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N₂ Fixation of Common and Hairy Vetches when Intercropped into Switchgrass

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Academic Editor: Bertrand Hirel

Received: 22 February 2017; Accepted: 6 June 2017; Published: 8 June 2017

Abstract: Interest in sustainable alternatives to synthetic nitrogen (N) for switchgrass (*Panicum virgatum* L.) forage and bioenergy production, such as biological N₂ fixation (BNF) via legume-intercropping, continues to increase. The objectives were to: (i) test physical and chemical scarification techniques (10 total) for common vetch (*Vicia sativa* L.); (ii) assess whether switchgrass yield is increased by BNF under optimum seed dormancy suppression methods; and (iii) determine BNF rates of common and hairy vetch (*Vicia villosa* L.) via the N-difference method. Results indicate that chemical scarification (sulfuric acid) and mechanical pretreatment (0.7 kg of pressure for one minute) improve common vetch germination by 60% and 50%, respectively, relative to controls. Under optimum scarification methods, BNF was 59.3 and 43.3 kg·N·ha⁻¹ when seeded at 7 kg pure live seed ha⁻¹ for common and hairy vetch, respectively. However, at this seeding rate, switchgrass yields were not affected by BNF ($p > 0.05$). Based on BNF rates and plant density estimates, seeding rates of 8 and 10 kg pure live seed (PLS) ha⁻¹ for common and hairy vetch, respectively, would be required to obtain plant densities sufficient for BNF at the current recommended rate of 67 kg·N·ha⁻¹ for switchgrass biomass production in the Southeastern U.S.

Keywords: biological nitrogen fixation; legume intercropping; biomass sustainability; N-difference method

1. Introduction

Legumes are agronomically beneficial because they fix atmospheric nitrogen (N₂) through a symbiotic relationship with *Rhizobia* bacteria, which form nodules in leguminous roots. These beneficial bacteria enhance soil fertility by increasing N through rhizodeposition, which reduces the amount of synthetic N fertilizer needed for switchgrass growth [1]. However, biological N₂ fixation (BNF) can be affected by weather, inorganic-N present in soils, as well as legume vigor [2,3]. Furthermore, the decay of legumes may not be in synchrony with peak N demand by the main crop [4], and matching legumes with companion crops can be challenging [5]. Annual switchgrass yields average 15.9 Mg·ha⁻¹ in

the upper Southeast [6], with only modest responses to greater N fertilization [7]. Consequently, switchgrass N fertilization is recommended at an annual rate of 67 kg·ha⁻¹ [8], or approximately half the rate for corn (*Zea mays* L.) [9].

Legumes interseeded into switchgrass may fix N required for biomass production [10]. Experiments with legume-switchgrass mixtures (e.g., red clover (*Trifolium pretense* L.)) reported yields that exceed those of N-only, even at inorganic-N rates of 240 kg·ha⁻¹ [11]. Similarly, common and hairy vetch are reportedly effective at increasing soil N and can fix N₂ required for a single biomass-cut system [12,13]. Specifically, common and hairy vetches have been reported to fix between 50 and 350 kg·N·ha⁻¹ and 25 and 190 kg·N·ha⁻¹, respectively, in aboveground growth [14–17]. Common and hairy vetches are cool-season legumes, and as such, peak photosynthesis and subsequent fixation occur from winter until switchgrass' spring green-up. There are several potential advantages of using common vetch in lieu of hairy vetch. Common vetch is frequently found growing throughout the Southeastern U.S. [18] and typically has fewer hard seeds than most varieties of hairy vetch [19,20]. Hairy vetch hard seeds can range between 5 and 30%, last 5+ years in the soil and be a noxious weed [20–22].

Myriad methods are used to determine BNF, including acetylene reduction and hydrogen evolution [2,3]. These techniques must be performed in a controlled environment and are therefore unsuitable for quantifying N₂ fixation of field-grown legumes [23,24]. On the other hand, ¹⁵N isotope dilution, ¹⁵N natural abundance, N-balance and N-difference methods all are suitable for in situ experiments; however, each technique has inherent advantages and disadvantages. The N-difference method estimates amounts of N supplied from symbiosis by comparing N₂-fixing legumes to neighboring non-fixing reference plants. This method is simple and inexpensive and works best under low soil-N conditions [25,26]. The disadvantages are that the N-difference method assumes that legumes and non-fixing plants exploit equal amounts of soil N [2,27] and that plant sizes and/or root morphologies do not differ [28,29]. However, estimates obtained by the N-difference method are comparable to those from more expensive techniques [30,31].

Proper seeding rates for interseeding legumes into lowland switchgrass stands are not well defined. Rates used for previous studies with upland switchgrass have been for frost-seeding into grass pastures, and a reduction of rates has been recommended [32]. Therefore, legume seeding rates need to be developed to establish persistent legume stands that increase N availability without inducing spatial and resource competition with switchgrass. Consequently, legume symbiotic relationships and their interaction with the soil environment were assessed via a comparison of switchgrass dry matter yields to help determine the effectiveness of N₂-fixation by legume hosts. The specific objectives of this study were to: (i) determine the efficacy of physical and chemical seed scarification for common vetch germination; (ii) determine whether or not switchgrass yields are increased by vetch intercrops; and (iii) determine N-fixation rates of common and hairy vetch via the N-difference method in switchgrass production systems.

2. Materials and Methods

2.1. Switchgrass Stands and Site Descriptions

Switchgrass cv. Alamo was planted in spring 2007 at 9 kg·ha⁻¹ pure live seed (PLS) at three field sites, two at the East Tennessee Research and Education Center (ETREC): the Plant Sciences Unit [(ETREC-PS (35°8' N, 83°9' W)] and the Holston Unit [(ETREC-H (35.53° N 83.57° W)], as well as at the Plateau Research and Education Center (PREC), Grasslands Unit in Crossville, TN (36.1° N 85.8° W). Soils at the Plant Sciences Unit are classified as a Huntington silt loam (fine-silty, mixed, active, mesic Fluventic Hapludolls), and soils at the Holston Unit are classified as a Huntington silt loam (fine-silty, mixed, active, mesic Fluventic Hapludolls). The Plant Sciences Unit has a 30-year mean annual temperature of 14.4 °C, with average precipitation of 1240 mm. Soil PREC is classified as a Lily silt loam (fine-loamy, siliceous, semi-active, mesic Typic Hapludults), with 30-year average annual precipitation of 1400 mm and an average temperature of 12.6 °C. Switchgrass plots had no soil amendments applied during this study.

2.2. Nitrogen Fixation of Legume Intercrops

Nitrogen content of common and hairy vetch plants at ETREC-Plant Sciences Unit were compared to monocots [wheat (*Triticum* spp.) and switchgrass]. The authors previously found that switchgrass and wheat assimilate soil-N similar to other commonly-used non-N₂ fixing reference plants and therefore are adequate reference plants for the N-difference method [33]. N₂ fixation of vetches was determined by using the N-difference method. Sample shoots of common vetch, hairy vetch and non-N₂-fixing reference plants wheat and switchgrass were gathered by cutting plants flush to the soil with pruning shears in late spring 2010. Sample tissue-N (grass separated from legumes) was then analyzed with near-infrared reflectance spectroscopy (NIR) using a LabSpec[®] Pro Spectrometer (Analytical Spectral Devices, Boulder, CO, USA) by Land O'Lakes/Sure-Tech (Indianapolis, IN, USA). Equations were standardized and checked for accuracy using grass hay and alfalfa (*Medicago sativa* L.) equations (for each legume of interest), which were developed by the Near Infrared Spectroscopy (NIRS) Forage and Feed Consortium (NIRSC, Hillsboro, WI, USA).

Plant aboveground-N was determined by multiplying plant dry matter (DM) by its percent N content [Equation (1)]. Reference plant N yield (non-nodulating species) was then subtracted from legume plant N yield to obtain the amount of legume fixed N on a per ha basis (Equation (2); [25]). The N-difference between vetches and reference plants was multiplied by average plant weights of legume plants sampled in late spring to obtain the aboveground-N per vetch plant. Total aboveground legume-N mass was determined by legume-N (mass per plant) × plant density (plants·m⁻²) and expressed as kg·ha⁻¹ to determine fixed N accumulated in legume and potentially available to switchgrass (value assuming complete bioavailability).

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

$$\text{N-difference (N}_2\text{ fixed)} = [\text{legume N mass (g}\cdot\text{kg}^{-1})] - \text{reference plant N mass (g}\cdot\text{kg}^{-1}) \quad (2)$$

Estimated seeding rates required for common and hairy vetch to fix the recommended rate of 67 kg ha⁻¹ N fertilizer were obtained from N₂-fixation rates determined by the N-difference method in this study. Specifically, total vetch aboveground plant N·m⁻² was calculated by multiplying the average vetch density (planted at a seeding rate of 7 kg·PLS·ha⁻¹) by the aboveground, per plant vetch N and divided by 50% (assuming half is bioavailable, [34,35]). To calculate the seeding rate required for vetch to supply 67 kg·N·ha⁻¹ to the companion crop, target N level was divided by bioavailable vetch N, thus developing a ratio to multiply the current seeding rate that would give the suggested seeding rates of common and hairy vetch (Equation (3)).

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

2.3. Legume Seed Treatment and Establishment Techniques

Common and hairy vetches were seeded in fall 2009 into established (3-year-old) Alamo switchgrass stands at the two locations. Legumes were seeded into approximately 20-cm-tall switchgrass stubble on 22 and 29 October 2009 at PREC and ETREC, respectively, with a Hege[™] plot drill (Colwich, KS) at a planting depth ranging from 0.6 to 1.3 cm. At ETREC and PREC, plot sizes were 7.6 × 1.5 m and 7.6 and 1.8 m, respectively, with 18 cm-wide row spacing. Seeding rates for both common and hairy vetch were 7 kg·PLS·ha⁻¹, and the control was represented by a 0 kg·N·ha⁻¹ rate. Seeding rates of common and hairy vetch were lowered from the pure stand rates of 34 kg·ha⁻¹ used for forage [36], thus reducing competition with switchgrass early in the season.

Legume seed treatments were tested for germination efficacy in a one-factor (scarification treatment method) completely randomized design. Seeds used for common vetch plantings 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. Seeds collected from the Plant Science Unit were divided into two lots. Lot 1 was dried at room temperature (approximately 25 °C), and Lot 2

was dried at 49 °C in a batch oven (Wisconsin Oven Corporation, East Troy, WI, USA). Seeds collected from the Holston unit were dried at room temperature (Lot 3). The three seed lots were treated for dormancy by dry cold stratification in a cooler at an average of 8 °C for 1 to 6 weeks, plus a control treatment (7 treatments), resulting in 21 treatments (including controls). The stratified vetch was then seeded into sand trays in a greenhouse for germination assessment.

Common vetch seeds from the Holston Unit (Lot 3) were treated for dormancy by physical and chemical scarification with 10 different treatments. Treatments included a control; physical scarification with 100-grit sandpaper (0.5 kg for 30 s, 0.5 kg for 1 min, 0.7 kg for 30 s, 0.7 kg for 1 min, 0.9 kg for 30 s, 0.9 kg for 1 min); treatment with 3% bleach (sodium hypochlorite) for 10 min, treatment with 98% sulfuric acid (H₂SO₄) for 1 min; and treatment with 1% hydrogen peroxide (H₂O₂) for 24 h. Physical scarification was applied with sandpaper attached to wood boards while using a weigh scale to ensure that target pressure was applied.

Scarified common vetch seeds were seeded into sand trays in a greenhouse for germination testing. Because the sulfuric acid seed treatment resulted in the greatest germination rate among all chemical seed scarification methods (Table 1), remaining common vetch seeds (Lots 1, 2, and 3) were treated with sulfuric acid (98% for 1 min), rinsed for 15 min, force-air-dried for 10 min and direct-seeded into switchgrass plots.

In early-June, a frequency grid [37] was used to measure legume stand densities on switchgrass plots interseeded with vetches. Four density counts were taken in each legume treatment plot. Plant densities were averaged from three replications at each location to determine legume density (m²). The count was multiplied by 0.4 according to [37] based on the likelihood of one plant per cell to estimate plant density per m² and averaged over three blocks at each location. Switchgrass height was measured per frequency grid observation, with an average height calculated for each plot.

Table 1. Physical and chemical seed scarification methods, treatments and average germination rates (number and %) of common vetch seed.

Scarification Method	Treatment ^b	Mean ^a	%
Control	Air dried seed (Holston)	8 cde	16
Sandpaper ^c	0.5 kg for 30 s	10 bcd	20
Sandpaper	0.5 kg for 1 min	9 cde	17
Sandpaper	0.7 kg for 30 s	7 cde	14
Sandpaper	0.7 kg for 1 min	16 ab	31
Sandpaper	0.9 kg for 30 s	12 bc	23
Sandpaper	0.9 kg for 1 min	10 bcd	20
Chlorine Bleach	3% sodium hypochlorite/10 min	4 de	7
Sulfuric Acid	98% H ₂ SO ₄ /1 min	20 a	40
Hydrogen Peroxide	1% H ₂ O ₂ /24 h	2 e	4

^a Mean separations based on Tukey's test followed by the same letter are not significantly different at $p < 0.05$ level; ^b treatments had three replications consisting of 17 air-dried seeds, each from Holston Unit seed collection;

^c 100 grit sandpaper.

2.4. Switchgrass Yield Measurements

Two harvest systems were tested in a two-factor (harvest system and N source) randomized complete block design to determine how canopy removal affects legume intercrop vigor and included a single, post-dormancy harvest at ETREC on 8 November 2010 and a two-cut harvest system at PREC on 9 June 2010 (early-boot stage) and 21 October 2010 (post-dormancy). Switchgrass plots were harvested using a Carter™ plot harvester (Brookston, IN, USA). The harvested plot area was 0.9 × 7.6 m, and the cutting height was 20 cm. Grab samples (1 to 2 kg) of switchgrass were collected from all plots at harvest and were weighed, dried in a batch oven at 49 °C and re-weighed to determine moisture content.

2.5. Soil Tests

Preliminary (prior to experimentation) soil nutrient levels were quantified on a per-plot basis for both locations to a 0 to 15 cm depth to determine nutrient concentrations of P, K, Mg and Ca. Samples were ground to pass through a 1-mm sieve on a Wiley soil crusher (Thomas Scientific, Swedesboro, NJ, USA), and Mehlich-1 extractable nutrients were measured by inductively-coupled plasma (ICP) using a 7300 ICP-OES DV (Perkin-Elmer, Waltham, MA, USA), respectively.

2.6. Data Analysis

Switchgrass yields and common vetch seed germination following chemical and physical scarification treatments were analyzed using PROC Mixed with SAS v. 9.1.3 [38]. Tukey's honestly significant difference test was used to determine differences in switchgrass yields and seed germination rates at an alpha level of 0.05. Fixed effects were legume and seed treatments, and locations and replications were assigned as random effects.

3. Results and Discussion

3.1. Seed Treatment

Cold stratification and scarification were assessed for their abilities to break the dormancy of common vetch. Stratification by chilling seeds for one to six weeks did not greatly induce vetch germination (Table 2). Germination rates of oven-dried seed were $\leq 4\%$, while germination of air-dried seed averaged 7% (Holston Unit collection, Lot 3) and 6% (Plant Science Unit, Lots 1 and 2), indicating adverse effects from high temperatures and subsequent seed desiccation. Cold stratifying (8 °C and at 41% relative humidity) did not increase common vetch germination. Other studies involving seed chilling at constant temperatures have not shown accelerated legume seed germination, but increased germination in spring has been achieved by moderate winter temperatures [39]. Similarly, hairy vetch germination has improved when subjected to warmer temperatures [40].

Table 2. Average ^a seed germination rates (number and %) of dry and cold stratification treatments of common vetch seed.

Treatments ^b	Holston Unit		Plant Science Unit			
	Air-Dried		Air-Dried		Oven-Dried (49 °C)	
	Means ^a	%	Means	%	Means	%
1 Week	1.5	6	2.5	10	0	0
2 Weeks	3.5	14	1.5	6	1	4
3 Weeks	2.5	10	2.0	8	1	4
4 Weeks	2.0	8	1.0	4	0.5	2
5 Weeks	1.0	4	1.0	4	0	0
6 Weeks	0.5	2	1.5	6	0.5	2
Control ^c	2.5	10	1.5	6	1	4

^a Means across treatments and replications; ^b treatments used 25 seeds for each of two replications from three different seed collections [Holston Unit and Plant Science Unit (oven- and air-dried)] and were chilled at 8 °C with 41% humidity; ^c air-dried and control seed were stored at ambient temperature that averaged 24 °C with 58% humidity.

Conversely, mechanical and chemical seed scarification treatments did result in differences in germination (Table 1). Sulfuric acid (40% germination) and sandpaper treatments (0.7 kg of pressure for one minute (31% germination)) resulted in germination that exceeded that of the control (8%). Consequently, stronger solutions or longer soaking times may be required for the hydrogen peroxide and bleach treatments to become effective. Hydrogen peroxide, bleach and physical scarification with sandpaper are safer alternatives to sulfuric acid and should be considered further. Similarly,

Larson, J.A. et al. [4] found that sulfuric acid treatments of 15 and 30 min produced 100% germination rates of vetch seed.

3.2. Nitrogen Fixation

Nitrogen fixation rates of common and hairy vetches were similar at 59.3 and 55.2 kg·N·ha⁻¹ based on a plant density of 8.5 plants·m⁻² and 43.3 and 37.6 kg·N·ha⁻¹ based on 7.0 plants·m⁻² (using wheat and switchgrass as non-fixing reference plants, respectively; Table 3); which was less than the current recommended N rate of 67 kg·ha⁻¹ for switchgrass [7]. In previous studies, hairy vetch supplied 90 to 150 kg·N·ha⁻¹ to subsequent crops [41–44]. Common vetch also has the potential to supply 106 to 146 kg·N·ha⁻¹ to subsequent crops [41,43]. Given the preceding N₂-fixation rates and plant densities, estimated seeding rates of common and hairy vetch should be 8 and 10 kg·PLS·ha⁻¹, respectively, to achieve 67 kg·N·ha⁻¹ contribution. If achieved, these rates should supply the recommended N rate for switchgrass biomass production [7].

Table 3. Estimated seeding rates for common and hairy vetch to obtain the recommended rate of N fertilizer for switchgrass using the N-difference method to calculate N₂-fixation rates of common and hairy vetch at the East Tennessee Research and Education Center.

Reference Plant	Vetch Aboveground N·plant ⁻¹	Observed Average Vetch Density ^a	Total Aboveground Vetch N	Bioavailable Vetch N	Target N	Vetch Seeding Rate to Supply 67 kg·N·ha ⁻¹ to Switchgrass
	g	m ⁻²	g m ⁻²	kg·ha ⁻¹		kg·PLS·ha ⁻¹
Common Vetch						
Wheat	1.4	8.5	11.9	59.3	67	7.6
Switchgrass	1.3	8.5	11.0	55.2	67	8.2
Hairy Vetch						
Wheat	1.2	7.0	8.7	43.3	67	10.4
Switchgrass	1.1	7.0	7.5	37.6	67	12.0

^a Common vetch plant density was averaged from both East Tennessee Research and Education Center (ETREC) and Plateau Research and Education Center (PREC) locations in 2010. Hairy vetch plant density was taken from ETREC due to no seedling emergence at PREC. Both common and hairy vetch densities were obtained with seeding rates of 7 kg·ha⁻¹.

3.3. Legume Establishment

Recommended seeding dates for cool-season legumes are early fall or the last two weeks in February through the end of March [36]. In this study, legumes could not be planted into uncut, mature switchgrass stands and, thus, were not seeded until late fall following switchgrass biomass harvest. Late seeding, combined with harsh weather conditions, may have led to late germination, and thus, small seedlings that were not winter-hardy had reduced survival. Establishment of common and hairy vetch for seeding rates of 7 kg·ha⁻¹ at ETREC averaged 10 and 7 plants·m⁻², respectively, as measured on 12 and 13 May. At PREC, legume densities were 7 and 0 plants·m⁻², respectively, on 25 May 2010 (Table 4). In general, common vetch had greater mass than that of hairy vetch across both locations (Table 4).

Lower densities of vetch seedlings at PREC could have been caused by low soil nutrient levels, considering that P (phosphorus) is an essential element for legume nodulation. Levels of soil P and potassium were considerably lower at PREC than at ETREC (Table 5). Successful incorporation of legumes into switchgrass stands will require a soil test and amending nutrient and pH levels before planting. Therefore, the reduced vetch fixation rates observed in this study could, in part, be due to low soil test P levels and the late planting of legume seed.

Table 4. Average ^a common and hairy vetch legume (LG) plant densities ^b and heights and switchgrass (SG) plant heights at ETREC and PREC in 2010.

Location	Common Vetch			Hairy Vetch			Control		
	Plant Density ^b	Height		Weight	Plant Density	Height		Weight	Height
		LG	SG	LG		LG	SG	LG	
	No. m ⁻²	cm		g	No. m ⁻²	cm		g	cm
ETREC	10	59	119	9.9	7	54	84	7.5	110
PREC	7	43	86	9.6	0	4	109	7.8	87

^a Means across treatments and replications; ^b plant density = [(frequency of occurrence × 0.4) × 100] (Vogel and Masters, 2001).

Table 5. Soil nutrient levels of phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in common and hairy vetch plots at East TN (ETREC) and Plateau (PREC) Research and Education Centers (determined via Mechich-1 extractant).

Nutrient	ETREC			PREC		
	Common Vetch	Hairy Vetch	Control	Common Vetch	Hairy Vetch	Control
	kg·ha ⁻¹					
P	86	117	63	6	7	9
K	170	102	218	86	81	127
Ca	3375	3408	3019	2439	2374	1276
Mg	437	482	427	205	182	72

3.4. Switchgrass Yield Impacts

Switchgrass yields did not vary among treatments or number of harvests after only one year when compared to the 0 N control (Table 6); considering that common vetch (13 Mg·ha⁻¹), hairy vetch (12.4 Mg·ha⁻¹) and grass-only yields (10.7 Mg·ha⁻¹) were not different ($p > 0.05$). Neither common nor hairy vetch seeds were inoculated prior to planting, although the presence of legume nodules following establishment indicated that *Rhizobia* were present in soils; however, concentrations prior to seeding were not determined. When seeding common or hairy vetches into established stands of switchgrass, it is advisable to inoculate seeds with appropriate species of *Rhizobia* prior to planting to ensure nodulation and effective legume stands to achieve target levels of N₂-fixation. In cases of low *Rhizobia* populations in soils and/or no inoculation when seeding, it may take two to three years to naturally develop proper soil *Rhizobia* levels for vetch to fix the N required to elevate switchgrass yield.

Based on the results herein, interseeding switchgrass with inoculated common or hairy vetch seed at rates of 8 and 10 kg·PLS·ha⁻¹, respectively, is expected to illicit a greater switchgrass yield response (Table 3). These estimated seeding rates are predicted to be necessary to achieve the recommended rate of 67 kg·N·ha⁻¹ for switchgrass.

Table 6. Average ^a dry matter yields of switchgrass per common vetch or hairy vetch treatment from a one-cut biomass harvest system at ETREC and a two-cut forage/biomass harvest system at PREC in 2010.

Treatment	ETREC		PREC		All Locations
	Biomass	Forage	Biomass	F + B ^b	Both Harvests
	Mg·ha ⁻¹				
Common Vetch	15.9 a ^a	3.8 a	6.2 a	10.0 a	13.0 a
Hairy Vetch	12.7 a	3.5 a	5.1 a	8.6 a	12.4 a
Control	11.6 a	3.3 a	5.4 a	8.7 a	10.7 a

^a Mean separations based on Tukey's test at $p < 0.05$ applied to individual columns across treatments; ^b summation of forage and biomass yields.

4. Conclusions

Under proper seeding rates and P-management, common and hairy vetches could potentially be viable alternatives for offsetting inorganic-N fertilizer inputs for switchgrass production. Common vetch germination can be increased through a sulfuric acid pretreatment before seeding, but such pretreatment may be unsafe and cost-prohibitive. A cost-effective alternative to breaking seed dormancy and increasing vetch seed germination is mechanical scarification (i.e., 100 grit sandpaper at 0.7 kg of pressure for one minute) or via a mechanical drum for large-scale systems.

Relatively similar aboveground N₂-fixation rates of common and hairy vetch plants (59.3 and 43.3 kg·N·ha⁻¹, respectively) were measured in this study. Both common and hairy vetch can theoretically supply 67 kg·N·ha⁻¹, the recommended rate of N fertilizer for switchgrass, if sufficient plant densities are achieved and adequate *Rhizobia* populations are present. Based on the results reported herein, it is estimated that switchgrass yield will increase beyond the control with common or hairy vetch seeded at rates of 8 and 10 kg·PLS·ha⁻¹, respectively. Proper legume management guidelines that address legume varieties compatible with switchgrass, appropriate bacterium for seed inoculation and seeding dates and rates that minimize the competition of vetch when intercropped with switchgrass need to be further developed to make this legume a viable option for displacing inorganic-N in switchgrass biofuel and forage production systems.

Acknowledgments: Mention of tradenames or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture or the University of Tennessee. The authors would like to thank The University of Tennessee Agricultural Experiment Station and the University of Tennessee Soil, Plant and Pest Center, (Nashville, TN, USA), as well as Stacy Warwick for providing technical assistance.

Author Contributions: Fred L. Allen conceived and designed the experiments; Kara S. Warwick performed the experiments; Patrick D. Keyser, Gary E. Bates, Don D. Tyler, Paris L. Lambdin and Dan H. Pote contributed expertise and helped with carrying out the experiments; Amanda J. Ashworth wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ashworth, A.J.; Allen, F.; Keyser, P.; Tyler, D.; Saxton, A.; Taylor, A. Switchgrass yield and stand dynamics from legume intercropping based on seeding rate and harvest management. *J. Soil Water Conserv.* **2015**, *70*, 375–385. [CrossRef]
2. Ledgard, S.F.; Steele, K.W. Biological nitrogen fixation in mixed legume/grass pastures. *Plant Soil* **1992**, *141*, 137–153. [CrossRef]
3. Graham, P.H. Biological Dinitrogen Fixation: Symbiotic. In *Principles and Applications of Soil Microbiology*; Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., Eds.; Pearson Education Inc.: Upper Saddle River, NJ, USA, 2005; pp. 405–432.
4. Larson, J.A.; Jaenicke, E.C.; Roberts, R.K.; Tyler, D.D. Risk effects of alternative winter cover crop, tillage, and nitrogen fertilization systems in cotton production. *J. Agron. Appl. Econ.* **2001**, *33*, 445–457.
5. Warwick, K.; Allen, F.; Keyser, P.; Ashworth, A.J.; Tyler, D.; Saxton, A.; Taylor, A. Biomass and forage/biomass yields of switchgrass as affected by intercropped cool and warm-season legumes. *J. Soil Water Conserv.* **2016**, *71*, 21–28. [CrossRef]
6. Lemus, R.; Parrish, D.J.; Wolf, D.D. Nutrient uptake by ‘Alamo’ switchgrass used as an energy crop. *Bioenerg. Res.* **2009**, *2*, 37–50. [CrossRef]
7. Mooney, D.F.; Roberts, R.K.; English, B.C.; Tyler, D.D.; Larson, J.A. Yield and breakeven price of ‘Alamo’ switchgrass for biofuels in Tennessee. *Agron. J.* **2009**, *101*, 1234–1242. [CrossRef]
8. Garland, C.D. *SP701-A-Growing and Harvesting Switchgrass for Ethanol Production in Tennessee*; University of Tennessee Extension publication: Knoxville, TN, USA, 2008; Available online: <https://extension.tennessee.edu/publications/Documents/SP701-A.pdf> (accessed on 7 June 2017).
9. Sanderson, M.A.; Reed, R.L.; McLaughlin, S.B.; Wullschleger, S.D.; Conger, B.V.; Parrish, D.J.; Wolf, D.J.; Taliaferro, D.D.; Hopkins, C.; Ocumpaugh, A.A.; et al. Switchgrass as a sustainable energy crop. *Bioresour. Technol.* **1996**, *56*, 83–93. [CrossRef]

10. Ashworth, A.J.; West, C.P.; Allen, F.L.; Keyser, P.D.; Weiss, S.; Tyler, D.D.; Taylor, A.M.; Warwick, K.L.; Beamer, K.P. Biologically fixed nitrogen in legume intercropped systems: comparison of N-difference and ^{15}N enrichment techniques. *Agron. J.* **2015**, *107*, 2419–2430. [CrossRef]
11. George, J.R.; Blanchet, K.M.; Gettle, R.M.; Buxton, D.R.; Moore, K.J. Yield and botanical composition of legume-interseeded vs. nitrogen-fertilized switchgrass. *Agron. J.* **1995**, *87*, 1147–1153. [CrossRef]
12. Opitz von Boberfeld, W.; Beckmann, E.; Laser, H. Nitrogen transfers from *Vicia sativa* L. and *Trifolium resupinatum* L. to the companion grass and the following crop. *Plant Soil Environ.* **2005**, *51*, 267–275.
13. Tyler, D.D.; Duck, B.N.; Graveel, J.G.; Bowen, J.F. Estimating response curves of legume nitrogen contribution to no-till corn. In *The Role of Legumes in Conservation Tillage Systems*; Power, J.F., Ed.; Soil Conservation Society of America: Ankeny, IA, USA, 1987; pp. 50–51.
14. Clark, A.J.; Decker, A.M.; Meisinger, J.J.; Mulford, F.R.; McIntosh, M.S. Hairy vetch kill date effects on soil water and corn production. *Agron. J.* **1995**, *87*, 579–585. [CrossRef]
15. Holderbaum, J.F.; Decker, A.M.; Meisinger, J.J.; Mulford, F.R.; Vough, L.R. Fall seeded legume cover crops for no-tillage corn in the humid east. *Agron. J.* **1990**, *82*, 117–124. [CrossRef]
16. Ranells, N.N.; Waggoner, M.G. Grass-legume bicultures as winter annual cover crops. *Agron. J.* **1997**, *89*, 659–665. [CrossRef]
17. Blanchet, K.M.; George, J.R.; Gettle, R.M.; Buxton, D.R.; Moore, K.J. Establishment and persistence of legumes interseeded into switchgrass. *Agron. J.* **1995**, *87*, 935–941. [CrossRef]
18. UC SAREP. *Common Vetch*; University of California Sustainable Agriculture Research & Education Program: Oakland, CA, USA, 2006; Available online: http://www.sarep.ucdavis.edu/cgi-bin/ccrop.EXE/show_crop_14 (accessed on 12 May 2011).
19. Matic, R.; Nagel, S.; Saunders, R. *Vetch Variety Sowing Guide 2015*; National Vetch Breeding Program and SARDI: Australia, 2015. Available online: http://www.sardi.sa.gov.au/__data/assets/pdf_file/0010/45964/vetch.pdf (accessed on 12 February 2015).
20. Sattell, R.; Luna, D.J.; McGrath, D. *Hairy Vetch (Vicia villosa)*; EM 8704; Oregon Cover Crops, Oregon State University: Corvallis, OR, USA, 2004; Available online: http://forages.oregonstate.edu/php/fact_sheet_print_legume.php?SpecID=41 (accessed on 12 February 2015).
21. Hanaway, D.; Larson, C. *Hairy Vetch (Vicia villosa Roth)*; Oregon State University: Corvallis, OR, USA, 2004; Available online: <http://ohioline.osu.edu/agf-fact/0006.html> (accessed on 26 May 2012).
22. Myers, D.; Underwood, J. *Hairy Vetch as An Ohio Cover Crop*; OSU Extension publication. AGF-006; Ohio State University: Columbus, OH, USA, 1990. Available online: <http://ohioline.osu.edu/agf-fact/0006.html> (accessed on 26 May 2012).
23. Myrold, D.D.; Ruess, R.W.; Klug, M.J. Dinitrogen fixation. In *Standard Soil Methods for Long-Term Ecological Research*; Robertson, G.P., Coleman, D.C., Bledsoe, C.S., Sollins, P., Eds.; Oxford University Press: New York, NY, USA, 1999.
24. Minchin, F.R.; Witty, J.F.; Mytton, L.R. Reply to ‘Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay’ by J.K. Vessey. *Plant Soil* **1994**, *158*, 163–167. [CrossRef]
25. Danso, S.K.A. Assessment of biological nitrogen fixation. *Fert. Res.* **1995**, *42*, 33–41. [CrossRef]
26. Ebelhar, S.A.; Frye, W.W.; Blevins, R.L. Nitrogen from legume cover crops for no-till corn. *Agron. J.* **1984**, *76*, 51–55. [CrossRef]
27. Zuberer, D.A. Biological Dinitrogen Fixation: Introduction and Non-Symbiotic. In *Principles and Applications of Soil Microbiology*; Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., Eds.; Pearson Education Inc.: Upper Saddle River, NJ, USA, 2005; pp. 373–404.
28. Segundo, S.U.; Boddey, R.M. Theoretical considerations in the comparison of total nitrogen difference and ^{15}N isotope dilution estimates of the contribution of nitrogen fixation to plant nutrition. *Plant Soil* **1987**, *102*, 291–294.
29. Boddey, R.M.; Chalk, P.M.; Victoria, R.L.; Matsui, E. Nitrogen fixation by nodulated soybean under tropical field conditions estimated by the ^{15}N isotope technique. *Soil Biol. Biochem.* **1984**, *16*, 583–588. [CrossRef]
30. Chalk, P.M. Dynamics of biologically fixed N in legume-cereal rotation: A review. *Aust. J. Agric. Res.* **1998**, *49*, 303–316. [CrossRef]
31. Phillips, D.A.; Jones, M.B.; Centre, D.M.; Vaughan, C.E. Estimating symbiotic N fixation by *trifolium subterraneum* L. during regrowth. *Agron. J.* **1983**, *75*, 736–741. [CrossRef]

32. Bell, M.J.; Wright, G.C.; Peoples, M.B. The N₂-fixing capacity of peanut cultivars with differing assimilate partitioning characteristics. *Aust. J. Agric. Res.* **1994**, *45*, 1455–1468. [[CrossRef](#)]
33. Gettle, R.M.; George, J.R.; Blanchet, K.M.; Buxton, D.R.; Moore, K.J. Frost seeding legumes into established switchgrass: Forage yield and botanical composition of the stratified canopy. *Agron. J.* **1996**, *88*, 555–560. [[CrossRef](#)]
34. Ashworth, A.J.; Keyser, P.D.; Allen, F.L.; Tyler, D.D.; Taylor, A.M.; West, C.P. Displacing inorganic-nitrogen in lignocellulosic feedstock production systems. *Agron. J.* **2016**, *108*, 1–8. [[CrossRef](#)]
35. Jorgensen, F.V.; Ledgard, S.T. Contribution from stolons and roots to estimate the total amount of N₂ fixed by white clover (*Trifolium repens* L.). *Ann. Bot. (Lond.)* **1997**, *80*, 641–648. [[CrossRef](#)]
36. McNeill, A.M.; Zhu, C.; Fillery, I.R.P. Use of *in situ* ¹⁵N-labelling to estimate the total below-ground nitrogen of pasture legumes in intact soil-plant systems. *Aust. J. Agric. Res.* **1997**, *48*, 295–304. [[CrossRef](#)]
37. Bates, G.; Harper, C.; Allen, F. *PB 378 Forage and Field crop Seeding Guide for Tennessee*; The University of Tennessee Agricultural Extension Service, UT Extension publication: Knoxville, TN, USA, 2008. Available online: <http://forages.tennessee.edu/Page%204-%20Planting/pb378.pdf> (accessed on 26 May 2014).
38. Vogel, K.P.; Masters, R.A. Frequency grid—A simple tool for measuring grassland establishment. *J. Range Manage.* **2001**, *54*, 653–655. [[CrossRef](#)]
39. SAS Institute. *SAS/STAT 9.3 User's guide*; SAS Institute, Inc.: Cary, NC, USA, 2007.
40. Van Assche, J.A.; Debucquoy, K.L.A.; Rommens, W.A.F. Seasonal cycles in the germination capacity of buried seeds of some Leguminosae (Fabaceae). *New Phytol.* **2003**, *158*, 315–323. [[CrossRef](#)]
41. Ortega-Olivencia, A.; Devesa, J.A. Seed set and germination in some wild species of *Vicia* from SW Europe (Spain). *Nord. J. Bot.* **1997**, *17*, 639–648. [[CrossRef](#)]
42. Hargrove, W.L. Winter legumes as a nitrogen source in no-till grain sorghum. *Agron. J.* **1986**, *78*, 70–74. [[CrossRef](#)]
43. Peoples, M.B.; Ladha, J.K.; Herridge, D.F. Enhancing legume N₂ fixation through plant and soil management. *Plant Soil.* **1995**, *174*, 83–101. [[CrossRef](#)]
44. Ranells, N.N.; Waggoner, M.G. Nitrogen release from grass and legume cover crop monocultures and bicultures. *Agron. J.* **1996**, *88*, 777–782. [[CrossRef](#)]



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