site.

Despite differences in legume establishment success, all tested legumes could be recommended for interseeding into switchgrass (with the exclusion of arrowleaf clover, as it never established well in our study). The abundance of legumes fluctuated from establishment year to the third study year, consequently producers would need to re-establish every 3-5 years (depending on intercrop species) to maintain the legume component, with the possible exceptions of ladino clover, red clover, and partridge pea. More research is needed to determine persistence long-term of legumes, particularly of annual re-seeding species whose densities may increase over-time.

Considering neither legume density nor persistence was impacted by seeding rate, the lower seeding rates could therefore be used for proper legume establishment; albeit, red clover densities may benefit from a higher seeding rate. However, the higher seeding rate is not recommended due to the observed competition from red clover and partridge pea (particularly in bottomland areas). In addition, economic assessments of legume intercropping at lower seeding rates to determine breakeven points are needed for switchgrass biofuel and forage cropping systems to ascertain economic feasibility of intercropping systems.

There were some indications that legume intercropping may improve switchgrass forage quality results (reduced ADF, NDF, and increased CP levels), even with legume tissue removed before quality analysis. More beneficial legume intercropping results were observed during the second year, suggesting more cumulative beneficial forage quality and quantity impacts from legume integration. Specifically, there is a slight indication that greater digestibility and intake of switchgrass-forage may occur when intercropped with partridge pea, hairy vetch, and red clover after the establishment year.

Similarly, yield during the second harvest year was more positively impacted compared to that of the first legume establishment year, with the majority of significant legume intercropping harvest regime location combinations occurring during forage harvests. Consequently, legume intercropping might be considered more remunerating for switchgrass-forage systems. Hairy vetch, ladino clover, and partridge pea in some cases had the greatest efficacy for improving yields when compared to medium inorganic-N levels (33 kg N ha^{-1}; 30 lbs N ac^{-1}), and in other instances did not differ from 0 kg N ha^{-1}. However, arrowleaf clover consistently induced the least yields, generally not different than the 0 kg ha^{-1} rate.

Overall, across all seeding rates and all species, in some cases there were marginal trends suggesting a positive relationship between switchgrass yield and legume frequency for selected legumes. For integrated and biomass yields, relationships with legume density were generally positive with increasing legume density until reaching approximately 10 plants m^{-2} (3 plants ft^{-2}). Consequently, red clover, partridge pea, and ladino clover intercrops may enhance forage quality and yield (equivalent to 33 kg N ha^{-1}; 30 lbs N ac^{-1}) thereby minimally reducing fertilizer costs and carbon-positive inputs in the Mid-South.

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APPENDIX

Table 2.1 Mehlich 1 soil baseline (2010) test results at Research and Education Centers in Tennessee (East Tennessee Research and Education Center [ETREC], Plateau Research and Education Center [PREC], and Milan Research and Education [RECM]).

Location	**pH**	**P** (kg ha^{-1})	**K** (kg ha^{-1})	**Ca** (kg ha^{-1})	**Mg** (kg ha^{-1})
RECM	7.06	30.24 M†	148.96 M	2,222 S	191.52 S
ETREC	6.72	29.12 M	50.40 L	1,676 S	293.44 S
PREC	6.83	35.84 H	40.32 L	2,177 S	26.88 D

†Note: L=low; M=medium; H=high; S=sufficient; D=deficient

Table 2.2 Switchgrass forage (early June) quality results (switchgrass tissue only) by legume and nitrogen treatments by location [East Tennessee Research and Education Center (ETREC), Plateau Research and Education Center (PREC), and Research and Education Center at Milan (RECM)] and by year (2011 and 2012).

Year	Location	Treatments	Crude protein (%)	ADF (%)	NDF (%)	Ash (%)	Hemicellulose (%)
2011	ETREC	ACˆ	10.55 a†	38.58 a	66.52 a	6.24 a	27.94 a
		RC	10.03 a	40.96 a	66.51 a	6.28 a	25.55 a
		LC	9.99 a	39.21 a	67.48 a	5.94 a	28.28 a
		PP	10.80 a	37.34 a	69.17 a	5.95 a	31.83 a
		HV	10.09 a	43.44 a	68.14 a	6.50 a	24.70 a
		IF	11.21 a	41.75 a	64.33 a	6.06 a	22.58 a
	PREC	AC	8.77 a	41.05 a	66.96 a	5.49 a	25.91 a
		RC	9.32 a	37.26 a	66.45 a	5.07 a	29.19 a
		LC	9.22 a	40.21 a	66.67 a	5.32 a	26.46 a
		PP	9.17 a	39.61 a	65.84 a	5.41 a	26.22 a
		HV	10.92 a	36.71 a	64.20 a	5.15 a	27.49 a
		IF	10.58 a	35.01 a	64.25 a	4.85 a	29.24 a
	RECM	AC	8.4 b	44.03 a	78.96 a	7.21 a	34.92 ab
		RC	9.62 ab	44.26 a	78.18 a	7.38 a	33.92 ab
		LC	7.50 b	46.68 a	78.33 a	6.73 a	31.65 b
		PP	8.89 b	45.51 a	79.26 a	7.25 a	35.75 a
		HV	11.91 a	46.17 a	70.57 b	6.82 a	24.40 c
		IF	8.63 b	43.58 a	79.30 a	7.02 a	35.72 a
2012	ETREC	AC	5.58 ab	46.19 c	83.05 ab	4.47 ab	36.86 a
		RC	5.21 ab	48.27 a	85.44 a	3.83 b	37.16 a
		LC	4.78 b	48.63 a	85.31 a	3.85 b	36.67 a
		PP	5.95 a	45.78 c	81.96 b	5.01 a	36.18 a
		HV	5.51 ab	46.40 bc	83.64 ab	4.58 ab	37.24 a
		IF	5.14 ab	48.08 ab	84.14 ab	3.81 b	36.06 a
	PREC	AC	7.66 a	40.17 a	79.62 a	2.82 a	39.45 a
		RC	7.86 a	40.03 a	79.73 a	2.90 a	39.69 a
		LC	7.88 a	39.76 a	79.13 a	3.22 a	39.36 a
		PP	7.68 a	39.63 a	78.72 a	3.13 a	39.08 a
		HV	7.75 a	39.63 a	78.96 a	3.03 a	39.27 a
		IF	7.83 a	39.90 a	79.00 a	2.95 a	39.08 a
	RECM	AC	1.33 a	52.51 a	91.12 a	6.73 a	38.60 a
		RC	1.77 a	52.69 a	89.88 a	7.38 a	37.21 a
		LC	0.36 a	58.90 a	87.50 a	7.25 a	28.59 a
		PP	1.59 a	52.97 a	90.01 a	7.02 a	37.04 a
		HV	1.43 a	52.90 a	90.74 a	6.82 a	37.83 a
		IF	1.23 a	53.67 a	91.77 a	7.21 a	38.10 a

†Note one: different letters indicate differences within a given location and year combination at the $P<0.05$ level using LSD.

* Note one: legume intercrop treatments: arrowleaf clover (AC), red clover (RC), ladino clover (LC), partridge pea (PP) hairy vetch (HV), and inorganic fertilizer [(IF), combined across inorganic-N rates: 67 & 33 kg ha^{-1}].

Table 2.3 Switchgrass forage (early June), biomass only (mid-November), biomass, and integrated (forage + biomass) yield by treatment at the East Tennessee Research and Education Center (ETREC), Plateau Research and Education Center (PREC), and Research and Education Center at Milan (RECM) for 2011 and 2012.

		2011				2012			
Location	Treatment	Biomass only (Mg ha^{-1})	Forage (Mg ha^{-1})	Biomass (Mg ha^{-1})	Integrated (Forage + Biomass) (Mg ha^{-1})	Biomass only (Mg ha^{-1})	Forage (Mg ha^{-1})	Biomass (Mg ha^{-1})	Integrated (Forage + Biomass) (Mg ha^{-1})
ETREC	AC	11.15 a[§]	5.72 a	8.54 a	14.26 a	11.80 ab	4.70 a	5.58 ab	10.29 a
	RC	11.06 a	5.47 a	9.14 a	14.61 a	11.16 ab	5.30 a	5.88 a	11.19 a
	LC	9.912 a	5.26 a	7.85 a	13.11 a	11.15 a	4.20 a	5.92 a	10.12 a
	PP	10.93 a	6.08 a	7.82 a	13.90 a	10.88 ab	4.22 a	5.58 ab	9.78 a
	HV	10.26 a	5.37 a	9.33 a	14.70 a	12.05 b	4.59 a	5.83 a	10.43 a
	N-0	9.27 a	6.07 a	8.74 a	14.81 a	12.00 a	4.59 a	5.05 ab	9.65 a
	N-33	9.44 a	6.04 a	10.41 a	16.45 a	12.10 a	4.20 a	4.15 b	8.36 a
	N-67	12.86 a	6.84 a	8.13 a	14.97 a	10.60 a	5.22 a	5.12 ab	10.34 a
PREC	AC	9.02 bc	3.51 bc	2.79 a	6.30 a	5.99 d	1.82 cd	3.32 a	5.14 a
	RC	8.68 bc	3.10 c	2.65 a	5.75 a	6.89 bcd	1.69 d	3.30 a	5.00 a
	LC	8.41 c	3.99 bc	2.81 a	6.64 a	6.09 cd	2.34 abc	3.42 a	5.75 a
	PP	9.73 b	3.33 bc	2.59 a	5.93 a	7.42 b	1.88 cd	3.34 a	5.23 a
	HV	8.78 c	3.18 bc	2.88 a	6.05 a	7.05 bc	1.96 bcd	3.50 a	5.46 a
	N-0	8.15 bc	3.44 bc	2.35 a	5.79 a	5.85 bcd	1.51 d	3.02 a	4.54 a
	N-33	9.95 ab	4.24 ab	3.12 a	7.36 a	8.84 a	2.69 ab	3.54 a	6.23 a
	N-67	11.61 a	5.10 a	3.23 a	8.33 a	9.48 a	2.78 a	2.88 a	5.67 a
RECM	AC	10.02 a	4.15 a	3.97 a	8.13 a	8.38 a	3.47 cd	3.76 c	7.23 d
	RC	9.97 a	5.34 a	4.54 a	9.89 a	8.06 a	4.33 bc	3.76 c	8.10 bcd
	LC	9.67 a	5.41 a	3.98 a	10.16 a	7.89 a	3.56 cd	4.40 abc	7.96 cd
	PP	8.68 a	5.4 a	3.95 a	9.36 a	7.57 a	4.95 ab	4.64 ab	9.60 ab
	HV	9.91 a	6.33 a	4.12 a	10.45 a	7.59 a	4.14 bcd	4.73 ab	8.88 bc
	N-0	8.15 a	5.54 a	2.66 a	8.11 a	6.97 a	2.71 d	4.03 bc	6.73 d
	N-33	9.92 a	5.7 a	4.28 a	10.0 a	7.13 a	6.13 a	5.25 a	11.39 a
	N-67	7.99 a	9.01 a	3.87 a	12.89 a	7.53 a	6.22 a	4.99 ab	11.22 a

[§] Note one: different letters indicate a significant difference within a given harvest, location, and experimental year at the $P<0.05$ level using LSD.

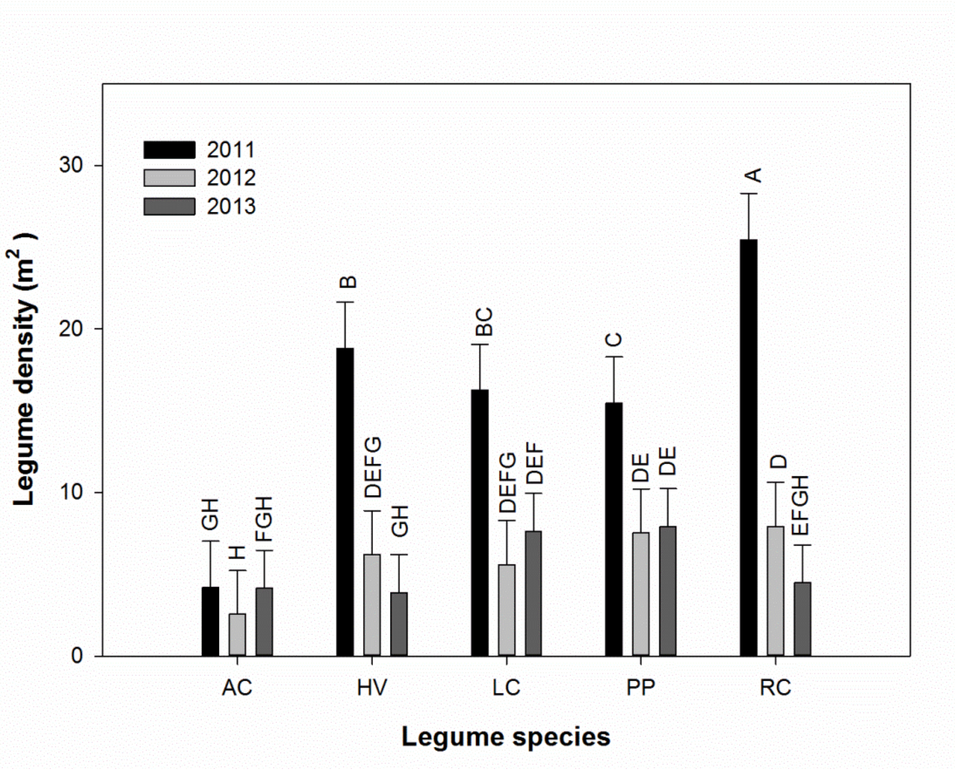

Figure 2.1 Legume density by species (arrowleaf clover [AC], hairy vetch [HV], ladino clover [LC], partridge pea [PP], and red clover [RC]) combined across locations (East Tennessee, Plateau, and Milan Research and Education Centers) and seeding rates (low, medium, and high) per species during 2011-2012. Different letters indicate significant differences at $P \leq 0.05$, using LSD compared across years and species. Vertical bars are one standard error.

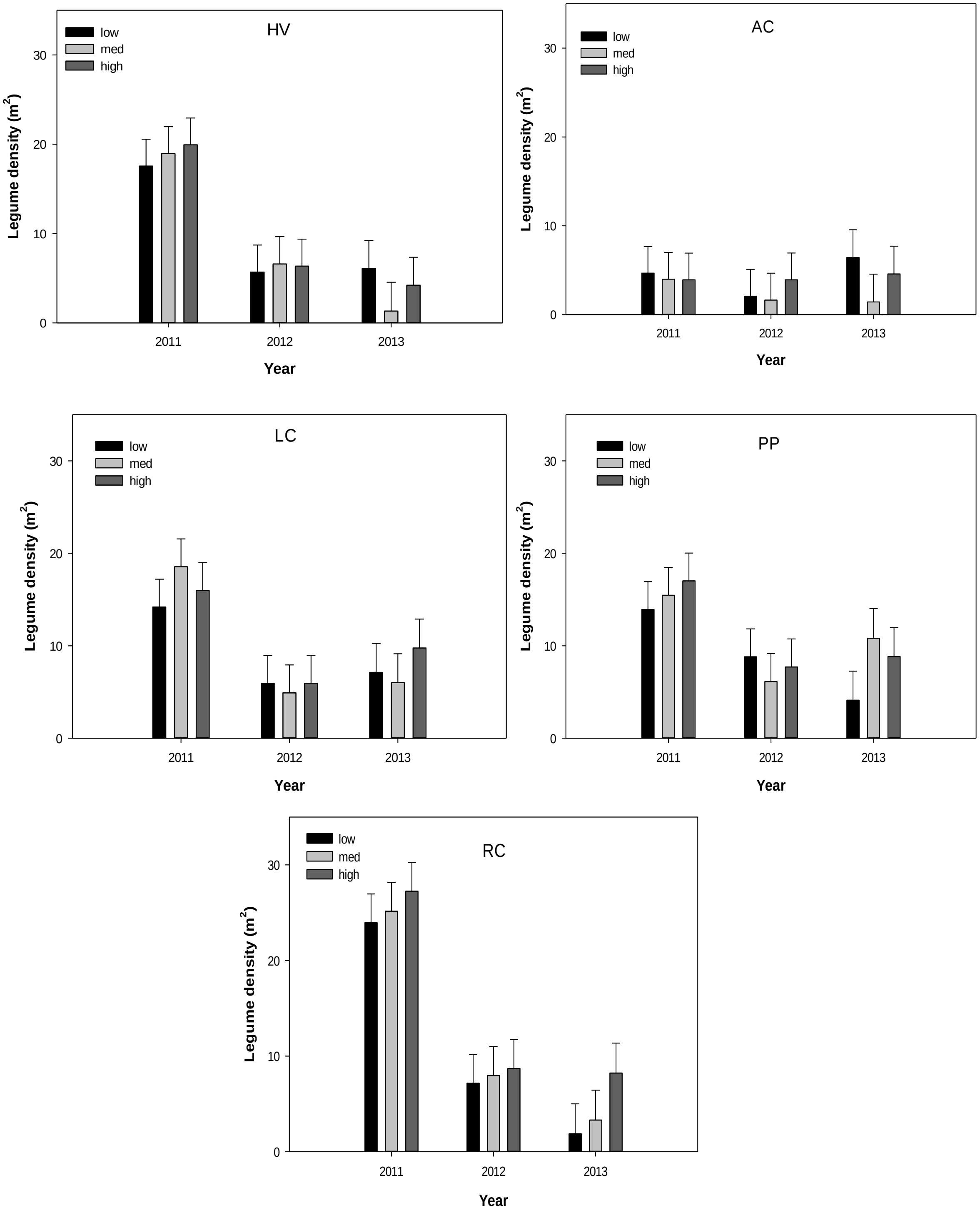

Figure 2.2 Legume density by seeding rate (low, medium, and high) and species (arrowleaf clover [AC], hairy vetch [HV], ladino clover [LC], partridge pea [PP], and red clover [RC]) combined across locations (East Tennessee, Plateau, and Milan Research and Education Centers) during 2011-2013, and analyzed within years. Vertical bars are one standard error.

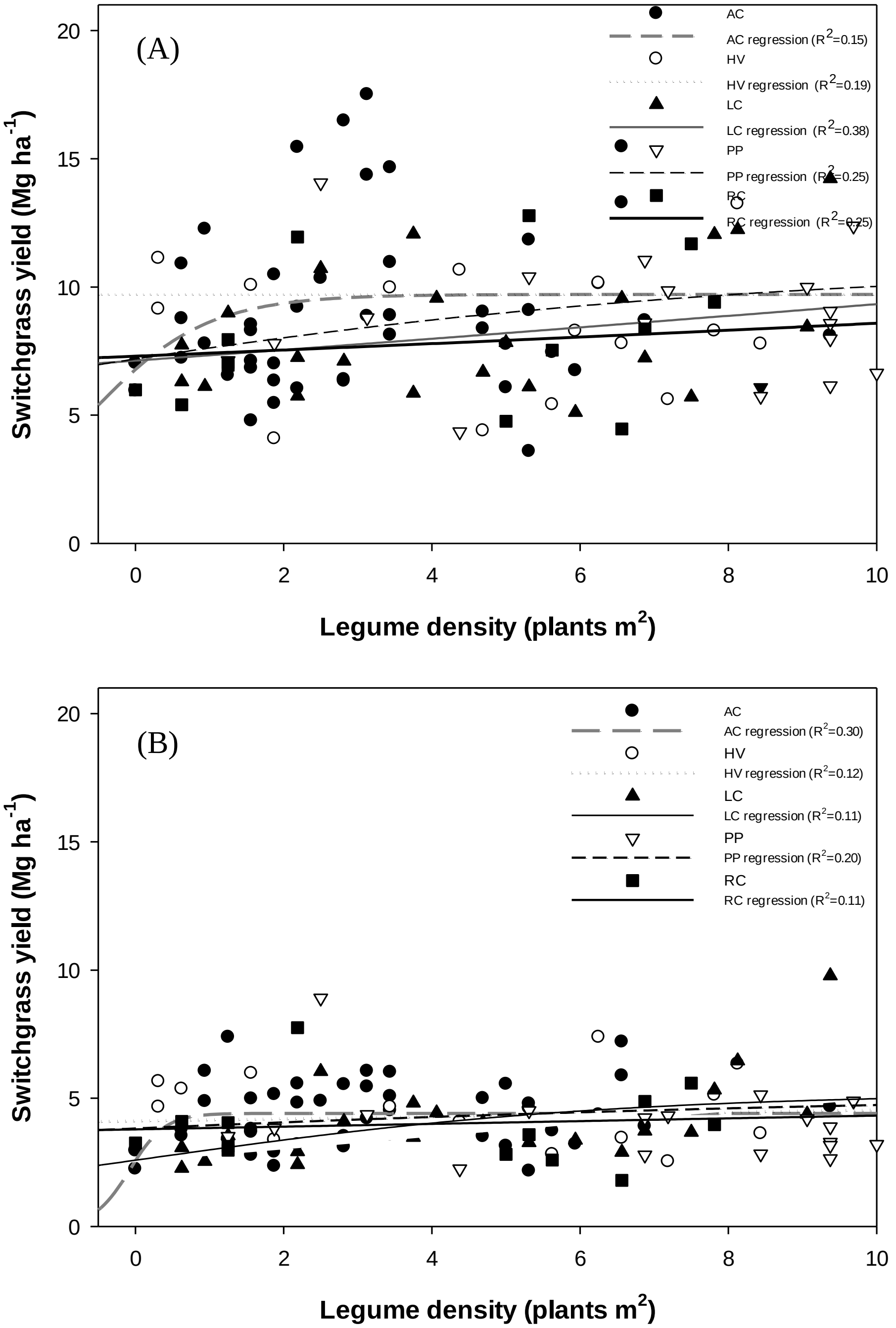

Figure 2.3 Switchgrass integrated (early June + biomass mid- November [a]) and biomass-only (one-cut; mid- November [b]) yields regressed against legume density by species (arrowleaf clover [AC], hairy vetch [HV], ladino clover [LC], partridge pea [PP], red clover [RC]). Data were collected from the East Tennessee, Plateau, and Milan Research and Education Centers from 2011-2012, and regressed with non-linear regression (equation: Sigmoidal, Sigmoid 3 Parameter).

CHAPTER III

Biologically Fixed Nitrogen in Legume Intercropped Systems: A Comparison of N-Difference and ^{15}N Enrichment Techniques

Abstract

Alternative sources to synthetic nitrogen (N) inputs could reduce costs and environmental impacts of cellulosic bioenergy and forage production systems. Biological N_2 fixation (BNF) via legumes may serve those functions when interseeded into switchgrass (*Panicum virgatum* L.). Data are needed on amounts of nitrogen derived from the atmosphere by diverse legumes and supplied to *Panicum* spp. and on validation of techniques for such measurements. Our objectives were to i) verify the use of switchgrass as a non-N_2-fixing reference plant for distinguishing between soil- and atmosphere-derived N in companion legumes; ii) determine BNF levels based on the N-difference and ^{15}N enrichment methods for one cool-season and two warm-season legumes; and iii) evaluate the validity of the N-difference method by comparing it against the ^{15}N enrichment technique. Interseeded legumes included one cool-season legume, red clover (*Trifolium pretense* L.), and two warm-season legumes, partridge pea (*Chamaecrista fasciculate* L.) and sun hemp (*Crotalaria juncea* L.) in a field trial at Knoxville, TN (humid, temperate climate). The same experiment was carried out in the semi-arid tropics (U.S. Virgin Islands) and included a near relative of switchgrass, guinea grass (*P. maximum* L.) and sun hemp and pigeon pea (*Cajanus cajan* L.) as the only legume intercrops. Results revealed little difference in N-assimilation rates of legume and the non-N_2-fixing reference plants, suggesting switchgrass may be an appropriate reference plant. Annual fixation rate for red clover was greatest, followed by partridge pea, and then sun hemp in temperate systems (averaging 86, 68, and 25 kg ha^{-1}, respectively), all of which tended to supply greater amounts of N in subsequent seasons. Considerably greater BNF occurred in pigeon pea and sun hemp in tropical (exceeding 240 kg ha^{-1}) intercrop systems compared to temperate locations, perhaps because these legumes are better adapted to tropical conditions. Therefore, N input via BNF of selected legumes can meet or exceed recommended N fertilizer application rates (67 kg N ha^{-1}) in both humid temperate and semiarid tropical pasture/feedstock systems. The N-difference method may also be used to measure BNF, as it estimated comparable fixation rates to that of ^{15}N enrichment derived values.

Key words: biological nitrogen fixation—15N enrichment method —nitrogen difference method—switchgrass—legume intercropping.

1. Introduction

As human population increases, so does the importance of biological nitrogen fixation (BNF) by leguminous species. Agriculture has become increasingly dependent on fossil fuel-derived inputs such as fertilizers. The modern green revolution promotes agricultural and energy sustainability by reducing environmental impacts [e.g., greenhouse gases (GHG), groundwater contamination, and fossil energy consumption] and producing renewable biofuels. Use of nitrogen (N)-fixing legume species to replace fossil fuel-based fertilizers exemplifies the convergence of agricultural and energy sustainability. The manufacture and use of synthetic N fertilizers (namely ammoniacal and nitrate forms) are significant causes of GHG emissions and off-field movement of nutrients to natural waters. Nitrous oxide is a byproduct of nitrification of ammonium and denitrification of nitrate in soils during synthetic N fertilizers transformations and is approximately 300 times more potent as a GHG than CO_2 (Schlesinger, 1991). Although interseeding N-fixing legumes has been used with many tropical crops and temperate pastures, no legumes have been identified as promising companion species for supplying N to switchgrass. While switchgrass has been touted as an environmentally friendly feedstock for biofuels, it still requires N inputs to promote satisfactory yields. Recommended annual N applications of 67 kg ha^{-1} for switchgrass growth (Mooney et al., 2009) indicate that potential exists to further improve environmental benefits if inputs are replaced with BNF (Ashworth et al., 2015).

Legumes fix atmospheric N_2 into plant-assimilable NH_4^+ in root nodules via symbiosis with *Rhizobium*-type bacteria, but legumes also take up plant-available soil N, resulting in a mixture of soil-derived and atmosphere-derived N in the total N mass of the plant. The amounts and proportions of N fixation in legumes is affected by agronomic practices, environmental factors, and microbial and soil conditions (Peoples and Baldock, 2001; Peoples et al., 2009). Therefore, the amounts and proportions of N fixed by legumes vary widely with many interacting conditions (Rennie and Rennie, 1983). Several methodologies aim to fractionate the plant N mass into atmospheric and soil sources, or directly measure the activity of nitrogenase, the *Rhizobium* enzyme directly responsible for fixing N^2 that resides in legume nodules. Each methodology has inherent drawbacks; however, some techniques provide data that are more useful than others depending on the research objectives and legume growth habit. Therefore, there is no single acceptable technique for measuring N_2 fixation under all conditions and for all purposes. Nevertheless, quantifying N fixation is valuable for assessing the potential for replacing synthetic fertilizer N with more environmentally benign legume N in relation to growth restraints. Methodologies for BNF determination are described in more detail below.

In nature, N atoms predominantly carry an atomic mass of 14 (^{14}N), but 0.3663% of N atoms possess atomic mass of 15, which is a stable (nonradioactive) isotope. Changes in the enrichment (or depletion) of the 15N isotope can be used to quantify transformations of N such as BNF. A 15N-enrichment (atom percentage excess) technique has been applied to measuring BNF in grass-legume mixtures in which the plant-available N pool in the soil is artificially enriched with 15N, a non-N-fixing reference plant (in this case the grass) takes up the 15N-labeled soil-derived N, and unlabeled N from the atmosphere is fixed via BNF into the legume (Peoples et al., 2009). Since legumes take up N from both the soil and atmosphere pools, the degree to which unlabeled atmospheric N dilutes labeled N uptake from soil allows calculation of the percentage of N uptake derived from atmosphere (%Ndfa) and therefore the mass of N from atmosphere (N fixed, or BNF). This dilution of 15N label may be challenging to monitor due to variation in legume and reference plant ^{15}N uptake ratios (Danso et al., 1993; Unkovich and Pate, 2000). A major advantage to this method is that it distinguishes between soil-derived and atmosphere-derived N in the harvested portion of the legume (Peoples et al., 2009). However, a major constraint to this method is that when the %Ndfa is low, the error in this method will be high (Danso et al., 1993). Limitations also exist due to the high variability of the natural abundance of ^{15}N in soils, thus the accuracy of this technique is largely dependent on the uniformity of the ^{15}N label in the labile N pool (Ledgard and Steele, 1992). For those reasons, the method works best if the labile soil N pool is highly enriched relative to background 15N levels.

Two agronomic-based methods for determining legume fixation levels are N-balance and N-difference methods. The N-balance method requires all inputs and losses of N to be quantified, and then the increase in N balance is used to estimate N input from BNF (Peoples et al., 2009). One major limitation is that losses (e.g., leaching and volatilization) are difficult to measure, thus diminishing the accuracy of BNF estimates. The N-difference method is an inexpensive and simple way to estimate BNF of a legume population. This is done by calculating the difference of legume total N uptake to that of an adjacent non-N_2-fixing reference species, such as a grass or some other non-legume. This method assumes that the total N uptake of the reference species is solely derived from soil and that both species assimilate equal amounts of labile soil N during the growing season (Danso, 1995). The BNF calculation also assumes that both species translocate the same amounts to the harvested portion of the crops. This technique is carried out by growing the fixing and non-fixing crops under the same conditions and physically close together. One potential limitation in this method is that legume and reference species' morphology and rooting depth may vary and, therefore, capabilities of assimilating and translocating soil-derived N differ. The N-difference technique is considered most reliable when available soil N is low and where there are great differences in growth between the legume and the reference species (Bell et al., 1994). Statistical differences in total N uptake may only be seen when BNF levels exceed 20 kg N ha^{-1} (Weaver and Danso, 1994; Weaver, 1986; Zuberer, 2005).

The 15N-enrichment and N-difference techniques are predicated on legume and reference species to be grown under similar amounts of ^{15}N and labile soil N, respectively (Segundo and Boddey 1987), and that both species remove the same ratio of labeled N and non-labeled soil N. This principle holds even though the actual quantities of labeled and non-labeled N uptake may differ greatly between the species (Ledgard and Steele, 1992). Ideally, reference plants and N-fixing plants should be similar in size to potentially absorb similar amounts of total N for plant growth (Danso et al., 1992; Boddey et al., 1984). Shallow-rooting annual small grains [e.g., wheat (*Triticum* spp.)] are widely accepted as a reference plant, however, little is known about the use of deep-rooted perennial species as reference species. Therefore, we will test switchgrass compared to other commonly used reference crops with various rooting morphologies.

Among available methods for field determination of legume N fixation, 15N enrichment and N-difference methods are considered the most suitable when analyzing BNF in soils inherently low in N and where fixation is expected to be high (Peoples et al., 2009). 15N-enrichment techniques, proposed more than 40 years ago, are still used to estimate BNF because of the advantage of distinguishing between soil-derived and atmosphere-derived legume N; however, the extra expenses of 15N application and assay limit its use in applied research. Witty and Ritz (1984) concluded that N-difference estimates of BNF agree with 15N-enrichment measurement, given that the assumptions and sampling techniques are not independent of each other. Since this has not yet been studied with switchgrass, we will test and verify the N-difference method by comparing its results with the ^{15}N enrichment approach and to acquire realistic estimates of the N supplying capacity of various legumes to switchgrass.

Legume symbioses fix approximately 70 million tonnes of N per year worldwide, with about half of this fixation occurring in temperature zones (cool and warm) and the remainder happening in the tropics (Brockwell et al., 1995). Soil temperature greatly impacts the activity of nitrogenase. Nitrogenase activity accelerates greatly over the range of 10-35ºC, with varying temperature responses occurring among legume species (Liu et al., 2011). In addition, soil moisture deficit may severely depress *Rhizobium* activity (Albrecht et al., 1994). Consequently, measuring BNF on tropical legumes in a tropical environment (bimodal annual rainfall and high soil temperatures) may provide greater insight into fixation ranges over diverse soils and climates.

To attain these goals, we estimated BNF rates of one cool-season and three warm-season legume species. No data to the authors' knowledge have been published on fixation rates of red clover (*Trifolium pretense* L.), partridge pea (*Chamaecrista fasciculate* L.), and sun hemp (*Crotalaria juncea* L.) when intercropped with *Panicum* spp. either by 15N or N-difference methods. Results will help identify an intercrop species that serve as a renewable source of labile-N for *Panicum* spp., while replacing fossil fuel-based N inputs and associated costs. Specifically, objectives are to: i) assess the legitimacy of using switchgrass as the non-leguminous reference plant for estimating BNF; ii) determine levels of BNF using the N-difference and ^{15}N-enrichment methods for sun hemp, red clover, and partridge pea; and iii) evaluate the validity the N-difference method by comparing it against the ^{15}N enrichment technique.

2. Materials and Methods

2.1. *Field site descriptions*

Legumes were interseeded into 3-yr old stands of switchgrass at Holston Unit, East Tennessee Research and Education Center (ETREC) located near Knoxville, TN on a soil mapped as a Sequatchie silt loam (fine-loamy, siliceous, semiactive, thermic Humic Hapludults), and at the Greenville Research and Education Center (GREC), TN on a Dunmore silty clay loam (fine, kaolinitic, mesic Typic Paleudults). Legume treatments included one cool-season legume: red clover (cv. Cinnamon Plus), and two warm-season legumes, partridge pea (cv. Lark), and sun hemp (cv. Tropic Sunn). The three legume species were compared for their potential to host N fixation when intercropped with switchgrass via the N-difference and^{15}N enrichment methods as described below. Mean annual precipitation for the duration of the study (2011-2013) at ETREC was 1450 mm, with mean annual temperature at 15.6^{o}C (NOAA, 2013). This site was under orchardgrass (*Dactylis glomerata* L.) hay production for 4 yr prior to experimentation. Mean GREC precipitation was 1190 mm, with mean annual temperature at 14.7^{o}C (NOAA, 2013). The site was previously under tall fescue (*Schedonorous arundinaceus* [Schreb.]) hay production.

An additional site located near the inter-tropical convergence zone in St. Croix, USVI (STX) and was included to determine BNF of selected legumes under endemic climates and soils, and ascertain the consistency of fixation measurements under varied environments. The experimental site was located on the southwestern and south-central coastal plain physiographic region (Kemp, 1927), corresponding to the major land resource area (MLRA 273) region Z (Caribbean Region, Semiarid Mountains and Valleys). Rainfall in this region has a bimodal nature, with the initial early rainy season beginning in May and extending until June, with a brief dry period in July and the second half of the rainy season spanning from August to November (concurrent with hurricane season). Mean temperature during the experimental period (July 2013-July 2014) was 28°C, with precipitation totaling 1080 mm. Field measurements were taken on a Sion clay (coarse-loamy, carbonatic, isohyperthermic Typic Calciustolls), which consists of very deep, well drained, and moderately slowly permeable soils, which are formed from alkaline marine deposits. At STX, BNF of sun hemp and pigeon pea, (*Cajanus cajan* L. cv. Caqui) were determined using switchgrass and guinea grass (*Panicum maximum* L. cv Mombasa) as the non-fixing reference crops.

2.2. *Data collection*

Preliminary and terminal soil nutrient levels were quantified on a per-plot basis for all locations. Preliminary basic soil test levels were conducted at 0-15 cm depths in 2010 to determine levels of pH, cation exchange capacity (CEC, meq 100 g^{-1}), and plant pre-sidedress nitrate (PSNT, ppm) and nutrient concentrations of P, K, Mg, and Ca. Samples were ground to pass a 1-mm sieve on a Wiley soil grinder (Thomas Scientific, Swedesboro, N.J.) and then analyzed with a Mehlich-1 extractant by University of Tennessee (UT) Soil, Plant, & Pest Center (Nashville, TN) and with Mehlich-3 at A&L Laboratory (Memphis, TN) for TN and STX locations, respectively.

Established Alamo switchgrass plots (1.5 x 7.6 m^2), planted at 9 kg pure live seed (PLS) ha^{-1} with 18 cm row-spacing in the spring of 2007 at ETREC and in spring 2008 at GREC were used in this study. Broadleaf weeds were controlled during the establishment year by 2,4-dichlorophenoxyacetic acid at a product rate of 0.9 L ha^{-1}. Legumes were no-till drilled annually into switchgrass stubble at ETREC using a 7-row Hege™ plot drill (Colwich, KS) and a 5-row Great Plains™ No-Till plot drill (Salina, KS) at GREC. Red clover was planted on 13, 28 February 2012 and 28 February and 13 March 2012 at ETREC and GREC, respectively, at the rate of 12 kg PLS ha^{-1}. Partridge pea and sun hemp were seeded on 12 April and 6 May 2012 and 5 and 16 May 2013, at the rate of 18 and 24 kg PLS ha^{-1} at ETREC and GREC, respectively. Planting depth ranged from 0.6 to 1.3 cm, depending on seed size. Partridge pea and sun hemp seeds were inoculated with the cowpea group of *Bradyrhizobium* spp., and red clover with the clover group inoculum of *Sinorhizobium meliloti*.

At the tropical location, switchgrass and guinea grass plots were seeded by hand on 60-cm centers on 1 November 2012 (onset of the rainy season). Plot size was 1.8 x 6.1 m and 1.8 x 7.6 m, for switchgrass and guinea grass, respectively. Pigeon pea and sun hemp were interseeded by hand at 24 and 46 kg PLS ha^{-1}, respectively, on 15 June, 25 November, 2013, and 27 March 2014, and inoculated with the cowpea group of *Bradyrhizobium*. Pigeon pea establishment failed in switchgrass, therefore pigeon pea was only included as an intercrop in guinea grass.

This experiment utilized a one factor randomized complete block design with three blocks per location with legume species as the fixed effect. In temperate locations, this included a single annual harvest (June-Augusts, depending on legume species maturity) throughout 2013-2014. Tropical location harvests included two dry-season harvests (March and November, 2013) and one during the onset of the rainy season (July, 2014). Pigeon pea was substituted for partridge pea due to its proven success for this environment, and red clover was eliminated from the STX trials because this species was not adapted to the hot tropics. Guinea grass, a tropical *Panicum* species and near relative of switchgrass, was included in the event that switchgrass establishment failed. Guinea grass is very similar physiologically to switchgrass; however, guinea grass is better adapted to the specific geographical and environmental conditions of STX.

2.3.*Validating the use of a deep-rooted perennial as a reference plant*

Nitrogen concentration of common (*Vicia sativa* L.) and hairy (*Vicia villosa* L.) vetch plants at ETREC-Plant Sciences Unit in Knoxville was compared to that of monocots wheat (*Triticum aestivum* L.), foxtail barley (*Hordeum jubatum* L.), and to dicot broadleaf weeds including wild geranium (*Geranium* spp.), marestail (*Conyza canadensis* L.), and Venus looking glass (*Tridanis perfoliata* L.). At the ETREC-Holston Unit, common and hairy vetch plants were compared to switchgrass, foxtail barley, daisy fleabane (*Erigeron strigosus* L.), wild geranium, marestail, and Venus looking glass in order to determine appropriate reference species for BNF techniques. Species tested herein were chosen based on previous work (Warwick, 2010), as the validation of switchgrass as a non-leguminous reference plant was needed prior conducting work in the proceeding sections.

Common and hairy vetches were seeded in fall 2009 into established 3-yr-old switchgrass stands of ‘Alamo’ at the two locations. The vetches were seeded into approximately 20cm-tall switchgrass stubble on 22 and 29 October, 2009 at the Plateau Research and Education Center (PREC) at Crossville, TN, on a Lily silt loam (fine-loamy, siliceous, semiactive, mesic Typic

Hapludults), and at the ETREC (Holston unit) on a Huntington silt loam (fine-silty, mixed, active, mesic Fluventic Hapludolls). Legumes were seeded with a Hege™ plot drill (Colwich, KS) at a planting depth ranging from 0.6 to 1.3-cm. At ETREC and PREC, the plot sizes were 7.6 x 1.5 m and 7.6 and 1.8 m, respectively, with 18-cm spacing between rows. The seeding rate for both vetch species was 6.7 kg PLS ha^{-1} and no N fertilizer was applied. The seed used for common vetch were collected from volunteer populations at ETREC Holston and Plant Science Units in early summer 2009 and treated by stratification and scarification to break dormancy. Wheat and barley were fall seeded, whereas broadleaf and monocots were volunteer species. Vetch plants were compared to wheat and other species that were harvested in late May 2010.

A frequency grid (Vogel and Masters, 2001) was used to quantify legume stand densities in reference plots interseeded with legumes in early summer to allow time for legume growth. Four density counts were made in each legume treatment plot and averaged over three blocks at each location.

Shoot samples of common vetch, hairy vetch, and non-N-fixing reference plant species of wheat, switchgrass, wild geranium, barley, marestail, Venus looking glass, and daisy fleabane were gathered by cutting the plants flush with the soil with pruning shears in late spring 2010. All species listed above were in their reproductive stages, with the exception of switchgrass and marestail, which were pre-anthesis. Reference plant sizes with the exception of Venus looking glass plants were all larger than vetch plants. Samples were weighed, oven-dried, reweighed, and ground to pass through a 1-mm sieve with a Wiley mill (Thomas Scientific, Swedesboro, NJ). Samples were then analyzed for N concentration by the UT Soil, Plant, and Pest Center

(Nashville, TN). Concentration of N was multiplied by plant dry matter (DM) yield to determine N yield (mass, or soil N uptake).

2.4. *Legume mediated fixation in switchgrass swards*

For N-difference determination, shoot tissue of red clover, partridge pea, sun hemp, and adjacent non-N-fixing reference plant was harvested at the soil level during reproductive stages or maximum N-fixation periods (i.e. between pod initiation and filling stage). Legume stand densities (plants m^{-2}) were quantified before sampling. For both N-fixation determination methods, swards were cut to a 20.3 cm stubble height in both intercrop and control (switchgrass only) microplots. Samples (legume and grass crop biomass) collected from all plots were weighed, dried at $49^{o}C$ in a batch oven (Wisconsin Oven Corporation, East Troy, WI), and re-weighed to determine moisture content. Samples were then ground through a 2-mm sieve on a Wiley mill (Thomas Scientific, Swedesboro, NJ). Sample tissue (grass separated from legumes) was then analyzed with near-infrared reflectance spectroscopy (NIR) using a LabSpec® Pro Spectrometer (Analytical Spectral Devices, Boulder, CO). Equations were standardized and checked for accuracy using the Grass Hay equation developed by the NIRS Forage and Feed Consortium (NIRSC [Hillsboro, WI]).

Plant aboveground N yield was determined by multiplying plant dry matter yield by its N concentration (Eq. 1 below). Reference plant N yield (non-legume, switchgrass) was then subtracted from legume plant N yield to obtain legume fixed N on a per-ha basis (Eq. 2). The N-difference of the legume and reference plants were multiplied by average plant weights of legume plants to obtain the aboveground-N per plant. Total aboveground legume N mass was determined by legume-N x plant density, and then converted to kg ha^{-1} to determine fixed N supplied by legume (value assuming complete bioavailability). Finally, required seeding rates to reach target N were determined by Equation 3.

$$\text{Plant N yield (kg ha}^{-1}\text{)= [(Plant DM) x (\%N)] / (100)} \quad [1]$$

$$\text{\% N-difference of legume (N}_2\text{ fixed) = (legume N) – (reference plant N)*100} \quad [2]$$

$$\text{Seeding rate for target N= [(Target N kg ha}^{-1}\text{) x (kg PLS ha}^{-1}\text{)] / (legume N kg ha}^{-1}\text{)} \quad [3]$$

2.5. *Nitrogen-15 enrichment method compared to N-difference for determining BNF of legumes interseeded into switchgrass*

One microplot (0.85 m^2 per species), replicated thrice was located randomly per block during 2012 and 2013, with care taken to avoid overlap with the previous year's locations. Two separate sets of reference plant-only microplots (one for cool-season and warm-season legumes) were located at each site with three replications, therefore, there were 15 microplots total per temperate location. Because this small amount of ^{15}N-labled N does not influence nutrition of the legume or reference plant, nor does it depress N fixation of legumes, ^{15}N was applied at the rate of 0.40 g $microplot^{-1}$ (4.7 kg ha^{-1} of ^{15}N) during mid-April to early-May (cool-season legume) and early-mid June (warm-season legumes). A solution of ^{15}N-enriched $(NH_4)_2SO_4$ (99 atom%) was added with 2000 mL of water, and randomly injected four times with a 50 mL syringe into soil to ensure soil-solution contact. Then remaining solution was added to each microplot to simulate 2.1 mm of rainfall during the application period. A barrier was constructed around microplots to reduce runoff and eliminate mobility of labeled-N outside treatment area.

Once ^{15}N was introduced into the soil, two pools of plant-available N were created (Chalk and Ladha, 1999), ^{15}N enriched and naturally occurring, unlabeled-N. Tissue analysis of ^{15}N then gave an estimation of both labeled and non-labeled pools taken up by reference plant; from here the fraction of the total plant-N uptake from the soil when the enrichment of the legume relative to the reference was measured. The premise of the ^{15}N excess method is that artificial enrichment of the labile soil-N pool with ^{15}N above natural background levels allows quantification of unlabeled atmospheric N_2 uptake by legume. Consequently, the greater the ^{15}N enrichment of the plant-available soil pool achieved, the greater the accuracy of the method (Peoples et al., 2009).

Shoot tissue of red clover, partridge pea, sun hemp, pigeon pea, and reference plant (switchgrass) were harvested from microplots after a period of growth as described in section 2.3. ^{15}Nitrogen was analyzed using a PDZ Europa ANCA-GSL elemental analyzer [interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK)] by the University of California-Davis Stable Isotope Facility (Davis, CA). Samples were combusted at 1000°C in a reactor; and following combustion, nitrogen oxides were converted to N_2 in a reduction reactor (reduced copper at 650°C). N_2 and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL min^{-1}) before entering the isotope ratio mass spectrometer (IRMS). Thereafter, the %Ndfa of the legume, atom% ^{15}N excess, and percent N transfer were calculated by Equations 4 and 5.

$$\%\ \text{Ndfa} = \frac{100[1-(\text{atom}\ \%\ 15\text{N excess of legume})]}{(\text{atom}\%\ ^{15}\text{N excess of switchgrass})} \qquad [4]$$

$$\text{Atom}\%\ ^{15}\text{N excess} = (\text{atom}\%\ ^{15}\text{N of sample}) - 0.3663 \qquad [5]$$

The percentage of grass shoot N derived from nitrogen transfer is expressed as 'potential %NT' and the uptake (or yield) of transferred N (per kg ha^{-1}) as 'TNY' from the below equations [Eqs. 6 and 7] in which, %^{15}N is the %^{15}N atom-excess in plant material and N yield is N yield of switchgrass or guinea grass grown in mixtures.

$$\text{Potential \%NT} = 1 - \frac{(\%^{15}\text{N of switchgrass in mixture})}{(\%^{15}\text{N of switchgrass alone})} \times 100 \qquad [6]$$

and

$$\text{TNY} = (\text{\%NT} \times \text{N yield})/100 \qquad [7]$$

Legume N yield was determined by multiplying microplot legume DM yield (shoot mass kg ha^{-1}) by legume shoot-N concentration. Finally, amount N fixed (kg ha^{-1}) was calculated by multiplying legume N yield by %Ndfa [Eq. 8].

$$\text{Amount N fixed (kg ha}^{-1}) = (\text{\%Ndfa} \times \text{legume N yield})/100 \qquad [8]$$

Nitrogen fixation estimate from the N-difference method was compared with the 15N-enrichment method using linear regression in Sigmaplot (Sigmaplot V12.5; Systat Software, Inc., San Jose, CA). In addition, a simple-correlation analysis was performed to examine the linear relationship between the fixation methods (SAS V9.3; SAS Inst. Cary, NC) by the SAS macro 'pdmix800' (Saxton, 1998), with a Type-I error rate of 5%. All residuals in the aforementioned models were normally distributed ($P \geq 0.05$; Shapiro-Wilk>0.85).

3. Results and Discussion

Fluctuations in soil chemical characteristics indicated minimal transformations when compared across years per intercrop species (Table 3.1). Initial macro and micronutrient (P, K, Ca, and Mg) levels at GREC were all considered 'high' or 'medium,' with the exclusion of a few plots testing 'low' for potassium and 'sufficient' for magnesium. Conversely, at ETREC, all plots tested 'low' in potassium, 'sufficient' in magnesium, and a variety of ratings ('low-high') for phosphorus (DNS), indicating adequate rhizosphere conditions. Temperate intercropping (after yr-3) increased pH, whereas P and K tended to decrease for both temperate sites. For the location in the tropics, across both companion grass crops, sun hemp indicated more favorable impacts on soil characteristics (Table 3.1), as P, K, Ca, Mg, and CEC increased from yr-1 to yr-2. However, pigeon pea intercrops only increased K, Ca, and Mg and decreased nitrate-N, and pH.

3.1. *Validating the use of a deep-rooted perennial as a reference plant*

The use of both dicot and monocot non-N-fixing reference plants revealed little difference in the amount of N-fixed of common and hairy vetch at both locations (Table 3.2). The use of switchgrass, wheat, and weeds as reference plants to determine N fixation of common and hairy vetch showed similar results. Marestail revealed the smallest amount of N fixation for vetch plants, and the most variability. This could have been a result of plant maturity levels, as Marestail was in vegetative rather than reproductive stage like the other dicot weed species. Results suggest switchgrass assimilates soil N similarly to the other non-N-fixing reference plants tested. Therefore, results indicate switchgrass can function as a reference plant for both

BNF determination techniques. Comparisons of utilizing dicots and switchgrass as reference plants have not previously been conducted.

3.2. *Legume mediated fixation in switchgrass swards*

Both methods of N-fixation measurement revealed that all tested legumes in this study promoted N enhancement for all years and environments. For both years, red clover supplied companion crops with greater than the current recommended N recommended rate of fertilizer N (67 kg ha^{-1}), whereas partridge pea supplied an excess amount in 2013 at both temperate locations (Tables 3). However, if one assumes 50% bioavailablity, red clover in 2013 at ETREC was the only legume location combination to supply N-rates competitive with the recommended rate of fertilizer N. Specifically, red clover N fixation amounts ranged from 81 to 96 kg ha^{-1} (Table 3.4) across all locations and years from the $^{15-}$N technique, which was similar to values reported by others (54 to 373 kg ha^{-1}; Peoples et al., 1995). However, the majority of high fixation observations occurred in a green manure-cover crop setting with no canopy competition, consequently, when dense grass swards exist, fixation rates are likely greatly reduced. Fixation values of partridge pea ranged from 65 to 105 kg ha^{-1} (no fixation values for partridge pea are currently reported in literature). In addition, the %Ndfa was generally greatest for partridge pea, followed by red clover, and sun hemp (77, 72, and 61% Ndfa, respectively, averaged over years and locations), suggesting greater *Rhizobium* activity by the native legume host. Similar trends were found for theoretical percent N transfer in that partridge pea resulted in the greatest amount supplied (50% TYN), followed by sun hemp (45%), and then red clover (38%; Table 3.4).

Given the preceding N fixation results and plant densities, and depending on location and year, estimated seeding rates would need to be 15-24, 21-60, and 4-12 kg PLS ha^{-1} for partridge pea, sun hemp, and red clover, respectively, to achieve 67 kg N ha^{-1} contributions in temperate regions (Table 3.3). If achieved, these rates should supply the recommended rate of N for switchgrass. As evidenced by the high seeding rate requirement, sun hemp generally was the lowest N-yielding legume (yield dependent variable) in this study, likely a result of poor establishment and vigor when grown as a N-fixing crop with switchgrass.

Across methods, 2013 N contributions were greater, which was likely attributable to modest N additions from the previous year, which are known to stimulate BNF. Specifically, as decomposition and release of legume N from green tissue during the subsequent growing season results in substantial N contributions. In addition, belowground N contributions from legume to grass may be substantial in that previous studies have stated that belowground legume mass may provide as much as 50% of plant N after decomposition (Peoples and Baldock, 2001; Jorgensen and Ledgard, 1997; McNeill et al., 1997). Mallarino et al. (1990) has shown that theoretical transfer of legume N to grass contributed to only 20 to 40% of plant N early in the seeding year, with more than 60% occurring later in the season and subsequent years for re-seeding annuals.

At the tropical location, N fixation was substantially greater than in the temperate locations for both ^{15}N and N-difference methods (Tables 5 and 6). This was likely due to enhanced plant growth with higher temperatures and longer growing season (Brockwell et al., 1995; Liu et al., 2011). Consequently, based on N-difference results, all legumes would supply enough N for adequate *Panicum* growth when compared to the current recommended rate, even assuming 50% bioavailability. Based on seeding rate calculations, all seeding rates could be reduced to achieve target fixation rates, some substantially so (Table 3.5), likely due to the self-re-seeding habit of some of these species. In addition, the N-difference from legume (%) and the aboveground N yield per plant was also substantially greater (3-5 fold increase) for tropical compared to temperate location. Results suggest that legume N fixation in the tropics can offset substantial amounts of inorganic fertilizer N.

Fixation rates via the ^{15}N enrichment method were generally greatest for partridge pea and lowest for sun hemp in the temperate locations (Table 3.4). Among all legume species at temperate locations; sun hemp performed the poorest, providing less than target levels (67 kg N ha^{-1}). Consequently, sun hemp does not function as a practical N-contributing legume to switchgrass growth, likely due to the overlap in growth of these species. Conversely, at the tropical location, this equatorially adopted species supplied excess N to companion crops (guinea grass and switchgrass; Table 3.5). In addition, potential N transfer, N yield, total N yield (TNY), and amount of N fixed were greater in the tropics due to a longer growing season. Excluding the final fixation measurement (July 2014), switchgrass-sun hemp intercropping resulted in high levels of N fixation (313 and 303 kg N ha^{-1} November 2013 and March 2014, respectively, Table 3.6). Such high performance was likely due to the poor establishment of switchgrass and its photoperiod sensitivity resulting in reduced vegetative growth and consequently, less competitive with sun hemp, thereby allowing strong growth in support of N fixation. Other studies have substantiated the high fixation rates of sun hemp (135 to 285 kg ha^{-1}; Schomberg et al., 2007) and pigeon pea (235 kg ha^{-1}; Peoples et al., 1995). In addition, greatest sun hemp %Ndfa occurred when grown with guinea grass compared to %Ndfa with switchgrass in the tropics (80 vs 60 %Ndfa, respectively); however, %Ndfa of sun hemp was less than that measured in pigeon pea growing with guinea grass (93%; Table 3.6). Conversely, sun hemp had greater theoretical N transfer to guinea grass compared to that of pigeon pea in November 2013 and March 2014, but not in July 2014.

3.3. *Nitrogen-15 enrichment method compared to N-difference for determining BNF of legumes interseeded into switchgrass*

The N-difference technique adequately measured BNF in legume-switchgrass swards when compared with 15N enrichment, considering that when N fixation was averaged across temperate locations, species, and years, means were both near 65 kg N ha^{-1}. Results that the N-difference method can provide comparable estimates to that of more expensive and sophisticated isotope techniques have been supported by others (Høgh-Jensen and Schjoerring, 1994; Phillips et al., 1983; Bell et al., 1994). Some studies have suggested only 30% of total grass N yield was derived from legume transfer (Hardarson et al., 1988; Mallarino et al., 1990). However, results from this study suggest that a value upwards of 40% may be more appropriate in temperate locations (Table 3.4). Data in Tables 3 and 5 illustrate the great amount of variability (SD up to 33% of mean) among microplot fixed-N for the N-difference technique, likely due to the spatial heterogeneity of plant growth and % species composition; with generally less variability occurring for the ^{15}N method. The highest spread of fixation occurred in lower yielding plots [e.g. sun hemp (approx. 40% SD of mean)]. This variability is likely due to the yield dependence of this measurement, as the percentage of legume-supplied N from fixation (i.e., NDFa and potential N-transfer) are yield-independent and had lower variation, thus suggesting main sources of error from estimating legume fixation comes from the variable legume and grass yields. In addition, percent potential N transfer values reported here generally agree with others, in that up to approximately 70% of N supplied to the grass can be derived from BNF (Mallarino et al., 1990).

Correlation results suggest a moderately positive relationship (Pierson correlation coefficient= 0.71; *P*<0.0001) between the N-difference and ^{15}N techniques. In addition, linear regression of ^{15}N enrichment and N-difference techniques indicated adequate N-fixation estimation by the N-difference method; [R^2=0.51; F=54.12 (Fig. 1)]. Generally, when estimated fixation rates were below 75 kg N ha^{-1}, N-difference method tended to overestimate amount of N fixation when using 15N as the standard for accurate estimation of N fixation. However, beyond that threshold the N-difference method tended to equally underestimate and overestimate BNF. Additionally, the majority of observations were higher than the current recommended N application rate (averaging 79 and 72 kg N ha^{-1}, ^{15}N enrichment and N-difference across all locations and years, respectively). Consequently, the less expensive and time consuming method (N-difference) may be used in lieu of the more sophisticated method (^{15}N enrichment) for determining biological N fixation rates. Further verification with more site-years is still needed to confirm the ability of the N-difference method to accurately estimate BNF in switchgrass-legume swards.

4. Conclusions

Elucidating renewable nitrogen contributions from symbiotic fixation is important for understanding N balances in order to maximize sustainable N production in perennial grass-legume systems. The use of both dicot and monocot non-N-fixing reference plants revealed switchgrass assimilates analogous N compared to other reference plants and can therefore function as a non-leguminous reference plant for N-determination techniques used in this study (N-difference and 15-N enrichment).

If 50% bioavailability is not assumed, both red clover and partridge pea fixed more N than the current recommended N rate (67 kg N ha^{-1}) at temperate locations when intercropped in switchgrass (85 and 86 kg N ha^{-1}, respectively, via the ^{15}N enrichment procedure). Potential nitrogen transfer tended to increase with both N fixation measurement techniques during yr-2 of this study (12% increases from yr-1); however, legume density thresholds should be considered, in that companion crop competition may negatively affect N-fixation rates.

Considerable inputs of legume-derived N can be obtained in tropical intercrop systems. Nitrogen yields (via the ^{15}N enrichment method) were lowest for sun hemp in temperate regions (48 kg N ha^{-1}), but greatest between the two legume intercrops in the tropics (321 kg N ha^{-1}). Pigeon pea also had substantial fixation (246 kg N ha^{-1}), exceeding recommended fertilizer-N levels in pasture/feedstock systems. Therefore, tropical environments may promote greater levels of N fixation due to enhanced biological activity, a longer growing season, and higher temperatures.

^{15}N-enrichment tracers have proven capabilities at isolating various N cycling processes and quantifying nitrogen supplied during BNF. One of the main advantages to the ^{15}N- enrichment method is that an estimate of %Ndfa is provided (integrates all changes in %Ndfa during the experimental period). However, when averaged across temperate locations, species, and years the less sophisticated N-difference techniques comparably measured BNF in legume-switchgrass swards compared to the isotopic techniques (65.5 versus 64.9 kg N ha^{-1} for N-difference and ^{15-}N techniques, respectively). Consequently, this method may be used as a surrogate for isotopic ^{15}N-

based BNF estimations; however, choice of measurement technique may depend on study objectives, location, and legume host species.

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APPENDIX

Table 3.1 Soil characterization of baseline [yr1 (2012)] and final year (yr3 (2014)]- results (averaged on a per plot basis) for switchgrass and guinea grass, based on legume intercrop at the East Tennessee Research and Education Center (ETREC) and Research and Education Center at Greeneville (GREC) in TN, and at the St. Croix, USVI, Agricultural Experiment Station (STX). Soil test results from Mehlich-1 extractant and Mehlich-3 extractant at TN and STX, respectively.

Location	**Legume intercrop**	**pH**	**P**	**K**	**Ca**	**Mg**	**NO$_3^-$[*]**	**CEC**
			------------------------kg ha^{-1}----------------------				mg kg^{-1}	Meq 100 g soil^{-1}
ETREC								
1[‡]	RC	5.2	25.7	56.7	1363	200.0	0.5	8.7
	PP	5.1	26.8	48.2	1278	164.0	0.3	8.5
	SH	5.2	30.0	57.0	1317	191.2	0.6	8.7
3	RC	6.0	19.2	35.0	1074	172.5	0.5	6.5
	PP	5.9	17.1	39.5	1113	183.8	0.7	6.8
	SH	5.8	18.8	38.5	1047	168.7	0.5	6.6
GREC								
1	RC	6.5	39.7	90.3	1642	410.3	0.4	9.2
	PP	6.4	39.7	94.2	1690	426.7	0.5	9.1
	SH	6.4	45.0	109.2	1669	424.2	0.5	9.2
3	RC	7.2	43.7	76.83	1688	506.3	0.4	9.3
	PP	7.1	44.8	91.2	1805	525.0	0.5	9.7
	SH	7.0	45.8	89.0	1825	537.2	0.5	9.8
STX								
1-SG[†]	SH	8.1	6.0	303.0	12497	307.7	4.0	52.6
	PiP	7.9	11.0	313.7	12355	307.3	6.0	52.2
2	SH	7.8	7.3	294.0	13631	336.7	3.7	57.3
	PiP	7.5	8.3	317.0	13724	343.7	2.0	57.8
1-GG	SH	8.2	5.3	209.3	14132	228.0	2.3	58.2
	PiP	8.2	7.3	218.7	13393	214.3	2.3	55.2
2	SH	7.9	7.0	179.3	16899	278.7	2.0	69.6
	PiP	7.6	7.7	228.3	18472	278.3	2.0	75.8

[‡] 1= initial sampling period per location; 2 or 3=terminal sampling per location.

[†] companion crops: SG=switchgrass; GG=guinea grass. Intercrops: RC=red clover; PP=partridge pea; SH=sun hemp; PiP= pigeon pea.

[*] PSNT: plant preside-dress nitrogen; CEC: cation exchange capacity.

Table 3.2 Nitrogen fixation rates for common vetch versus non fixing species using the N-Difference method at Plant Science and Holston Units of the East Tennessee Research and Education Center (ETREC) in Knoxville, TN, and hairy vetch using the N-Difference method at Holston Unit of the ETREC in 2010.

Reference Plants	**N from Vetch**	**N from reference plant**	**% N-Difference of vetch**	**Average[†] vetch plant weight**	**Vetch Aboveground N plant^{-1}**
	----------g kg^{-1}----------			----------g----------	
		Plant Science Unit			
Common vetch					
Wheat	24.7	10.1	14.6	9.6	1.4
Wild Barley	24.7	13.1	11.6	9.6	1.1
Geranium spp.	24.7	13.4	11.3	9.6	1.1
Marestail	24.7	14.4	10.3	9.6	1.0
Venus Looking Glass	24.7	11.6	13.1	9.6	1.3
		Holston Unit			
Switchgrass	25.8	12.2	13.6	9.6	1.3
Wild Barley	25.8	8.5	17.3	9.6	1.7
Daisy Fleabane	25.8	9.0	16.8	9.6	1.6
Geranium spp.	25.8	12.1	13.7	9.6	1.3
Marestail	25.8	17.1	8.7	9.6	0.8
Venus Looking Glass	25.8	10.7	16.1	9.6	1.5
Hairy vetch		Holston Unit			
Wheat[‡]	26.0	10.1	15.9	7.8	1.2
Switchgrass	26.0	12.2	13.8	7.8	1.1
Wild Barley	26.0	8.5	17.5	7.8	1.4
Daisy Fleabane	26.0	9.0	17.0	7.8	1.3
Geranium spp.	26.0	12.1	13.9	7.8	1.1
Marestail	26.0	17.1	8.9	7.8	0.7
Venus Looking Glass	26.0	9.7	16.3	7.8	1.3

[†] Means of vetch plant weights across all samples

‡N-difference values using wheat were taken from wheat samples from the Knoxville Plant Science Unit

Table 3.3 Nitrogen fixation rates for partridge pea, sun hemp, and red clover using the N-difference method at the East Tennessee Research and Education Center (ETREC) in Knoxville, TN, and Greeneville Research Education Center (GREC) in Greeneville, TN, in 2012 and 2013.

ETREC

	Above-ground N $plant^{-1}$	% N-difference of legume	Average legume density	Total aboveground legume N	N fixed (±se)	Seeding rate for target N
	g	g kg^{-1}	pl m^{-2}	g m^{-2}	kg ha^{-1}	kg PLS ha^{-1}
2012						
Partridge pea	0.9	5.9	11.7	10.5	52.1± 8.4[†]	23.1
Sun hemp	0.6	8.3	14.7	9.4	43.2±6.2	37.3
Red clover	1.8	22.6	8.3	14.8	76.2±5.4	10.6
2013						
Partridge pea	0.9	6.0	14.6	13.8	68.5 ±7.3	17.6
Sun hemp	1.1	5.9	4.9	5.3	26.2 ±2.0	61.3
Red clover	1.8	11.7	20.9	37.3	185.6±32.2	4.3

GREC

	g	g kg^{-1}	pl m^{-2}	g m^{-2}	kg ha^{-1}	kg PLS ha^{-1}
2012						
Partridge pea	1.1	7.7	8.2	9.5	47.4±13.1	25.4
Sun hemp	0.8	10.5	12.2	10.3	58.8±18.8	27.3
Red clover	1.6	11.9	8.6	13.8	68.6±16.1	11.7
2013						
Partridge pea	0.8	4.3	17.6	15.3	75.6±1.6	15.9
Sun hemp	1.1	8.7	13.6	15.7	78.0±9.9	20.6
Red clover	1.2	22.6	10.7	18.3	92.9±22.2	8.7

[†] Values reported after means represent standard error

Table 3.4 Nitrogen fixation rates for partridge pea, sun hemp, and red clover using the N-15 enrichment method at the East Tennessee Research and Education Center (ETREC) in Knoxville, TN, and Greeneville Research Education Center (GREC) in Greeneville, TN, in 2012 and 2013.

ETREC

	Ndfa[‡]	Potential N transfer	TNY[††]	N yield	N fixed
	%	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
2012					
Partridge pea	64.9 ±27.4[†]	47.3±13.1	51.6±5.1	112.2±14.7[†]	76.0±40.2
Sun hemp	69.0±33.4	40.0±5.1	24.9±1.4	67.0±9.2	18.3±17.9
Red clover	80.3±14.5	30.9±10.1	29.0±12.8	133.1±10.9	95.9±39.8
2013					
Partridge pea	80.15±3.3	56.8±0.7	38.6±17.5	84.6±20.4	65.1±20.9
Sun hemp	37.1±31.3	48.1±8.9	22.7±7.6	48.3±20.1	18.0±15.5
Red clover	70.2 ±13.7	51.6±9.8	59.6±16.0	114.6±9.9	80.7±19.4

GREC

	%	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
2012					
Partridge pea	84.2±4.0	47.1±11.5	27.5±22.2	111.0±30.7	93.1±23.6
Sun hemp	85.6±10.2	54.3±6.8	25.6±20.3	50.2±43.9	40.0±30.5
Red clover	70.9 ±24.4	28.1±5.7	15.7±10.3	103.9±8.4	82.7±9.8
2013					
Partridge pea	78.2±10.3	53.9±10.9	73.2±25.2	133.7±23.1	105.6±29.7
Sun hemp	51.0±15.7	35.9±4.8	23.1±4.0	69.4±12.2	34.2±4.1
Red clover	68.8±6.4	40.5±0.5	44.5±13.7	124.4±8.7	85.9±13.1

[†] Values reported after means represent standard error

[‡] Ndfa=nitrogen derived from atmosphere

[††] TNY= total nitrogen yield

Table 3.5 Nitrogen fixation rates for sun hemp, and pigeon pea intercrops in guinea grass and switchgrass using the N-difference method at the Agricultural Experiment Station, at St. Croix, USVI (STX) during 2013 and 2014.

Harvest date & Intercrop	Above-ground N $plant^{-1}$	% N-difference of legume	Average legume density	Total aboveground legume N	N fixed (±se)	Seeding rate for target N
Switchgrass						
	g	g kg^{-1}	pl m^{-2}	g m^{-2}	kg ha^{-1}	kg PLS ha^{-1}
2013 (Nov.)						
Sun hemp	4.1	20.1	10	40.5	201.4±173.8†	8
2014 (Mar.)						
Sun hemp	3.5	28.1	16	56.5	281.2 ±30.3	6
2014 (Jul.)						
Sun hemp	4.6	22.9	11	50.6	251.9±100.7	6
Guinea grass						
	g	g kg^{-1}	pl m^{-2}	g m^{-2}	kg ha^{-1}	kg PLS ha^{-1}
2013 (Nov.)						
Sun hemp	4.0	20.1	14.3	57.6	286.9±45.9	5
Pigeon Pea	7.7	38.4	5.3	40.9	203.6±40.9	15
2014 (Mar.)						
Sun hemp	4.1	20.2	13.3	53.8	268.0±128.8	6
Pigeon Pea	5.3	26.4	10.3	54.6	271.8±62.7	11
2014 (Jul.)						
Sun Hemp	2.9	14.5	17.6	51.1	254.6±163.2	6
Pigeon Pea	2.9	14.3	11.0	31.6	157.4±26.2	18

† Values reported after means represent standard error.

Table 3.6 Nitrogen fixation rates for sun hemp and pigeon pea intercrops in guinea grass and switchgrass using the N-15 enrichment method at the Agricultural Experiment Station, at St. Croix, USVI, (STX) during 2013, and 2014.

Switchgrass

	Ndfa‡	Potential N transfer	TNY††	N yield	N fixed
	%	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
2013 (Nov.)					
Sun hemp	56.3±27.3[†]	49.7±27.7	156.0±33.6£	313.7±20.4	140.3±89.8
2014 (Mar.)					
Sun hemp	49.3±21.4	30.3±7.1	92.1±31.8	303.9±105.0	146.6±74.8
2014 (Jul.)					
Sun hemp	79.2 ±13.3	50.3±17.1	49.6±13.3	98.7±30.3	80.7±36.1

Guinea grass

	%	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
2013 (Nov.)					
Sun hemp	71.9±11.4	59.5±13.7	54.0±22.2	120.7±37.1	93.1±23.6
Pigeon pea	86.9±8.2	44.1±30.0	60.5±29.8	135.2±43.9	40.0±30.5
2014 (Mar.)					
Sun hemp	94.0±2.8	90.4±8.4	64.4±16.0	71.2±22.5	66.5±19.7
Pigeon pea	96.0±4.3	71.5±27.0	16.9±12.5	23.6±17.7	23.1±5.7
2014 (Jul.)					
Sun hemp	75.5±19.3	49.8±13.4	160.9±70.9	321.3±116.1	243.1±128.9
Pigeon pea	96.0±4.8	65.7±12.3	169.5±75.6	257.9±93.9	246.3±89.3

[†] Values reported after means represent standard error

‡ Ndfa=nitrogen derived from atmosphere

†† TNY= total nitrogen yield

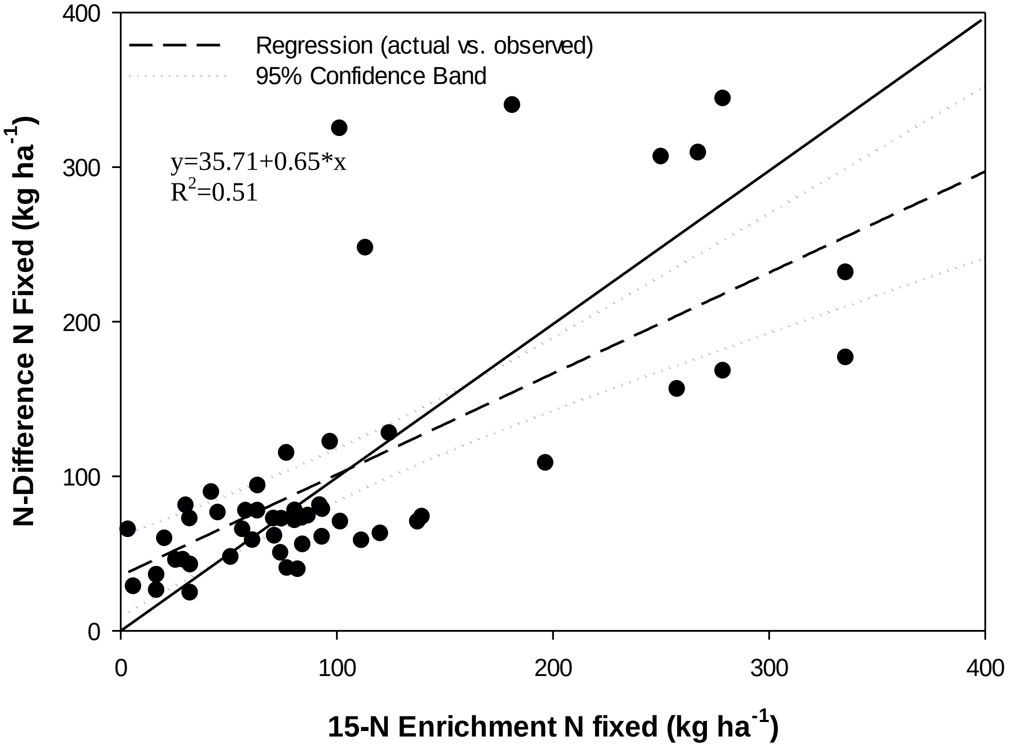

Figure 3.1 Correlation for 15-N enrichment and N-difference techniques for determining mean N fixation rates for partridge pea, sun hemp, red clover, and pigeon pea legumes for 2012 and 2013 at Knoxville and Greeneville, TN, and during 2013 and 2014 at the Agricultural Experiment Station, at St. Croix, USVI. Straight line represents 1:1 line.

CHAPTER IV

Environmental impact assessment of regional switchgrass feedstock production comparing nitrogen input scenarios and legume-intercropping system

Abstract

As the use of second-generation biofuel crops increases, so do questions about sustainability, particularly their potential to affect fossil energy consumption and greenhouse gas emissions. This study used a life-cycle approach to compare environmental impacts associated with three switchgrass (*Panicum virgatum* L.) production scenarios: i) regional production from a pool of Tennessee farmers based on in-field inputs and biomass yield; ii) varying nitrogen (N)-input levels from a replicated field study for 8-yrs i.e., a 100% and 9% decrease, and an 81% and 172% increase from 'baseline levels' of N inputs used under objective i; and, iii) a legume-intercrop system compared to baseline levels in order to determine effects of displacing synthetic-N with legumes. When compared across all agricultural inputs, nitrogen fertilizer production and breakdown resulted in the greatest environmental impacts. Although fertilization increased lignocellulosic yields, a 100% reduction in N-inputs from baseline levels reduced the formation of carbon, methane, and nitrous oxides *per unit of production,* (or dry tonne of biomass over 10-yrs) compared to a 172% increase. Switchgrass yield response indicated a 'less is more' scenario, as inputs beyond the current recommended input level (67 kg N ha^{-1}) are not environmentally remunerating. During switchgrass biomass production, inputs with lesser impacts included phosphorus, herbicides, pesticides, and diesel fuel. Legume-intercropping reduced greenhouse gas emissions and groundwater acidification (5% and 27% reduction in global warming potential and formation of acidifying species, respectively) compared with the 67 kg N ha^{-1}rate. Although N-fertilizers impact environmental sustainability of regional switchgrass feedstock production, environmental consequences can be reduced under proper N-management i.e., ≤67 kg N ha^{-1} or legume intercropping. However, given that the aim of second-generation feedstocks is to reduce the current reliance on fossil fuels, their production still requires fossil energy-based inputs. Consequently, greenhouse gas reductions and the extent of cleaner feedstock production during the agricultural biofuel supply chain is contingent upon input management and optimizing synthetic fertilizer usage.

Keywords

Switchgrass; Life cycle assessment; Nitrogen fertilizer; Legume inter-cropping; Biofuels

Acronyms

Life cycle assessment (LCA); global warming potential (GWP); greenhouse gas (GHG)

1. ***Introduction***

There are growing concerns about the environmental sustainability of feedstocks, specifically input requirements and their relationship to the amount and value of the outputs. One tool for evaluating system environmental sustainability is life-cycle assessment (LCA), which measures inputs to and emissions from production life cycles (Cherubini and Jungmeier, 2010). In addition, as much as 21.3 million ha of existing agricultural land in the U.S. may be converted to perennial grass feedstocks (McLaughlin et al., 2002), thereby making regionally-specific environmental sustainability measurements, such as LCA, critical before large-scale adoption of a second-generation feedstock.

Life-cycle assessments include life cycle inventories and life cycle impact assessments (ISO, 2006). Life-cycle inventory (LCI) is an accounting of the energy and raw material inputs, as well as emissions to air, water, and soil (ISO, 2006). The life cycle impact assessment (LCIA) process characterizes and calculates the effects of emissions identified in the LCI into generalized impact categories. Impact categories at the midpoint level characterize impacts using indicators located along (but before the end of) the mechanism chain [e.g., parameter in a cause-effect network between the inventory data and the category endpoints (Bare et al., 2000; ISO, 2006)]. Examples of midpoint impact categories that are commonly used are global warming potential (GWP), photochemical ozone creation potential [POCP (i.e., smog)], acidification, eutrophication, and ozone depletion (NREL, 2011). Impacts of various production scenarios can be compared and thus LCA can be used to identify options that reduce a system's environmental impacts .

The majority of biofuel-focused LCAs have found a significant reduction in greenhouse gas (GHG) emissions from second-generation feedstocks, such as switchgrass, when compared with their conventional fossil fuel or first-generation counterparts [i.e., corn-(*Zea mays* L.)-ethanol] (Blottnitz and Curran, 2007; Cherubini and Jungmeier, 2010; Kim and Dale, 2005). The Brazilian paradigm for ethanol production [i.e. fermentation of sugarcane (*Saccharurn officinarum* L.)] is reportedly the highest performing first-generation biofuel crop in terms of environmental sustainability, and may exceed that of some second-generation feedstocks (Kendall and Yuan, 2013). However, composited, peer-reviewed LCAs determined that among all fuel pathways expected to contribute substantially to the U.S's renewable fuel portfolio, switchgrass-ethanol offered the greatest reduction in GHG emissions when compared to other fuel pathways [i.e., corn-ethanol, soy-(*Glycine max* L.) based biodiesel, waste/grease biodiesel, and sugarcane -ethanol] (Adler et al., 2007; EPA, 2009). In these analyses, switchgrass-ethanol resulted in a 124% and 128% reduction in GHG emissions (compared to a gasoline baseline) under two scenarios: 30 year, 0% Discount Rate (i.e., values all emission impacts equally, regardless of time of emission impact); and 100 year, 2% Discount Rate (discounts future emissions annually at 2%), respectively (EPA, 2009).

Net energy analyses have been used to evaluate fossil fuel and cellulosic biofuel-energy efficiencies. Reed et al. (2012) constructed a LCI of switchgrass fuel pellets based on surveys of switchgrass farmers and wood pellet producers in the southeastern U.S. and concluded that energy produced in switchgrass pellets was five times greater than the total fossil energy consumed to create them. Schmer et al. (2008) reported that switchgrass biomass yields ranging from 5.2 to 11.1 Mg ha^{-1} resulted in an average estimated net energy yield (NEY; or energy output ha^{-1} minus fossil energy input ha^{-1}) of 60 GJ ha^{-1} yr^{-1} or 6.4 times more renewable energy than the non-renewable energy consumed. This study, as well as previous models [e.g., Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation Model (GREET) and the Energy and Resources Group (ERG) Biofuel Analysis Meta-Model (EBAMM)], assume a linear response of switchgrass yields to agricultural inputs. However, the majority of long-term field research indicates yield response reaches an asymptote before 134 kg nitrogen (N) ha^{-1}, with an inflection point being reached at 67 kg ha^{-1} (Mooney et al., 2009). Other studies have reported negative energy balances for switchgrass-based ethanol under assumptions of high levels of inputs (Pimentel et al., 2008); however, such assumptions are made without regionally measured plant-response values. Data are lacking that document specific environmental impacts from regional on-farm switchgrass production, especially impacts resulting from fertilization. Therefore, by evaluating impacts from varying fertilizer levels, the feasibility of environmentally sustainable feedstock production may be elucidated.

Production of second-generation biofuels may still result in acidification and ozone depletion due to use of synthetic fertilizers (compounds based on N and P) and pesticides (Larson, 2005; Zah et al., 2007). Nitrogen is an essential macronutrient in cropping systems but N emissions from its breakdown and production (e.g. nitrous oxide) are assumedly major contributors to GWP, acidification, and eutrophication in LCA simulations. Therefore, a positive result in one part of the cropping life cycle (i.e., increased crop yield) may be associated with a negative result (i.e. increased acidification) in another. However, there is a range of differences in production patterns and systems, as well as cultural practices, which makes regional and processing differences great (Ruviaro et al., 2012). Furthermore, the majority of LCAs conducted have been based on national or state-wide systems rather than on regional primary (enterprise-level) production data. Such regionally parameterized analyses of feedstock production are needed in order to more accurately model impacts of regional bioethanol production systems, albeit such specificity may preclude wide-scale assertions and recommendations.

A joint venture between the University of Tennessee, and DuPont Danisco Cellulosic Ethanol [i.e., University of Tennessee Biofuels Initiative (UTBI[1])] established the nation’s first pilot-scale, demonstration cellulosic biorefinery for the conversion of switchgrass to ethanol. This biorefinery, located in Vonore, Tennessee could require up to 154 tonnes of switchgrass per day and produce 19 million liters of ethanol per year (DOE, 2007). Since 2010, approximately 2,100 ha of switchgrass have been planted, all of which are located within 80 km of the Vonore plant, making this the nation’s first and largest switchgrass cellulosic ethanol venture. The study reported herein used: i) farm production-level field data from the UTBI project for modeling energetic efficiencies of switchgrass production, ii) an 8-yr, four sites field-study of four different N-fertilization rates on switchgrass biomass yield for a sensitivity analysis, and iii) a 4-yr replicated field study of intercropping legumes with switchgrass for biomass production as a replacement for the ‘baseline N fertilization rate’ used in objective i. Specifically, study objectives were to: i) identify the potential environmental impacts associated with farm-derived regional switchgrass production based on in-field agricultural input levels and biomass tonnage from a regional pool of farmers with the UTBI; ii) quantify environmental impacts as a function of decreasing or increasing N input levels. [i.e., a 100% and 9% decrease, and a 81% and 172% increase] compared to the baseline level of N input under objective i; and, iii) compare legume-

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[1] http://www.tennessee.edu/media/kits/biorefinery/docs/utbis_overview.pdf

intercrop systems to baseline results in order to determine the effects of displacing synthetic-N with intercropped legumes that host N-fixation.

2. *Methods*

This study applied LCA principles to evaluate environmental impacts of switchgrass grown under various scenarios. Cradle-to-gate LCI was developed (Reed, 2012) and LCIA used to compare production scenarios. This report is not however intended to present a complete LCA in full compliance with the ISO standards.

The agricultural production processes for both conventional and intercrop systems (Fig. 4.1, A1) include the basic flows and inputs in this analysis of farm-level switchgrass feedstock production. The 'baseline switchgrass production system' (A1) consists of the boundary assumed under objectives i and ii. The second production process is listed as 'legume-intercrop production system' in Figure 4.1 (A1). The industrial conversion and processing system (A2) were outside the model boundary.

Assumptions for basic flow processes (depicted in Fig. 4.1) assumed that during harvesting, biomass is cut and field cured (<20% moisture). Drying beyond this point is considered an externality in this cradle to farm-gate LCA, as scenarios would likely not vary. Furthermore, this study did not consider storage facility or machinery construction. Soil carbon fluxes for all scenarios were also considered outside the system boundary. Finally, as fertilization increases both yields and impacts, the net impact per unit, or functional unit, allows for standardization. Therefore, this LCIA used 1 tonne (or 1 Mg) of dry weight switchgrass biomass as the functional unit (and is the standard unit in commerce).

Life cycle impact assessment was conducted using the Tool for the Reduction and Assessment of Chemical and other environmental Impacts (TRACI 2.0) model to determine midpoint indicators (Bare et al., 2003; USDA, 2013). TRACI is a LCIA program developed by the U.S. Environmental Protection Agency (EPA) specifically for the U.S. that uses input parameters for nine regions in the U.S. (Bare et al., 2003; EPA, 2002). All mid-point impact categories were included that were provided and supported by TRACI and SimaPro LCA software. Specifically, the environmental mid-point impact categories of global warming potential (kg CO2-eq), acidification potential (H^+ moles-equivalent per kg of emission deposited), carcinogens (kg benzene-eq.), non-carcinogens (kg toluene-eq.), respiratory effects [particulate matter (PM 2.5-eq)], eutrophication potential (kg N-eq.), ozone depletion [chlorofluorocarbons (kg CFC-11-eq.)], ecotoxicity (2, 4-D-eq.), POCP (kg NOx-eq.), and GWP (kg CO2-eq) were examined in this study. A range of environmental parameters were analyzed and described below.

Eutrophication characterization factors take into account the potential release of chemicals containing N or P into air or water per kilogram of chemical released, relative to 1 kg chemical discharged directly to surface freshwater (Bare et al., 2003). Acidification aims to predict impacts resulting from excess H^+ ions in soil and water systems based on deposition in watersheds (Bare et al., 2003). The POCP midpoint category characterizes formation of ozone molecules in the troposphere and their resulting impacts on ecosystem and human health. An additional impact category reported is ozone depletion, which is the reduction of protective ozone in the stratosphere, generally caused by ozone reducing pollutants (e.g., CFCs, and halons); such reductions may result in greater ultraviolet radiation reaching earth’s surface (Bare et al., 2003). In addition, GWP was examined or the potential change in earth's climate from a buildup of greenhouse gasses based on the gas heat retention capacity. In this study, the 100-year time horizon was used rather than GWP 500, which gives greater weight to longer-lived gases, as recommended by the Intergovernmental Panel on Climate Change (IPCC) and used by the U.S. EPA for policy-making.

2.1. *Regional baseline switchgrass production scenario*

Localized preliminary life cycle inventory (LCI) data were based on research from the UTBI (Reed, 2012). The LCI in-field data were derived from a survey sent to 61 participating producers (19% response rate or 12 respondents) in the southeastern region of Tennessee and provided input [e.g. diesel fuel (to operate machinery used during life cycle), seed, herbicide, fertilizer, etc.], and switchgrass yield data for 2008, 2009, and 2010 (Table 1). Means from this survey established baseline yield and N-fertilization levels. Because some inputs (e.g., herbicide) decrease after perennial stand establishment, and yields increase, data were weight-averaged across surveyed farms to make up a ten-year life cycle period (assumed stand rotation). Switchgrass cv. 'Alamo' seeding rate (only for yr-1) was 7.8 kg pure live seed (PLS) ha^{-1}. Per standard production protocols, no fertilization occurred during year one, as perennial bunchgrasses are establishing during this time and fertilization promotes weed growth and interspecific competition. Therefore, 2008 [stand establishment] data were weighted once, 2009 once and 2010 eight times because switchgrass yields reach an asymptote in year three, (attaining only 33 to 66% of its maximum yield potential in years one and two, respectively), with very little yield variation thereafter due to physiological maturity; Mooney et al., 2009).

Farmer-derived agricultural input survey data (Table 1) for the inventory (LCI) collection was modeled with SimaPro LCA software (7th edition), which calculated the overall cradle-to-gate emissions associated with production processes (Fig. 4.1), by using a network of related inventories associated with regional inputs (Reed, 2012). Input data used in this study represent a ‘moderate’ scenario and are consistent with other energy consumption values for switchgrass biomass production (EUBIA, 2007; Pimentel and Patzek, 2005), and were collected in accordance with the Consortium for Research on Renewable Industrial Materials (CORRIM) research guidelines for life cycle inventories (CORRIM, 2001). Surveyed agricultural inputs included fertilizer e.g., N [urea (CH_4N_2O)], phosphorus [triple superphosphate ($CaH_4P_2O_8$) (K)] and potassium [muriate of potash (KCl) (K)], diesel fuel, herbicide, surfactant, seed, and machinery for transportation and application of the aforementioned inputs. Other upstream processes in the reference system not explicitly examined in this research (e.g., equipment-use hours, electricity, diesel, and production of fertilizer, seed, herbicide, and surfactant, as well as GHG emissions emitted during these processes) were taken from the US LCI Database in SimaPro (Pré Consultants SimaPro, 2012; USDA, 2013). Producer yields were determined by biorefinery managers and University personnel, as switchgrass producers were paid on a per-tonne basis.

2.2. *Switchgrass feedstock production under nitrogen input sensitivities*

Because of the direct and indirect effects on the environment associated with fertilizing, sensitivity analyses were conducted to quantify environmental impacts at various input levels based on a replicated, 8-yr small plot study on switchgrass N-input response data [e.g., a range of levels from low to a maximum deemed high enough to have surpassed switchgrass yield response; input values are considered typical nutrient response levels for this species (McLaughlin et al., 2002)]. Switchgrass cv. ‘Alamo’ nutrient response data used in the inventory analysis (objective ii) were collected by Mooney et al. (2009) at four different field sites at the University of Tennessee Research and Education Center at Milan (RECM; Table 2) for an 8-yr period (i.e. 2004-2011). The four sites were chosen to represent a range of soil types and landscape positions including two Grenada silt loam sites (well drained, level upland), a Vicksburg silt loam (a well-to moderately well-drained flood plain), and a Collins silt loam (a moderately-to somewhat poorly drained eroded sloping upland) (Mooney et al., 2009). Biomass yield data were collected from each site each year following the first-killing frost and weighted as described for objective i. In order to compile a 10-yr simulation period, yield data were averaged from 2006-2011 to make up the final 2 life-cycle years. No fertilizer was applied to any treatments at any site during yr-1 (e.g. establishment year). Starting in yr-2 the annual fertilizer treatments (yr-2 – yr-10) in this study were 0 (Sensitivity #1), 67 (Sensitivity #2; 60.5 kg N ha^{-1} yr^{-1} avg. over 10 yr; Table 2), 134 (Sensitivity #3; 121 kg N ha^{-1} yr^{-1}avg. over 10 yr), and 202 kg N ha^{-1} (Sensitivity #4; 181.5 kg N ha^{-1} yr^{-1}avg. over 10 yr) in the form of urea. From the results of this study, UT formulated the recommended rate of 67 kg N ha^{-1} yr^{-1} for switchgrass biomass production in Tennessee. Irrigation was not utilized in any field experiment and therefore, not included in any of the models, consequently data may not represent yield during years with extreme rainfall. Given the C4 photosynthetic pathway of this species, it is highly drought tolerant and therefore, irrigation is not needed for this crop in this region. In-field input data pooled and averaged from the 12 UTBI switchgrass farmer respondents (as described in section

2.1, or 66.6 kg ha^{-1} averaged over a 10-yr production cycle; Table 2) served as the moderate-N or baseline scenario. Minimal yield fluctuation (±10%) occurred within each sensitivity model. Each sensitivity data set (8-yr yield data) was averaged per input level (Sensitivity #1-4). Life cycle simulations for the sensitivity analysis based on the aforementioned agricultural input/output data were run separately and compared to baseline level results for objective i. Sensitivity #2 (i.e. 67 kg N ha^{-1} per year after the establishment year or 60.5 kg N ha^{-1} avg. for the complete 10-yr life cycle) yielded 1.45 Mg ha^{-1} more dry matter compared to baseline results (66.6 kg N ha^{-1}) likely due to robustness of data across climatic and phenotypic variation (i.e., 3 versus 8 data collection years). This default N-level used under objective i. was then compared to N-input sensitivities 1-4 i.e., a 100% and 9% decrease, and an 81% and 172% increase in order to monitor environmental impacts of agronomic inputs on a regional switchgrass biomass production basis.

2.3. *Environmental assessment of switchgrass-legume intercropping scenario*

Due to interest in N-input alternatives, a third scenario, or legume-intercropping was included. For the legume [i.e., red clover (*Trifolium pratense*)] intercrop system, several assumptions were made based on measured regional in-field data (Ashworth et al., 2012; Warwick, 2011). For example, based on local field studies by authors, every four years post-establishment, red clover requires re-seeding due to lack of persistence, and density being below proper levels (<4 plants per m^2; Warwick, 2011) for targeted N fixation (67 kg N ha^{-1}; biological nitrogen fixation determined via the 15-N enrichment method, data not shown). Consequently, for a 10-year simulation period, legume planting would occur two and a half times. Further, based on red clover-switchgrass intercropping studies, at proper legume densities switchgrass biomass yield does not vary from the 67 kg N ha^{-1} application (Ashworth et al., 2012). Consequently, the legume simulation assumed yield did not differ from the baseline scenario (objective i) when red clover was seeded at 13.4 kg ha^{-1} PLS. As such, input assumptions were as follows: legume seed and diesel for a single planting were multiplied by 2.5; a 0% yield reduction compared to LCA in objective i; a 13.4 kg ha^{-1} PLS legume seeding rate; same P inputs were required under objective i; and, 0 kg N ha^{-1} were needed over the entire simulation period (Ashworth et al., 2012). Other published clover inventory data with the SimaPro database (i.e. Ecoinvent v2.0; Jungbluth et al., 2007) were used for the upstream simulation of legume cropping systems under objective iii.

3. ***Results and Discussion***

Figures illustrating the contributions to the total environmental impact *per unit of production* (Figures 4.2 and 4.3) show the relative contribution rather than the importance of impacts [i.e. a small input can make a large contribution to a category that has a low total impact (such as grass seed input impacts for ozone depletion)]. The following figures show contribution analyses for the cradle-to-grave impacts of each system and have not been externally normalized (importance of impact category relative to external reference).

3.1. *Environmental assessment of regional baseline switchgrass production*

Greater than 50% of the total impact of inputs on regional agricultural switchgrass production inputs came from N and P fertilizer, diesel fuel, and glyphosate herbicide (Fig. 4.2).

Of those inputs, nitrogen created the greatest deleterious impact on regional switchgrass production when compared across all impact categories, particularly respiratory effects, acidification, and global warming. This is largely due to the upstream manufacture of synthetic nitrogen fertilizer (i.e., Haber-Bosh) being energy-intensive, as breaking the trivalent bond of nitrogen ($N \equiv N$) requires high pressure (100-200 atm), temperature (400-500°C), and energy (8,000 kcal kg^{-1} N) (USDA, 2008). In this process, nitrogen gas is combined with hydrogen to form ammonia (NH_3), and hydrogen in this reaction primarily comes from natural gas, one of the largest fuel inputs during this process. Current ammonia and urea production requires approximately 35 and 38 GJ per metric ton of N, respectively, in modern nitrogen plants (USDA, 2008). In addition, P and N inputs greatly influenced the eutrophication impact category (Fig. 4.2) by accelerating algal and aquatic weed growth and subsequent O_2 limitations due to their decomposition, as P and N-limited aquatic systems respond strongly to minor nutrient increases. Such impacts from fertilization have been observed by others that have assessed environmental impacts from farmer-level inventories (Bojacá et al., 2014). Additionally, diesel inputs into switchgrass systems impacted POCP and ecotoxicity (approximately 50% of total impact per impact category) due to emission release to the air, water, and soil. Glyphosate production and utilization greatly impacted ozone depletion (i.e. 47% of total impact). Upstream processing and on farm utilization of both 2, 4-D and surfactant contributed less than 2.6 and 0.5% across all mid-point categories surveyed per unit of production, respectively. Production processes associated with grass seed inputs (e.g., largely upstream impact of diesel for harvesting, planting, and transport) resulted in ca. 20% of total ozone depletion relative to other inputs (Fig. 4.2).

3.2. *Switchgrass feedstock production compared under nitrogen input sensitivities*

Relative environmental impacts from N-input sensitivities compared to the UTBI Farmers (i.e. internally normalized to the baseline scenario) indicate multiple trade-offs on total cradle-to-farm gate impact categories (Fig. 4.3). In the figure, the various sensitivities were internally normalized (i.e. impacts for each sensitivity are expressed as a percentage of the highest-impact sensitivity).

Sensitivity #4 or the 181 kg N ha^{-1} rate (172% N increase relative to baseline level) resulted in the greatest (55% greater impacts relative to baseline scenario) acidification, carcinogenic, and eutrophication effects on a per-tonne basis, followed by Sensitivity #3 compared across all N-input levels relative to the baseline scenario (Table 2). Eutrophication reductions from the zero fertilization rate, compared to fertilized scenarios was also observed in previous studies (Börjesson and Tufvesson, 2011). As was expected based on the relative large impact of N fertilizer, Sensitivity analysis #1 (or a 100% decrease from baseline levels of 67 kg N ha^{-1}) resulted in lowering the impacts across all categories relative to baseline except ecotoxicity, ozone depletion, and slightly positive impacts for smog production (+1.8 kg NOx-eq.) and non carcinogenics (+0.8 kg toluene-eq.); thus indicating N inputs result in greater yields (up to a point) but with disproportionate increases in environmental impacts. Consequently, inputs beyond the 67 kg N ha^{-1} level (Sensitivity #2), and perhaps even the 0 kg N ha^{-1} level (Sensitivity #1) may result in diminishing returns in terms of yield response and deleterious environmental impacts. Therefore, the current recommended rate of 67 kg N ha^{-1} is corroborated by environmental and agronomic efficiencies.

3.3. *Environmental assessment of switchgrass-legume intercropping scenario*

Results for the legume-intercropping life cycle assessment indicate a substantial portion of environmental disturbance from diesel fuel, phosphorus, and seed inputs on impact categories tested (Fig. 4.4). Namely, diesel inputs resulted in greater than 50% of the total impacts for acidification, non-carcinogenics, ecotoxicity, and smog; whereas under the baseline scenario, diesel was 20-51% less of a total constituent for the aforementioned mid-point categories, due to fossil-N contributing a greater portion of the total impact. Additional midpoint categories such as ozone depletion and GWP were affected by upstream seed inputs (approximately 60% and 42% of total impact, respectively). One likely explanation for seed input impacts were the inoculant coating and pre-treatment of legume seeds with water adsorbing polymers and limestone or rock phosphate that combat unfavorable soil and ambient conditions. Phosphorus inputs resulted in the greatest eutrophication constituent, likely due to P-limited algal systems and the resulting impacts of algal bloom proliferation under minor P increases, albeit phosphorus is not an overall large contribution in other impact categories and production systems at-large. Herbicide-related inputs (e.g., 2, 4-D, glyphosate, and surfactant) resulted in minor constituents [≤14%) excluding glyphosate for ozone depletion (i.e. 35%)] of total impact for legume-intercropping systems (Fig. 4.4). Herbicide inputs for the legume intercropping simulation differed slightly compared to baseline results, as ozone depletion impacts from glyphosate decreased due to greater legume seed proportional impacts; whereas, impacts from glyphosate were less for ecotoxicity due to greater diesel fuel requirements for legume planting. Also, relative to farmers with the UTBI, legume-intercropping resulted in favorable impacts on acidification, carcinogens, and eutrophication, due to fewer N inputs and therefore less oxide of nitrogen emissions. However, non-carcinogens, respiratory effects, ozone depletion, ecotoxicity, and smog were greater under this system, likely because of greater diesel (upstream and downstream impacts) and legume seed (red clover) preparation and processing (mainly upstream impacts) during the 10-yr life cycle.

3.4. *Climate forcing from regional baseline, nitrogen input, and switchgrass-legume intercropping scenarios*

Global warming potential (kg CO2-eq) is among the most widely studied categories due to interest in climate change, renewable fuels, carbon credits and carbon sequestration. A 0 kg ha^{-1} of N (Sensitivity #1) rate over the 10-yr life cycle resulted in the least production of greenhouse gas emissions on a per-tonnage biomass basis over its life cycle compared across all analyzed scenarios (Fig. 4.5). Sensitivity #3 (or an 81% increase in N-inputs) resulted in an 18% increase in GWP compared to UTBI, because yields were not greatly increased from the additional N (Fig. 4.5). Even though increasing fertilization generally increases crop production up to a point, the greatest N-rate input level (Sensitivity #4; or a 172% increase from baseline levels) had the highest formation of carbon, methane, and nitrous oxide on a per-tonne basis. Further, breakdown of synthetic-N in soils involves formation of N_2O and consequently, this input level resulted in the greatest GWP (e.g. a 39 fold increase in N_2O from sensitivity #1 to sensitivity #4). Secondly, the legume-intercropping simulation resulted in fewer stratosphere-warming gases, albeit only slightly less than the Sensitivity #2 (or the current recommended N-rate for Tennessee), because the legume-intercropping resulted in a substantial portion of CO_2-equivalents from diesel fuel, P, and seed inputs. Further, diesel emissions (during planting and harvesting processes) and legume seed (including coating) was a major carbon-source for the legume-intercropping scenario compared to baseline results. Therefore, based on results, lower input levels resulted in a more carbon-neutral end-product on a per-tonne biomass basis.

Since nitrogen dioxide formation impacts the greenhouse effect and the formation of H^+ ions, results from the GWP and aquatic acidification potential followed similar trends (data not shown). Formations of acidification species (i.e., sulfur and nitrogen oxides, hydrochloric and hydrofluoric acid, and ammonia) were greatest for the 202 kg N ha^{-1} rate. Conversely, the zero and current recommended nitrogen input rate had reduced acidification of groundwater potential on a per-tonnage basis. Furthermore, the legume-intercrop system caused less acid deposition than the UTBI simulation and the current recommended N rate (reductions of 54% and 27%, respectively).

4. ***Conclusions***

The need for improved biofuel crop yields on a dwindling landmass due to population pressures has created conflicting solutions for environmental sustainability, considering nitrogen fertilization promotes enhanced biomass production, but is a carbon-positive input. Therefore the environmental sustainability of raw feedstock production is contingent upon agricultural production practices, and reducing the conventional reliance on fossil fuels in these systems can corroborate cleaner production. Environmental consequences still exist for switchgrass production, but could be augmented with management practices that sustain crop yields (e.g. sustainable nutrient management and legume intercropping), thus increasing greenhouse gas savings.

When compared across all inputs, the fossil-carbon-intensive process of producing nitrogen fertilizers resulted in the greatest deleterious impacts on all regional switchgrass production scenarios, resulting in greater respiratory effects, acidification, and global warming potential. This is due to the energy-intensive industrial N_2 fixation process. Across all midpoint categories, there was a positive feedback when external inputs were reduced on a per-unit of biomass production basis. However there are still environmental consequences associated with low input ($\leq$67 kg N ha^{-1}) switchgrass production systems, albeit likely still fewer consequences than first-generation feedstocks such as corn (which requires upwards of 168-250 kg N ha^{-1}) for ethanol production (Kendall and Yuan, 2013), or fossil fuels such as diesel or gasoline (Bai et al., 2010). Consequently, there are potentially significant benefits offered by using switchgrass (with either the current recommended inorganic-N level, or legume intercropping nutrient regimes) as a bio-feedstock, particularly in terms of GHG emissions. However, these benefits may be offset by impacts in other categories such as respiratory effects, ecotoxicity, and photochemical creation, which have also been observed in other assessments (Cherubini and Jungmeier, 2009). Consequently, management precision of switchgrass and other lignocellulosic crops requires consideration, and reduction of agricultural inputs could be pathways for advanced environmental impact reduction. Moreover, life cycle assessments of additional second-generation feedstocks are needed at the farm-scale to target biofuel crops with enhanced environmental performance in efforts to reduce the reliance on fossil fuel derived agricultural inputs.

Awareness of trade-offs, the diversity of cultural and processing practices, and sensitivities and assumptions are important in LCA models, and should be considered by policy makers.

When compared across all N-input sensitivity scenarios, increasing fertilization in switchgrass feedstock production systems was less benign to human and environmental health on a per-tonne of feedstock production basis. Sensitivity #1 (no N fertilization) resulted in the lowest negative impacts across all categories, indicating that ‘less is more’ in terms of the environmental sustainability of switchgrass as a biofuel feedstock. However this was affected by switchgrass’ minimal N-response beyond a modest threshold. Despite this result, Sensitivity #1 input level is not recommended due to the potential for long-term soil-nutrient depletion, likely resulting in reduced soil fertility and even yield loss over-time (>10 yrs. or beyond the life cycle scope of this study). Based on inventoried field data, plant productivity began to reach a plateau (beyond Sensitivity #2) indicating increases in fertilizer inputs are not economically remunerating beyond the 67 kg N ha^{-1} nutrient input level for plant response (Mooney et al., 2009). This suggests a point of diminishing returns beyond this rate in terms of switchgrass N response and subsequent environmental impacts. Therefore, from an environmental and plant performance standpoint, sustainability of switchgrass feedstock production can be improved under proper N-management (i.e., ≤67 kg N ha^{-1}; current recommended rate) or legume intercropping (lower potential GWP, carcinogenics, eutrophication, and acidification) at the farm-gate level. However economic assessments of legume intercropping to determine breakeven points of this management practices are needed for switchgrass biofuel cropping systems to ascertain economic feasibility.

Data from this study can be included in regional databases for others interested in conducting LCAs, net energy ratios, or modeling feedstock systems. Producer surveys may allow for greater data representation and resolution of systems being modeled, however, there may be a tradeoff with lower sample sizes and lack of precision, as well as the inference space in which results may apply. Consequently, both wide-scale and localized environmental assessments of second-generation feedstocks are imperative going forward. Results herein provide regional guidelines for producing feedstocks sustainably, and may be used to identify potential improvements for production efficiency. Such data are needed to substantiate the claim that cellulosic-feedstock production systems are low-input and therefore environmentally sustainable.

Acknowledgements

Special thanks go to the USDA-NIFA Southeastern Region Sun Grant Program for funding the preliminary LCI on wood and switchgrass pellet production in the Southeast that was used in this project. We also extend our gratitude to the UTBI-contracted switchgrass farmers for their cooperation in providing vital in-field data.

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APPENDIX

Table 4.1 Cradle-to-(farm) gate life cycle inventory inputs per year per tonne of dry matter of switchgrass in the southeast. Input values are averaged over a 10-year production cycle (adopted from [Reed et al., 2012]).

Inputs	**Units**	**Average value**[a]	**Coefficient of variation**[b]
Diesel (tractor use)	L	3.98	46%
Nitrogen (fertilizer)	kg	4.77	84%
Phosphorous (fertilizer)	kg	0.49	224%
2, 4-D (pesticide)	L	0.05	219%
Glyphosate (herbicide)	L	0.05	213%
Surfactant	L	0.03	332%
Seed[c]	kg	0.56	n/a

[a] Average value is the weighted average over a 10-year switchgrass stand rotation, where inputs and yields are assumed to be constant in years 3-10.

[b] Coefficient of variation is standard deviation/average of the reported data, without any weighting for stand age.

[c] Seed input values were not reported by farmers, however, a 7.84 kg ha^{-1} PLS rate was assumed once over a 10- yr life cycle.

Table 4.2 Measured annual switchgrass dry matter yield (± standard error) for sensitivity analyses (1-4) averaged across N-inputs for 10 yrs. of production at University of Tennessee Research and Education Center (REC) locations (adopted from (Mooney et al., 2009), at producer farms in conjunction with the University of Tennessee Biofuels Initiative (UTBI; Reed et al., 2012) consisting of the baseline scenario, and REC centers for the legume intercropping scenario (Ashworth et al., 2012; Warwick, 2011) **.**

Scenario	**N-input**	**Relative to baseline**	**Yield**	**Data source**
	----kg a^{-1}----		----Mg ha^{-1}---	
Sensitivity[a] # 1	0	-100%	5.68 ± 1.9	REC
Sensitivity # 2	60.5	-9%	8.43 ± 2.6	REC
Sensitivity # 3	121.0	+81%	9.18 ± 2.9	REC
Sensitivity # 4	181.5	+172%	9.83 ± 3.1	REC
Baseline level	66.6	0%	6.98 ± 2.9	UTBI
Legume intercropping[b]	0	-100%	6.98 ± 2.9	REC

[a] Nitrogen input scenario sensitivities 1, 2, 3 and 4 (or 0, 67, 134, and 202 kg N ha^{-1}, respectively, with 0 fertilizer in yr-1 for all scenarios) and baseline N-levels weight-averaged over 10 years of production (yr-1 received 0 kg ha^{-1} N for all sensitivity scenarios) and divided by yield data for 10-yrs in order for functional unit standardization.

[b] Legume intercropping scenario assumed: a 0 kg N ha^{-1} rate during the life cycle, yield was consistent with baseline results, seeding occurred every four years (2.5 times during a 10-yr life cycle) at 13.4 kg ha^{-1} PLS, and diesel inputs per planting period.

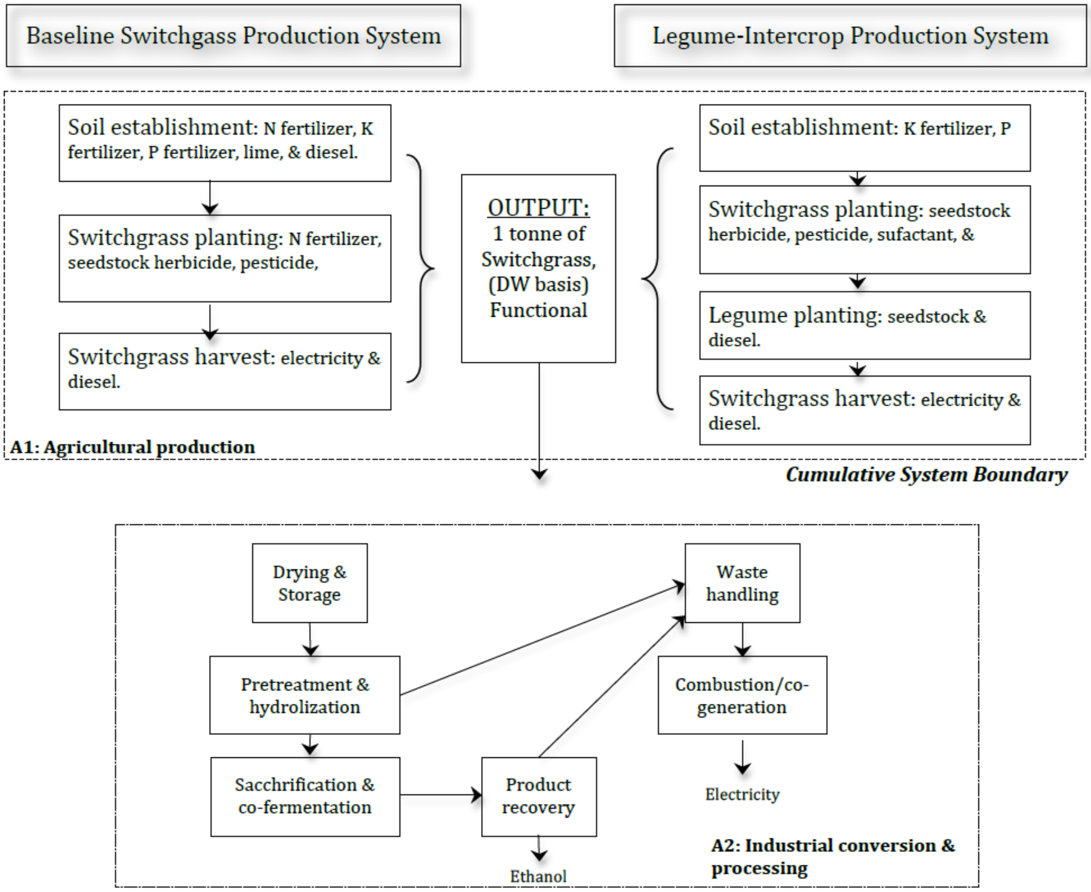

Figure 4.1 Life cycle analysis system boundary, including both the agricultural production (A1) phases and the industrial conversion and processing (A2) steps.

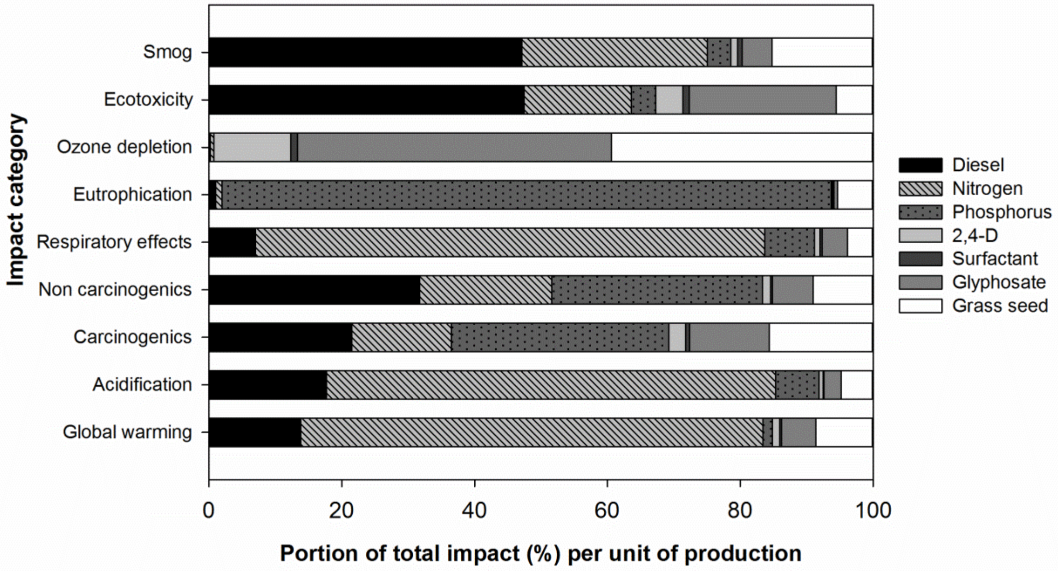

Figure 4.2 Impact categories and their relative impact proportion per Mg of harvestable biomass based on system inputs for baseline production (UTBI switchgrass farmers, over a 10 yr. simulation period).

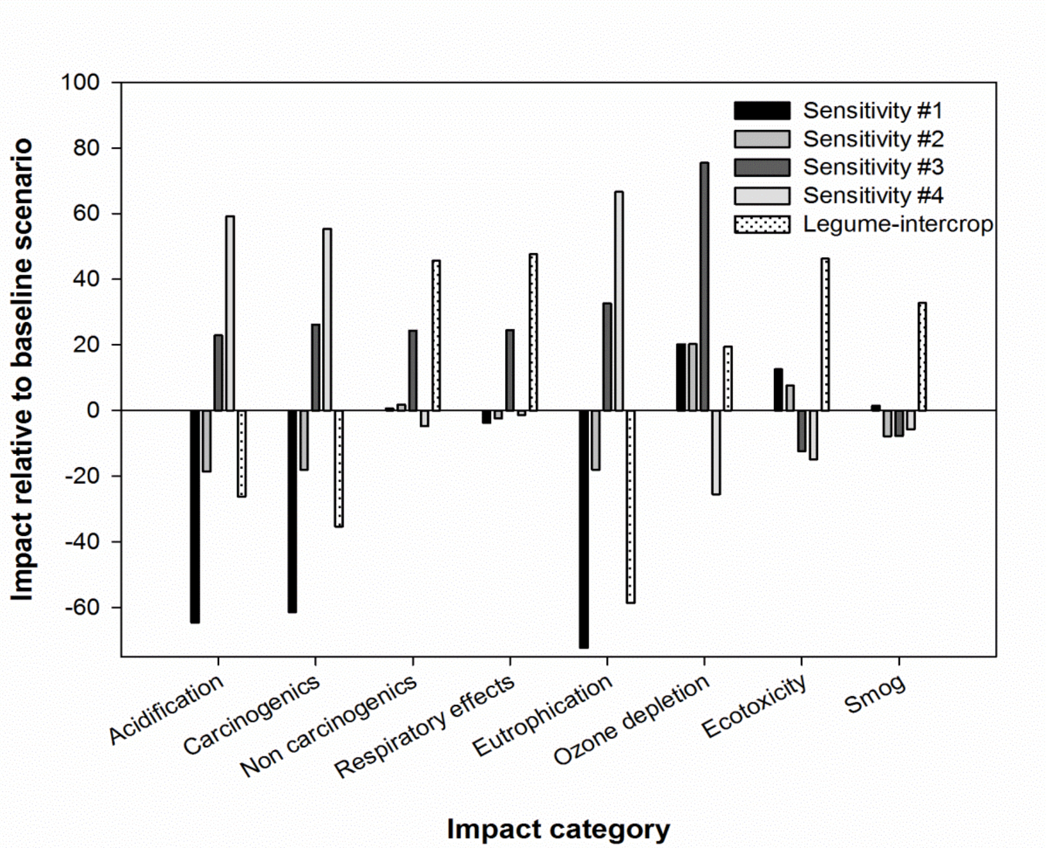

Figure 4.3 Impact categories and their relative impact proportion for nitrogen input sensitivities 1, 2, 3 and 4 (or 0, 67, 134, and 201 kg N ha^{-1}, respectively), legume intercrop, and baseline production scenarios from farmers with the University of Tennessee Biofuels Initiative (based on 10 yr simulation period and per Mg of harvestable biomass). A negative relative impact indicates a positive impact.

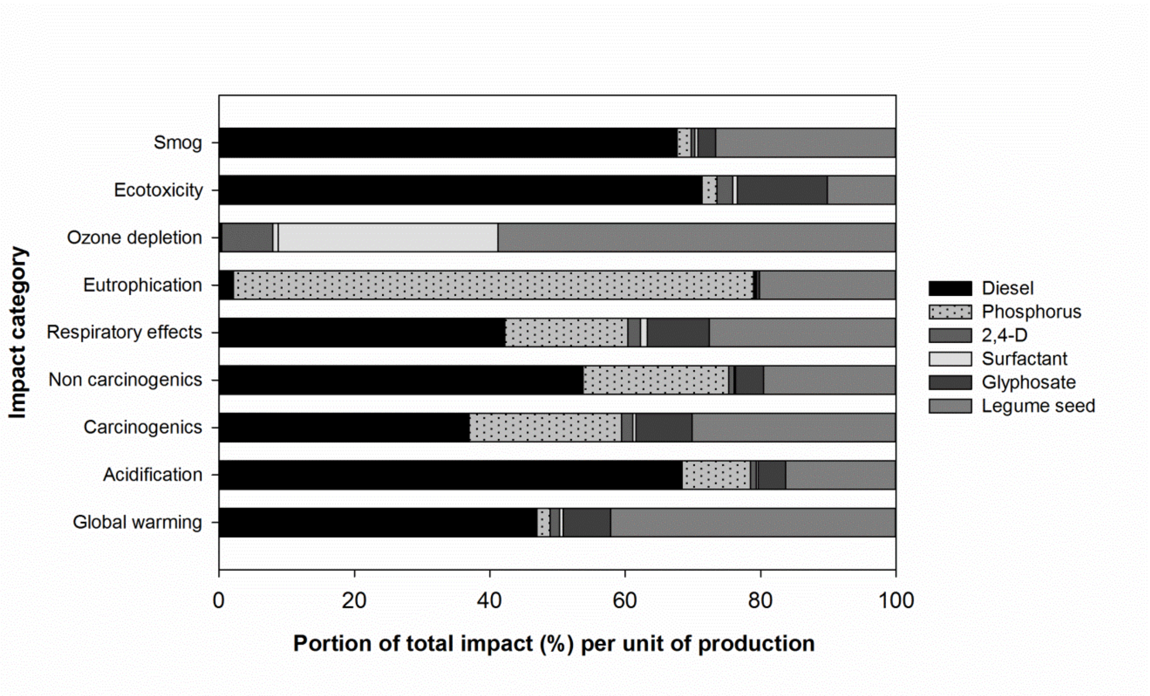

Figure 4.4 Impact categories and their relative proportion of impact per Mg of harvestable biomass based on system inputs for legume-intercropping in switchgrass feedstock production systems in the Southeast U.S. (over a 10-yr simulation period). Seed inputs include both grass seed and legume seed.

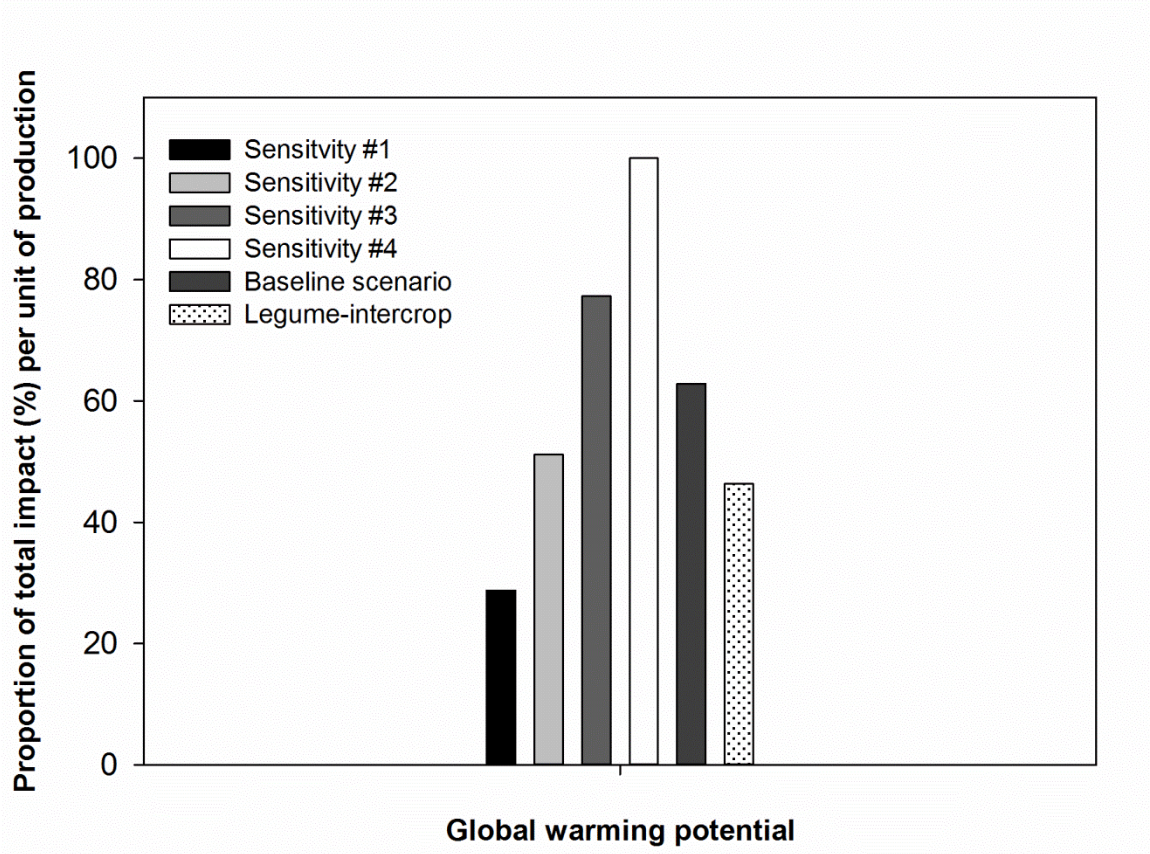

Figure 4.5 Global warming potential (kg CO2-equivalents) of switchgrass production over a range of input levels (0, 67, 134, and 202 kg N ha-1, Sensitivity #1-4, respectively), regional production from area growers, and a legume-intercropping scenario [internally normalized (divided by largest GWP score for all scenarios) and over a 10-yr simulation period and per Mg of harvestable biomass].

CONCLUSION

Switchgrass is a perennial, drought-tolerant biofuel feedstock with high yield and carbon (C) sequestration potential on soils considered marginally economical for row crops, thereby mitigating any direct food vs. fuel issues. However, such second-generation feedstocks have been touted as ecological alternatives to fossil fuels although they still require non-renewable energy for their production. Consequently, from an environmental and plant performance standpoint, sustainability of switchgrass feedstock production can be improved under proper nitrogen (N)-management (i.e., $\leq$67 kg N ha^{-1}; current recommended rate) or legume intercropping (lower potential GWP, carcinogenics, eutrophication, and acidification) at the farm-gate level.

A sustainable management practice for temperate growers could be to delay harvests until after maturity during the subsequent winter months to allow further moisture loss in the standing crop for optimum moisture storage conditions and feedstock alteration, depending on conversion technology. Further, forage quality (switchgrass only) in some cases was positively influenced by legume treatments, notably hairy vetch, red clover, and partridge pea ($P<0.05$). Nutrient (P & K) translocation, ethanol yield, hemicellulose, and in-field dry down was maximized by delaying harvests in temperate locations where senescence occurs. Such harvest delays may allow for greater presence of digestible fibers and fermentable sugars (5 and 6 carbon), due to in-field breakdown of waxy cuticles that inhibit sugar availability. However, biomass yield losses were observed (22%) when harvests were delayed due to weathering, thereby lowering ethanol yield on a per-biomass tonnage basis. Consequently, desired feedstock characteristics can be manipulated by harvest management, however, a trade off with yield reductions must be considered.

Varying forage and biomass yield response occurred when legumes were interseeded into switchgrass. Post-senescence harvests were impacted by soil amendments in that the 0 kg N ha^{-1} rate (as well as sun hemp and arrowleaf clover intercrops) resulted in the lowest biomass yields, whereas the low biochar rate (1 Mg ha^{-1}) was not different than the 67 kg N ha^{-1} rate, nor was it different than partridge pea, red clover, the high biochar rate (2 Mg ha^{-1}) soil amendments. Further, there is some indication that the second harvest year after legume establishment may result in more positive legume-intercropping effects (e.g. greater carry over N-rates, yield, and forage quality effects).

Switchgrass adaptation based on the SGWI indicated varying weed coverage results (5-30%) across all harvest periods, likely due to switchgrass photoperiod sensitivity, establishment success, and lack of biochemical adaptations. Consequently, results from the tropical location indicate that switchgrass may be produced under intensified climatic changes due to this species drought tolerance, genetic diversity, and competitive growth habit post-establishment on marginal soils. To be produced in the tropics, more aggressive weed measures and photoperiod insensitive varieties would be required.

There are a multitude of potential benefits from introducing legumes into pasture and monoculture biofuel systems in the humid east, including reduced fertilizer inputs, increased soil carbon additions from green manure, reduced weed pressure due to niche differentiation, and reduced leaching potential of soil nitrate to groundwater. Switchgrass pastures and biofuel swards can be interseeded successfully with cool-season legumes (ladino and red clovers) and the warm-season legume, partridge pea, without re-seeding (≥3 yrs; depending on soil texture, soil fertility, and rainfall). In addition, considering neither legume density nor persistence was impacted by seeding rate, the lower seeding rates could therefore be used for proper legume establishment.

The use of both dicot and monocot non-N-fixing reference plants revealed switchgrass assimilates analogous N compared to other reference plants and can therefore function as a reference plant for N-determination techniques used in this study (N-difference and 15-N enrichment). Both red clover and partridge pea fixed greater amounts of N than the current recommended N rate (67 kg N ha^{-1}) at temperate locations when intercropped in switchgrass (85 and 86 kg N ha^{-1}, respectively, via the ^{15}N enrichment procedure), albeit only about 50% of the fixed amount is bioavailable. Considerably greater inputs of biologically derived-N can be obtained in tropical intercrop systems, likely due to diazatropic species being less abundant and less adapted in temperate Tennessee compared to those in tropical systems. Therefore, tropical soils may promote greater levels of fixation due to enhanced biological activity, and tropical legumes may be more adept at hosting such symbioses. However, such high levels of N-fixation could be anticipated in the southeast under an intensified climate. In addition, N-difference techniques adequately measured fixation in legume-switchgrass swards compared to isotopic techniques (i.e., when averaged across temperate locations, species, and years means were 65.5 and 64.9 kg N ha^{-1} for N-difference and $^{15-}$N techniques, respectively). Consequently, this method may be used as a surrogate for indirect isotopic ^{15}N dinitrogen fixation estimations.

When compared across all inputs in a regional life cycle assessment, the fossil-carbon-intensive process of producing nitrogen fertilizers resulted in the greatest deleterious impacts on

East TN regional switchgrass production. Although fertilization increased lignocellulosic yields, a 100% reduction in N-inputs from baseline levels predicted reduced formations of carbon, methane, and nitrous oxides *per unit of production*, (or dry tonne of biomass over 10-yrs) compared to a 172% increase. Switchgrass yield response indicated a 'less is more' scenario, as inputs beyond the current recommended input level (67 kg N ha^{-1}) are not environmentally and economically remunerating. During simulated switchgrass biomass production, inputs with lesser impacts included phosphorus, herbicides, pesticides, and diesel fuel. Although N-fertilizers impact environmental sustainability of regional switchgrass feedstock production, environmental consequences can be reduced under proper N-management i.e., ≤67 kg N ha^{-1} or legume intercropping. Consequently, greenhouse gas reductions and the extent of cleaner feedstock production during the agricultural biofuel supply chain are contingent upon reducing synthetic fertilizer usage.

VITA

Amanda is a native of Fayetteville, AR and grew up in the Ozark Mountains. She had the fortune to attend the University of Arkansas for an undergraduate degree in Environmental, Soil, and Water Sciences. During her time as an undergraduate she was a recipient of two Americorp Education Awards through her completion of two Student Conservation Association internships. One internship included mapping invasive-plant populations in Montana with the USDA Forest Service, and the second internship was with the Bureau of Land Management in California working on safeguarding native dry-land species against extinction. Also during her undergraduate tenure she spent one year attending the University of Granada, Spain, through Academic Programs International, where she completed minors in Spanish and International studies of Agriculture. Pre-Masters degree she was also an intern for the Renewable Resources and Clean Technology International Program at the National Polytechnic Institute of France, as she received an Integral Valorisation of Bio-production EU-US Consortium Grant to conduct a life cycle assessment. These internships and educational experiences were the seeds that grew into personal satisfaction regarding her professional path, and propelled her to seek fulfillment through research and learning.

Ms. Ashworth received a Masters of Science in Agronomy from the University of Arkansas Crop, Soil and Environmental Science Department in 2010, where her research focused on switchgrass biomass production and nutrient removal as a bioenergy feedstock. Thereafter she relocated to the University of the Virgin Islands Agricultural Experiment Station where she conducted agronomy research on cover crops and dry-land forage systems. In the fall of 2010, Ms. Ashworth joined the staff as a Research Associate at the Center for Native Grasslands Management in the Department of Forestry, Wildlife and Fisheries at the University of Tennessee-Knoxville where her research has been focused on enhancing the establishment and management of native warm-season grasses in the Mid-south. During this time, she had the privilege making major contributions to over five multi-disciplinary, multi-institutional proposals totaling over $1.5 million. In the summer of 2014, she joined the Variety Test Program as a Research Associate housed in the Plant Sciences Department at the University of Tennessee. Amanda initiated the doctorate degree in Plant Sciences at the University of Tennessee January, 2011 and is expected to finish December of 2014.