Rapid Assessment of Nitrogen Concentration of Two Bioenergy Feedstock Grasses Using Hyperspectral Reflectance Spectroscopy

by
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Serve my country. Contributing research for the sustainable intensification of our national food, fuel, and fiber supply is my primary way of serving society. I have put forth my best efforts and I am proud to contribute to the scientific community with this study.

Uncommon effort equals uncommon results.

To Dr. Song Cui:

Thank you for your continual guidance. You are my mentor, professor, employer, thesis advisor, and friend. I owe my "roots" in agronomy to you and I look forward to conducting more research together.

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Abstract

Rapidly and accurately monitoring crop nitrate concentration is critical for both plant nutrient management and animal health, but can be difficult. Traditional methods are laborious and require destructive plant samplings followed by chemical analyses, thus, alternative methods are warranted. The objective of this research was to design a rapid nitrate assessment method using two native warm-season grass (NWSG) species, including ‘Alamo’ switchgrass (*Panicum virgatum* L.) and ‘Cheyenne’ indiangrass [*Sorghastrum nutans* (L.) Nash]. Both grass species were planted in a greenhouse and fertilized with urea at control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) rates. Plant height and leaf chlorophyll data were recorded weekly. Hyperspectral spectroscopy analysis and machine learning-based simulation approaches were used for building prediction models. The models accurately estimated botanical nitrate concentration even at its low level using data acquired from both years ($R^2 = 0.88$ and RMSE = 0.358).

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1. Introduction

Population experts predict that the world will be inhabited by an additional three billion people in the next 40 years, which will significantly increase the demand for food (Barnosky *et al.*, 2012). Additionally, U.S. legislation mandates ethanol production from non-grain sources reach 80 Gl per year by 2022, which will be equal to 25% of predicted transportation fuel needs by 2050 (Gelfand *et al.*, 2013). Therefore, modern U.S. agriculture is undergoing radical change to secure the food supply and America's energy future. Forage-based agriculture possesses great potential to address both challenges by supplying high quality feed to animals and producing the second-generation biofuel feedstocks, such as annual biomass sorghum (*Sorghum bicolor* L.) and perennial species such as switchgrass and indiangrass. Additionally, it can also provide great economic and ecological benefits such as seed production, C sequestration, and biodiversity.

Successful forage production depends greatly on an optimized nutrient management plan. Agronomists, soil scientists, and ecologists often work together on a system basis to optimize agronomic production, increase ecological resilience, and minimize environmental contamination caused by animal activities or nutrient losses. Additionally, great emphasis has been placed on mitigating greenhouse gas emissions, improving C sequestration potential, and controlling the fate of nutrients and pesticides within a forage production system. Precision nutrient management, particularly N, is one of the major concerns of producers and researchers. Insufficient N application or low N use efficiency of forages can lead to reduced biomass production, meanwhile, over-fertilization of N can cause nitrate (NO_3-) accumulation as well as severe toxicity problems when grazed by animals. Therefore, the principal questions of this research were whether a remotely-sensed method could be derived to assess botanical nitrate concentration of forages *in vivo* and if so, what spectral bands should be used and what mathematical models would be able to quantify this pattern accurately.

2. Literature Review

2.1. Benefits of Grassland Agriculture and Bioenergy Forage Grasses

Forages and grassland-based agriculture have long been important for the food supply of humans, mainly through ruminant animals and wildlife (Hendershot *et al.*, 2012). Recognized as the backbone of forage production, grasslands are among the largest ecosystems in the world, covering 25% of the Earth's surface (Reid *et al.*, 2008). Land grazed by livestock is the largest single land-use type globally, occupying approximately 4 billion ha (Lambin and Meyfroidt, 2011), and forage is the most consumed livestock feed in the world (Peters *et al.*, 2013). Grasslands are also the single largest land type in the U. S. (Sanderson *et al.*, 2009), with grazing land totaling 316 million ha and hay land another 64 million ha (Lubowski *et al.*, 2006). Because of their abundance, achieving sustainable grassland agroecosystems is of great importance (Sollenberger, 2015). Green plants fix and sequester large amounts of C in leaves, roots, and stems (Sheaffer and Moncada, 2012). Root systems of temperate grassland species can grow to significant depths. In a global analysis, Canadell *et al.* (1996) found a maximum rooting depth of 2.6 ± 0.2 m for temperate grasslands. The characteristic expansive fibrous root system and substantial aboveground biomass of grasses are indicative of the plants' ability to stabilize soil structure while converting a significant amount of atmospheric C into soil organic C. Integrating forage and livestock systems into conventional row-crop systems provides a number of important benefits to farmers, the environment, and the society (Allen *et al.*, 2012). The majority of forage crops can also be managed as dual-purpose crops, which can be used as bioenergy crops for providing biofuel feedstocks or directly used as feedstuff for animals. Additionally, land dedicated for forage production can be easily converted to other uses or incorporated into a rotational cropping system with increased nutrient, organic concentration and improved soil structure and microbial diversity (Sanderson and Adler, 2008).

Native warm-season grasses provide peak forage production during summer and can complement the poor growth of cool-season grasses to improve year-long forage availability (Anderson *et al.*, 1988). Additionally, by incorporating warm-season species into a cool-season dominated production system in Middle Tennessee, toxicity and poor nutritive value from heat stressed cool-season species such as tall fescue (*Festuca arundinacea* Shreb.) and orchardgrass (*Dactylis glomerata* L.) can be amended during summer grazing. Most NWSG also have greater tolerance to moisture deficit than cool-season grasses and will survive extreme winter cold while introduced warm-season species such as bermudagrass [*Cynodon dactylon* (L.) Pers.] will not (Sheaffer and Moncada, 2012). Warm-season grasses typically out-perform cool-season grasses and legumes in terms of biomass production because of the C_4 photosynthetic pathway, which completely eliminates the photorespiration process. This physiological difference and the great adaptation of these NWSG to the southeastern climate make them ideal candidates for the second-generation biofuel production. Most importantly, using NWSG for biofuel feedstocks instead of corn (*Zea mays* L.), which could be directly consumed by humans, can greatly alleviate the pressure on food crop production.

Switchgrass and indiangrass are both C_4 perennial warm-season grasses indigenous to the southeast, which have demonstrated pronounced potential as cellulosic energy crops (Sheaffer and Moncada, 2012). When managed as bioenergy crops, both crops produced ethanol yields comparable to corn (*Zea mays* L.) grain, with greenhouse gas emissions averaging 94% less than gasoline (Schmer *et al.*, 2008; Varvel *et al.*, 2008). Particularly, switchgrass is a high biomass producer and is relatively more water efficient as well as N, P, and K efficient than cool-season grasses on account of its C_4 physiology (Brown, 1978; Sanderson and Reed, 2000; Lemus *et al.*, 2008; Barney *et al.*, 2009). Many studies have been conducted in the past decade investigating various environmental benefits associated with growing these NWSG, including reduced soil erosion, increased water quality, enhanced soil C sequestration, diversified wildlife habitat, and reduced greenhouse gas emissions (Sanderson *et al.,* 2006; Blanco-Canqui, 2010; Johnson and Barbour, 2016). I selected switchgrass and indiangrass for this study because of their pronounced agronomic feasibility in Middle Tennessee and their adaptation to the southern climate.

2.2. Nitrogen in Agroecosystems

Nitrogen (N) is the most abundant element in the atmosphere (78%) and the third most abundant constituent in plant biomass after C and O (Sheaffer and Moncada, 2012). As a major component of chlorophyll, amino acids, adenosine triphosphate (ATP), and nucleic acids; N is the most important nutrient in crop production, yet one of the most difficult to manage. Symbiotic-N_2 fixation and modern industrial fertilizer production provide the majority of the N sources for crop production. However, N is always the most limiting growth factor for plants because N use efficiency and availability are strongly controlled by various plant physiological and soil biochemical processes such as enzymatic reduction, soil leaching, denitrification, volatilization, mineralization, and immobilization (Dinnes *et al.,* 2002). Plants can directly uptake two ionic forms of N, including ammonium (NH_4^+), a positively charged ion (cation); and nitrate (NO_3-), a negatively charged ion (anion). Both ions are very soluble in water and nitrate is not attracted to the soil particles, making it subject to leaching (Sheaffer and Moncada, 2012). Particularly, drought-stressed warm-season grasses tend to accumulate nitrate in stems, which can cause severe nitrate toxicity problems when grazed by livestock. Bioenergy crops, including NWSG, generally respond well to N fertilization (increased yield and protein concentration). However, N inputs must be applied at the right place, at the right time, and in the right amount due to the high economic and energy costs associated with fertilizer production and application (Power and Schepers, 1989; Sanderson *et al.,* 2006). Insufficient N application can cause low biomass production and reduce economic profitability. In contrast, excessive N application can lead to high foliar N concentration such as accumulation of nitrate in feedstock material that can reduce hydrocarbon yield during the thermochemical process and increase NO_x emissions. If managed properly, this beneficial nutrient can provide high NWSG biomass yield with low N input, which is ideal for the second-generation cellulosic biofuel production. Due to the complex chemistry and behavior of N in soils and plants, developing an easy-to-use and rapid methods for assessing botanical N concentration and monitoring its dynamics within a cropping system are of paramount importance.

2.3. Nitrogen and Sensing

Plants utilize N for synthesizing important proteins or biomolecules such as chlorophylls (Chl, primarily Chl a and Chl b; Filella *et al.*, 1995; Moran *et al.*, 2000), which are essential pigments for the conversion of light energy to stored chemical energy. The amount of solar radiation absorbed by a leaf is a typically function of the photosynthetic pigment content (Hatfield *et al.,* 2008). Thus, Chl concentration serves as a good indicator for plant N status and it also directly determines crop photosynthetic potential and primary production (Curran *et al.*, 1990; Filella *et al.*, 1995). Foliar Chl content is also closely related to plant stress and senescence (Hendry *et al.*, 1987; Peñuelas and Filella, 1998; Merzlyak *et al.*, 1999; Carter and Knapp, 2001). Therefore, developing a rapid and reliable method for assessing Chl status is crucial for plant need-based N applications. Traditionally, pigment analysis is conducted using wet chemical leaf extraction methods with organic solvents and spectrophotometric determination in solution (e.g. Lichtenthaler, 1987). This extraction technique, considered the standard method for chlorophyll assessment, requires destructive sampling (thus preventing developmental studies of single leaves) and is time consuming (Hatfield *et al.,* 2008). The SPAD (Soil Plant Analysis Development) meter (SPAD-502 Plus Chlorophyll meter, Aurora, IL), originally designed in the 1990s for *in vivo* Chl measurements, indicated strong correlation with leaf chlorophyll concentration and was therefore widely used in many agronomic studies for estimating crop N status (Hussain *et al.*, 2000; Singh *et al.*, 2002; Zhang *et al.*, 2008). However, SPAD meters can only provide relative values based on leaf Chl concentration, making it hard to use for cross-species or cross-date comparisons. Additionally, it has been reported that SPAD meters can have poor sensitivity when measuring leaves with similar Chl concentrations and its readings can be affected by crop variety, water status, and pathogen problems (Samborski *et al.*, 2009; Zhu *et al.*, 2011).

Spectroscopy analysis is one of the most successful and widely adopted methods for testing N concentration in forages and row crops (Sanderson *et al.*, 1996; Gislum *et al.*, 2004; Clay *et al.*, 2006; Foster *et al.*, 2013). The use of NIR spectroscopy for testing forage nutritive value is not limited to a single species, but can also be applied to an assortment of different vegetation mixtures. Most recently, Labbé *et al.* (2008) and Foster *et al.* (2013) both used an ASD FieldSpec spectroradiometer along with partial least square models (PLS) and showed satisfactory results in estimating total biomass N composition. In fact, PLS regression remains one of the most popular methods for biomass fiber, mineral, C, and N composition analysis (Sanderson *et al.*, 1996; Labbé *et al.*, 2008; Foster *et al.*, 2013). However, it requires a significant amount of testing and calibration and the performance on nitrate is still unknown.

3. Thesis Statement

Rapidly and accurately monitoring crop nitrate concentration is difficult. The objective of this research was to compile a rapid nitrate assessment and evaluation model for NWSG species using both remote sensing-based spectroscopic instruments and machine learning-based mathematical models. I hypothesized that plants fertilized differently would exhibit different nitrate concentration levels, which could consequentially incur different spectral reflectance patterns. Both plant height and foliar SPAD readings should be strongly affected by N treatment and harvesting date.

4. Materials and Methods

This experiment was conducted in the MTSU Plant and Soil Science Greenhouse in Murfreesboro, Tennessee from May 25th to July 24th 2015, and repeated from May 5th to September 14th 2016. Two NWSG species, including 'Alamo' switchgrass (*Panicum virgatum* L.) and 'Cheyenne' indiangrass [*Sorghastrum nutans* (L.) Nash] were planted in 46-cm diameter flower pots with standard potting soils (ProMix BX Growth Media, Premier Horticulture Inc. Quakertown, PA 18951). Soil samples were analyzed by the University of Tennessee Soil Plant and Pest Center (Nashville, TN 37211) and indicated neutral pH (pH = 6.87) and "Low" nutrient status on P (12.86 ppm), K (38.7 ppm), Ca (57 ppm), Mg (11 ppm), and NO_3- (9.3 ppm).

Irrigation water was supplied by an automated drip irrigation system controlled by a programmable digital timer. In each year, 16 pots of indiangrass and 16 pots of switchgrass were included in this study. Ten seeds were planted in each pot at a 0.5-cm seeding depth. For each NWSG, the pots were randomly divided into four treatment groups (four pots per treatment). Urea (46-0-0) fertilizer was applied one month after planting at four levels: control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}). All pots were irrigated from planting until one week after fertilization.

Foliar chlorophyll concentration was measured weekly using a SPAD 502-Plus Chlorophyll meter (Spectrum Technologies, Inc., Aurora, IL 60504) for five weeks immediately following fertilization. Three measurements were collected from each pot and average numbers were recorded. Meanwhile, plant height was recorded from three randomly selected plants out of each pot using a ruler. *In-vivo* foliar reflectance data was measured weekly following fertilization using the ASD FieldSpec®4 Standard-Resolution Spectroradiometer (ASD Inc., Boulder, CO 80301), which records spectral digital count data between the 350-to-2,500-nm range, yielding a 1.4-nm sampling interval in the 350-1,000 nm spectral range and 2.0-nm in the 1,000-2,500 nm range. A Plant Probe Kit (ASD Inc., Boulder, CO 80301) including an MR6 lamp (4.25V, 4.5 W), a contact probe, a leaf clip, and a white reference cap; was used for securing the plant leaf, providing the illumination light source, and the white reference standard. All spectral data were optimized, white-referenced, recorded, and converted to reflectance values using the ASD RS3 Spectral Acquisition Software (ASD Inc., Boulder, CO 80301). Then, reflectance data was converted to ASCII format using the ViewSpecPro Software (ASD Inc., Boulder, CO 80301) for further analysis using Matlab Programming Language (The MathWorks Inc., Natick, MA). The detailed description on leaf reflectance measurements and integration of the Plant Probe Kit can be found in the ASD FieldSpec®4 User Manual (ASD Document 600979). After the leaf reflectance measurements, all above-ground biomass was immediately cut and dried at 60°C to a constant weight, ground to pass a 1-mm screen in a Wiley Mill (Comeau Technique Ltd., Vandreuil-Dorion, Quebec, Canada) and then sent to the University of Tennessee Soil, Plant, and Pest Center for forage and plant tissue analysis. Particularly, a 2% acetic acid solution is used to extract nitrate from botanical tissues and all samples were analyzed using the TL-8000 Ammonia Analyzer (Timberline Instruments Inc., Boulder, CO 80308). Total N was analyzed using a combustion method and forage nutritive values were estimated using near-infrared (NIR) spectroscopy analysis.

Plant height, SPAD readings, and botanical nitrate were analyzed as a completely randomized design with four replications and repeated measure effect to control for autocorrelation of observations over time using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC). All ASCII spectral data were analyzed using machine learning algorithms implemented using the Matlab Programming Language based on a generalized regression neural network (GRNN) model to recognize the spectral pattern differences and predict the nitrate concentration across two grass species. A standard leave-one-out cross-validation procedure was used to evaluate the prediction performances.

5. Results and Discussion

5.1. Plant Height

Treatment effect on plant height depended greatly on years ($P < 0.05$), which was anticipated, because summer weather conditions (primarily solar radiation intensity and humidity) in both years were dramatically different from each other. The high humidity level from year one greatly increased N use efficiency as indicated in plant height data. Thus, data was analyzed by years. In year one, plant height data was strongly affected by species ($P < 0.05$), N treatment ($P < 0.001$), and harvesting date ($P < 0.0001$). Neither two-way nor three-way interactions were detected ($P > 0.1$). In year two, plant height data was again affected by species ($P < 0.0001$), N treatment ($P < 0.0001$), and harvesting date ($P < 0.0001$). A two-way interaction between species and treatment was detected ($P = 0.0412$). A three-way interaction was present, but apparently the interaction caused by a different harvesting date was due to magnitude of response only ($P = 0.017$).

Plant height responses were presented by years and species for consistency purposes (Fig. 1 and 2). For indiangrass, the high-rate of N fertilization increased plant height by more than 15-cm compared to control in both years (Fig. 1). In year two, both medium and high rates increased plant height more than the control and low rates. For switchgrass, all N fertilization treatments increased average plant height by almost 18-cm compared with no-N control during year one (Fig. 2). In year two, medium N fertilization rate increased height compared with no-N control and high N rate indicated even greater responses when compared to the medium rate. Plant height is one of the key indicators of plant growth and nutrient responses. The results from this study agreed well with other plant phenotyping studies involving crop canopy height measurements under different N management regimes (Yin *et al.*, 2011).

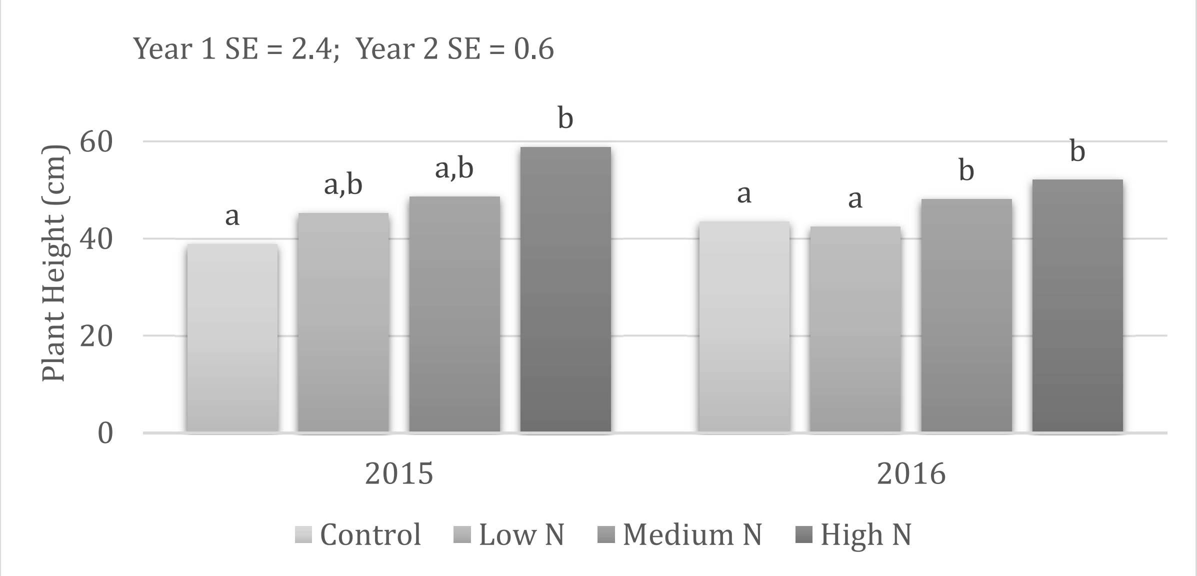

Fig. 1. Plant height of indiangrass affected by different N treatments, including control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) in two years (2015 and 2016). Letters separate means based on $P < 0.05$ level by pair-wise comparisons.

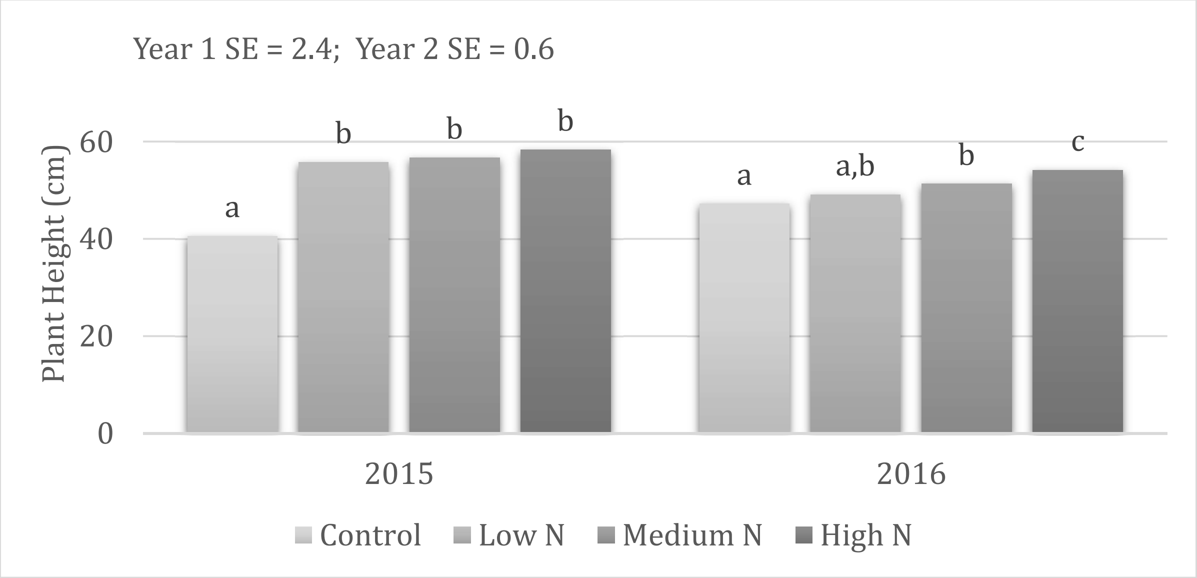

Fig. 2. Plant height of switchgrass affected by different N treatments, including control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) in two years (2015 and 2016). Letters separate means based on $P < 0.05$ level by pair-wise comparisons.

5.2. Foliar Chlorophyll Concentration

Foliar SPAD readings from year two were significantly lower than year one, leading to significant treatment-and-year interaction ($P < 0.05$). This difference was more than likely caused by the much lower humidity and more intense solar radiation in year two (cloudier conditions and precipitation in the summer season of year 1), leading to greater water stress and less N use efficiency. Thus, data was analyzed by years. In 2015, SPAD readings were strongly affected by species ($P < 0.01$), N treatment ($P < 0.0001$), and harvesting date ($P < 0.05$). Two-way interactions were detected between species and treatment ($P < 0.05$), between species and date ($P <$

0.05), as well as between treatment and date ($P < 0.05$). The two-way interactions that involved date appeared due to differences in magnitude of effect only. Therefore, data was averaged across different dates. Three-way interaction was not significant ($P = 0.8$). Interestingly, species effect ($P = 0.082$), two-way interactions (species and treatment, $P = 0.81$; species and date, $P = 0.534$), and three-way interaction ($P = 0.18$) were all insignificant in year two. Again, data were presented by years and by species for consistency purposes.

In year one, medium and high N treatments increased SPAD readings of indiangrasses (Fig. 3), meanwhile, fertilized switchgrasses indicated higher SPAD readings compared with no-N control (Fig. 4). Additionally, high N treatment increased SPAD readings when compared with low N and no-N control (Fig. 4). In year two, both medium and high N treatments increased SPAD readings when compared to no-N control of indiangrass and switchgrass. Results presented in this study provide strong evidence that SPAD readings could be used for quickly assessing N responses of NWSG. The use of a SPAD meter on many agronomic crops such as corn (*Zea mays* L.), rice (*Oryza sativa* L.), and sugar beet (*Beta vulgaris* L.) have been documented before (Singh *et al.*, 2002; Zhu *et al.*, 2011; Tsialtas *et al.*, 2014). Its use on forage species is limited.

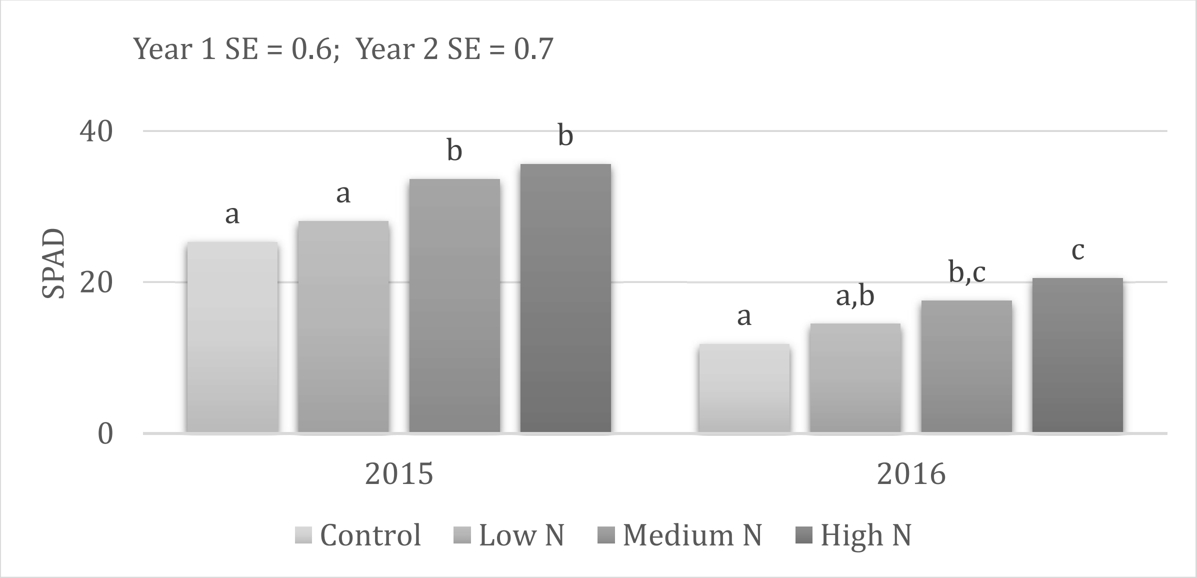

Fig. 3. Foliar SPAD readings of indiangrass affected by different N treatments, including control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) in two years (2015 and 2016). Letters separate means based on $P < 0.05$ level by pair-wise comparisons.

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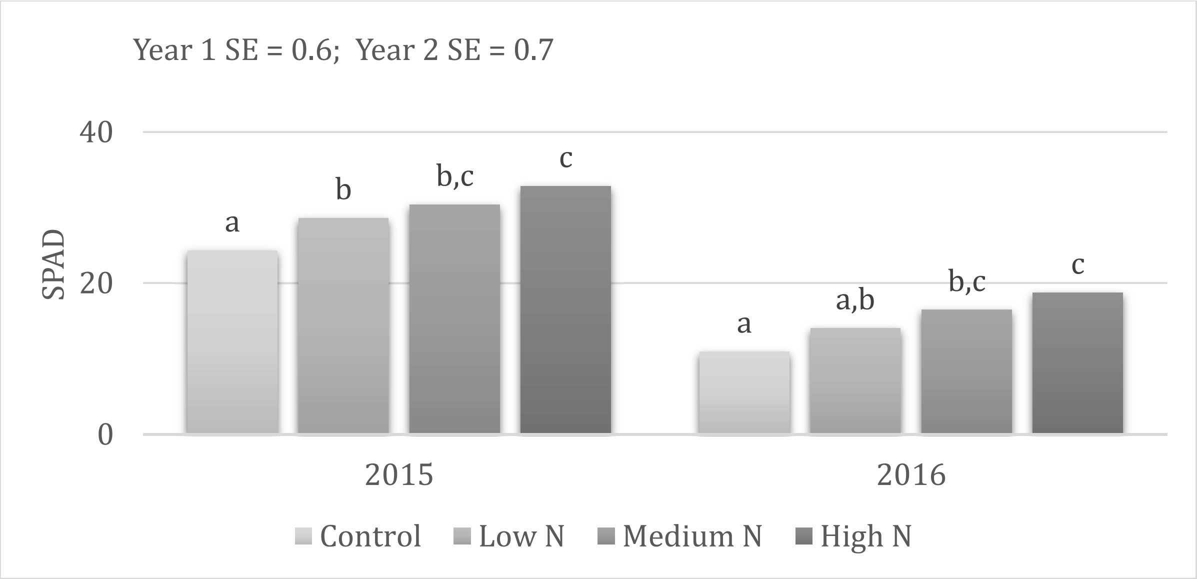

Fig. 4. Foliar SPAD readings of switchgrass affected by different N treatments, including control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) in two years (2015 and 2016). Letters separate means based on P < 0.05 level by pair-wise comparisons.

5.3. Botanical Nitrate Concentration

A strong year effect (P < 0.0001) and a treatment-year interaction (P < 0.005) on botanical nitrate concentration was observed from the result. Therefore, data was further analyzed by year. No treatment effects were present during year one (P = 0.13). In year two, botanical nitrate concentration was strongly affected by treatment (Fig. 5; P < 0.01). No species effect (P = 0.09) nor two-way interaction was present (P = 0.41). Both medium and high levels of fertilization treatment increased botanical nitrate concentration when compared with no-N control and low N treatments (Fig. 5).

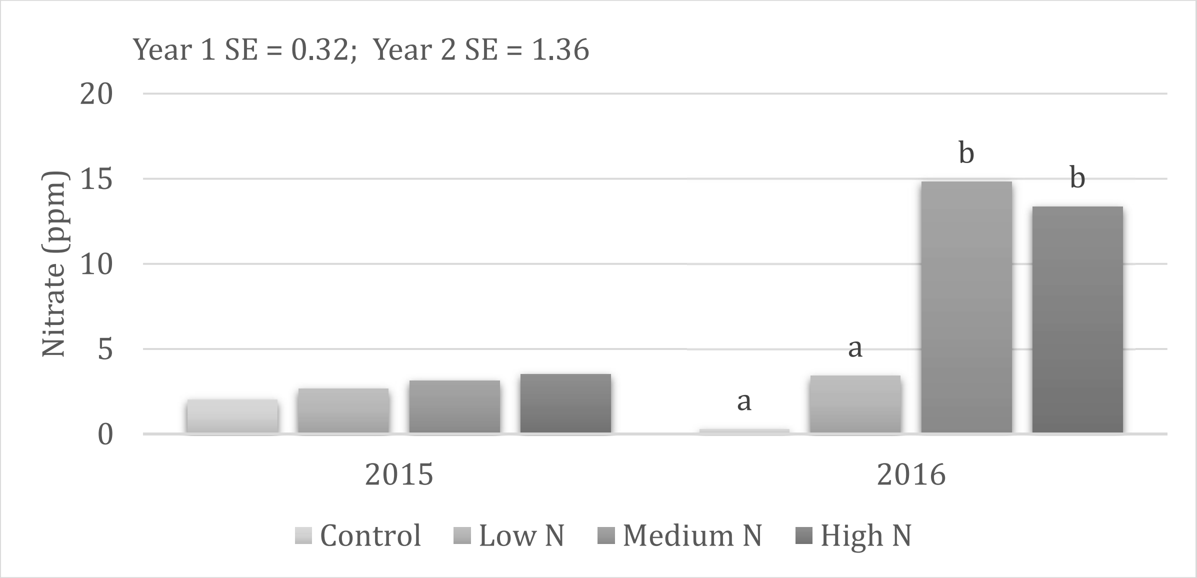

Fig. 5. Average botanical nitrate concentration of indiangrass and switchgrass affected by different N treatments, including control (0 kg N ha^{-1}), low (65 kg N ha^{-1}), medium (130 kg N ha^{-1}), and high (260 kg N ha^{-1}) in two years (2015 and 2016). Letters separate means based on $P < 0.05$ level by pair-wise comparisons.

Typically, botanical nitrate concentration tends to increase with increasing N fertilization, particularly in warm-season annual forages such as sorghum (*Sorghum bicolor* L.) and crabgrass (*Digitaria* spp.). However, the dynamics of nitrate in plant tissue are greatly affected by many factors such as stress, temperature, genetics, and soil conditions. The accumulation of nitrate is usually a result of high-level nitrate uptake and limited N incorporation for protein synthesis. In this study, irrigation water was removed one week after N fertilization to ensure limited N loss due to volatilization and moderate drought stress to promote nitrate accumulation (May *et* *al.*, 1990; Vetsch *et al.*, 1999; MacKown and Weik, 2004; Teutsch and Tilson, 2005). Nitrate concentration was greater in year two because of lower humidity and more intense solar radiation when compared to year one (average nitrate concentration: year two, 7.5 ppm; year one, 2.8 ppm). Limited range of nitrate concentration was observed across different N fertilization rates during these two years (0 to 30 ppm). The maximum nitrate concentration was not high enough to cause reductions in animal performances, but it could potentially affect biofuel conversion efficiency. This lack of response to drought stress could be caused by high humidity levels in the greenhouse environment as well as the high-drought tolerance of both NWSG species.

5.4. Spectral Reflectance Modeling

Spectral reflectance differences were detected between two grass species and between low nitrate and high nitrate concentrations within the same species (e.g. Fig. 6 and 7). We implemented computational algorithms using Matlab (The MathWorks Inc., Natick, MA) based on a generalized regression neural network (GRNN) model to recognize the spectral pattern differences and predict the nitrate concentration across two grass species. A standard model training, testing, and validation paradigm was followed. Particularly, four hundred spectral samples with nitrate concentration ranging from 0.1 to 27.5 ppm were selected for building the prediction model. The final model yielded satisfactory performances (Fig. 8; $R^2 = 0.88$ and RMSE = 0.358).

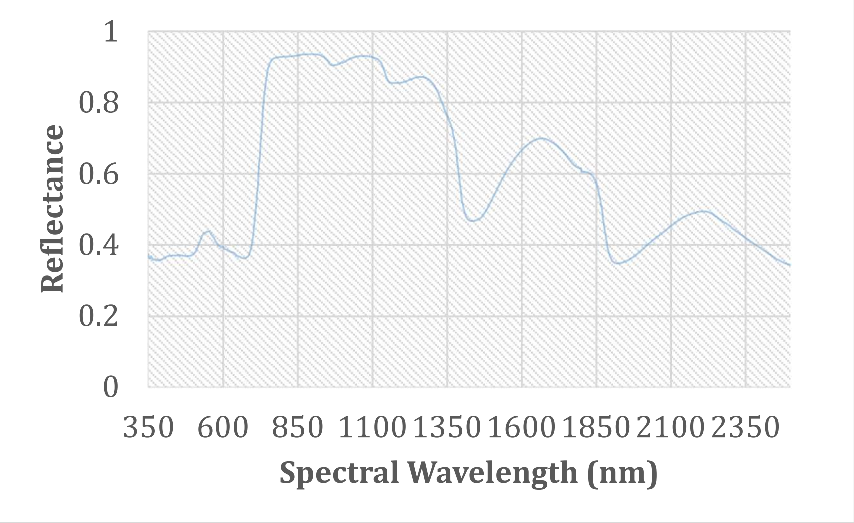

Fig. 6. Foliar reflectance pattern of indiangrass across 350 to 2,500 nm with an average leaf nitrate concentration of 3.23 ppm.

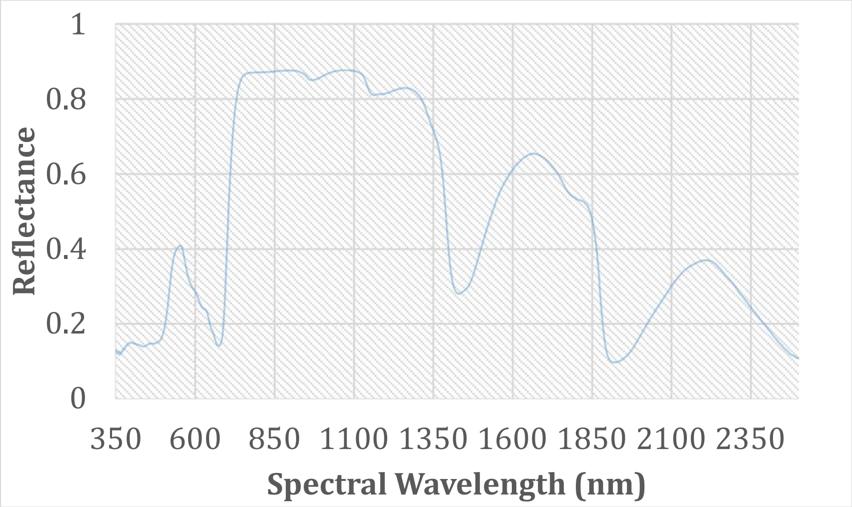

Fig. 7. Foliar reflectance pattern of switchgrass across 350 to 2,500 nm with an average leaf nitrate concentration of 12 ppm.

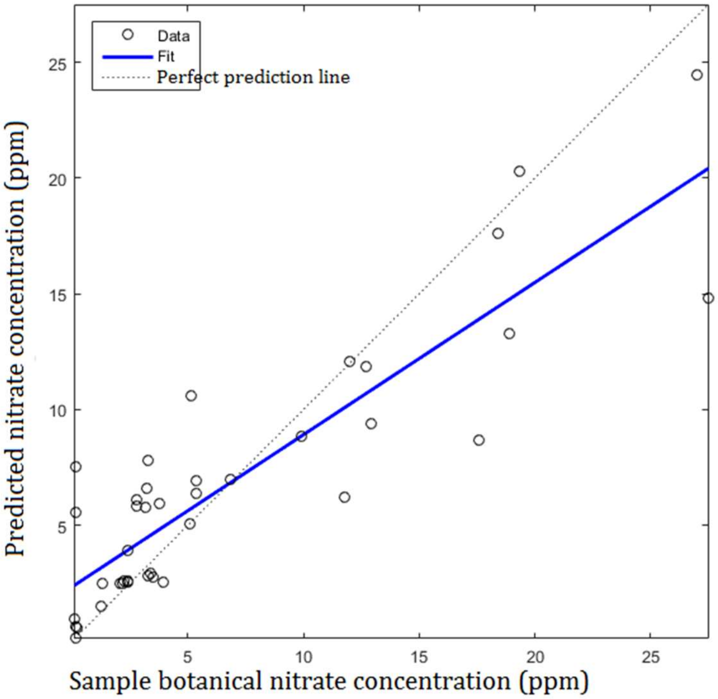

Fig. 8. Prediction performances of botanical nitrate concentration using a generalized regression neural network (GRNN) model.

6. Conclusions

Nitrogen is one of the most important nutrients for plant production, however, one of the most difficult to manage (Cui *et al*., 2013). Precisely applying N fertilizer on bioenergy feedstock grasses such as NWSG has great impact on hydrocarbon yield efficiency (Foster *et al*., 2013) and animal performance when managed as production forages. For animal grazing, forages containing less than 0.1% (dry matter basis) of nitrate are typically safe for grazing. However, botanical nitrate level can be easily increased by N fertilization to 0.25% (cautious level) or even higher than 1% (fatal level). For biofuel production, NWSG forages require N fertilization for optimal biomass production. However, over-fertilization can also cause increased botanical nitrate concentration which leads to low efficiency for biofuel production. Forage-based agriculture is mainly responsible for both supplying high-quality feed to animals and producing the second-generation biofuel feedstocks to meet the population demand. Therefore, increased production of biomass feedstock forages and improved N monitoring and management tools are greatly needed, especially in the southeastern region of the United States.

This study verified the biomass production responses (height and Chl concentration) of two NWSG species (switchgrass and indiangrass) affected by different N fertilization treatments. None of the NWSG's botanical nitrate concentrations exceeded 0.1%. A preliminary nitrate monitoring tool was successfully developed by integrating hyperspectral spectroscopy analysis and mathematical modeling, which can accurately predict botanical nitrate concentration even at its low level. Future studies are warranted to generate a broader range of foliar nitrate concentrations, which could increase the applicability and robustness of the prediction model. Additionally, field-level studies should also be conducted using multispectral sensors with the most informative spectral bands identified in this study to further validate the research findings.

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Definition of Terms

Adenosine triphosphate (ATP). A nucleotide used to transport chemical energy within cells for metabolism.

Agroecosystem. An ecosystem that has been modified by inputs of fertilizers, pesticides, energy, and human labor to produce fiber, food, and shelter. Plants and animals selected for specific traits are components of agroecosystems.

Amino acid. Molecules with a common structure consisting of a central carbon (C) with hydrogen (H), an amino acid group (NH_2), and a carboxyl group (COOH). Twenty different amino acids are used in protein synthesis.

Ammonium (NH_4^+). An ion derived from ammonia by combination with a hydrogen ion and known in compounds (as salts) that resemble in properties the compounds of the alkali metals.

Anion. A negatively charged ion.

ASCII. ASCII stands for American Standard Code for Information Interchange. Computers can only understand numbers, so an ASCII code is the numerical representation of a character such as 'a' or '@' or an action of some sort.

Autocorrelation. The correlation between paired values of a function of a mathematical or statistical variable taken at usually constant intervals that indicates the degree of periodicity of the function.

Bermudagrass [*Cynodon dactylon* (L.) Pers.]. An introduced, C_4, warm-season, perennial grass adapted to tropical locations and used for forage in the South.

Bioenergy. Renewable energy extracted from plant biomass or animal waste that contains energy originally derived from the sun. Bioenergy crops include grains such as corn, crop residue such as corn stover, woody biomass such as willow and timber wastes, and herbaceous crops such as switchgrass and prairie plants.

Biomass crop. A crop that is grown and harvested for the production of energy by fermentation, combustion, or gasification.

Biomolecule. An organic molecule present naturally in a living system.

Botanical N concentration. The amount of nitrate contained within leaves and stems of plants.

C_4 plants. Plants that use a type of carbon fixation in which the first stable compound formed is a four-carbon molecule (oxaloacetic acid). Examples include crops of tropical origin such as corn, sorghum, and sugarcane.

Calvin cycle. The second part of photosynthesis (carbon-fixation reactions) in which carbon dioxide is turned into simple sugars.

Carbon (C). An element that forms diamonds and coal that is found in petroleum and in all living plants and animals.

Carbon cycle. The movement of carbon through the different reservoirs of the earth's ecosystem.

Carbon fixation. The conversion of carbon dioxide into organic compounds by plants through the process of photosynthesis.

Carbon sequestration. The process of removing carbon from the atmosphere for storage in carbon reservoirs such as in plant tissues, the soil, or in the ocean.

Cation. A positively charged ion.

Cellulose. A long-chained, glucose polymer important to the structural support of plants. Cellulose is often linked to lignin and hemicellulose in plant cell walls.

Cellulosic biofuels. Cellulose is broken into glucose that is subsequently fermented into ethanol. One advantage of cellulosic ethanol as compared to corn grain ethanol is that perennial crops can be used, which have a lower impact on the environment.

Chlorophyll (Chl). Green pigment within chloroplasts that absorbs photons of light energy used in the process of photosynthesis.

Corn (*Zea mays* L.). A tall-growing, annual grass of tropical origin that is grown and harvested for its grain. Corn is the most economically important crop grown in the United States.

Crabgrass (*Digitaria* spp.). A warm-season, creeping grass that can become a serious weed if not controlled.

Denitrification. The process of nitrate being converted to N_2 under anaerobic conditions by soil bacteria.

Fibrous root system. A root system that is made up of several primary roots that branch and develop many lateral roots to form an interwoven mass.

Forage. Any crops whose vegetative parts – including stems, leaves, and sometimes attached seed or grain – are used for livestock feed. Animals can take forages directly from pastures by grazing, or forages can be fed following storage.

Forage quality. The potential forage feeding value of a crop. Features of forage quality are nutrient concentration, palatability, and antiquality components.

Generalized regression neural network (GRNN). A type of radial basis network that is often used for function approximation in a statistical analysis.

Grassland agriculture. The use of grasses as well as legumes to feed livestock, support wildlife, and maintain land resources.

Hydrocarbon. A compound of hydrogen and carbon, such as any of those that are the chief components of petroleum and natural gas. An organic compound whose molecules contain only carbon and hydrogen atoms.

Hyperspectral remote sensing. Also known as imaging spectroscopy, is a relatively new technology that is currently being investigated by researchers and scientists with regard to the detection and identification of minerals, terrestrial vegetation, and man-made materials and backgrounds.

Immobilization. The process of converting inorganic nitrogen to organic nitrogen by soil microbes.

***In vivo*.** A process performed or taking place in a living organism.

Indiangrass [*Sorghastrum nutans* (L.) Nash]. A C_4, native, perennial, warm-season grass with numerous environmental benefits.

Industrial fixation. The process of taking ionic nitrogen (N_2) from the air and converting it to nitrogen-bearing compounds to be used as fertilizers, such as anhydrous ammonia, urea, or ammonium nitrate.

Leaching. The loss of water-soluble plant nutrients from the soil, due to rain and irrigation.

Mineralization. The process of organic nitrogen being converted to inorganic nitrate by soil microorganisms.

Nanometer (nm). A metric unit of length equal to one billionth of a meter.

Nitrate (NO_3-). One nitrogen atom bonded with three oxygen atoms form (NO_3-). Nitrate is soluble in water and leached from the soil.

Nitrification. The process of ammonium being converted into nitrate by soil bacteria.

Nitrogen (N). Nitrogen is a major component of chlorophyll, the compound by which plants use sunlight energy to produce sugars from water and carbon dioxide (i.e., photosynthesis). It is also a major part of amino acids, the building blocks of proteins. Nitrogen is a component of energy-transfer compounds, such as ATP (adenosine triphosphate). Finally, nitrogen is a significant factor of nucleic acids such as DNA, the genetic material that allows cells (and eventually whole plants) to grow and reproduce.

Orchardgrass (*Dactylis glomerata* L.). A C_3, cool-season perennial grass used as forage.

pH. The negative logarithm of the hydrogen ion concentration in an aqueous solution.

Phenotype. The observable characteristics of an individual plant that can be due to genotype, environment, or the interaction of both.

Photorespiration. A light-dependent process in some plants resulting in the oxidation of glycolic acid and release of carbon dioxide that under some environmental conditions (as high temperature) tends to inhibit photosynthesis.

Photosynthesis. A process in which plants use the energy they capture from the sunlight to produce sugars that are stored or used in respiration by the plant.

Reduction. A chemical change in which an element gains electrons; the oxidation number of the element decreases.

Rice (*Oryza sativa* L.). One of the oldest and most widely cultivated crops in the world. It is a grass that grows best in extremely wet conditions.

Senescence. A gradual deterioration of the functional characteristics of a plant; aging.

Soil-Plant Analyses Development (SPAD) meter. A compact instrument that instantly measures chlorophyll content or "greenness" of your plants to reduce the risk of yield-limiting deficiencies or costly over-fertilizing. This device quantifies subtle changes or trends in plant health long before they're visible to the human eye. A non-invasive measurement; simply clamp the meter over leafy tissue and receive an indexed chlorophyll content reading (-9.9 to 199.9) in less than 2 seconds.

Sorghum (*Sorghum bicolor* L.). A vigorous, annual grass that resembles corn. It can be used for grain production, whole plant feeding to livestock, silage, ethanol production, or grazing. It is more tolerant of low rainfall than is corn.

Spectrophotometry. A method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through a sample solution.

Spectroradiometer. An optical instrument designed to measure spectral radiance or irradiance. It has a built-in optical measuring and targeting system with full-range detection capacity from 350nm to 2,500nm. Due to its high accuracy, it is often used as a reference instrument in research and development laboratories.

Spectroscopy. The art of using a spectroscopic instrument to collect spectral data. The branch of science concerned with the investigation and measurement of spectra produced when matter interacts with or emits electromagnetic radiation.

Sugar beet (*Beta vulgaris* L.). One of the leading sugar crops in the world. It is grown for its root, which when harvested, contains between 18-20% sugar.

Switchgrass (*Panicum virgatum* L.). A C_4, native, perennial, warm-season grass with numerous environmental benefits.

Symbiotic N fixation. The process that occurs when soil bacteria known collectively as *Rhizobium* fix dinitrogen (N_2) within organized structures (nodules) on the roots of plants.

Tall fescue (*Festuca arundinacea* Shreb.). A C_3, cool-season perennial forage grass native to Europe.

Urea. A colorless crystalline compound [$CO(NH_2)_2$] that is the main nitrogenous breakdown product of protein metabolism in mammals and is excreted in urine.

Volatilization. The process of urea in soils being converted to ammonium carbonate, then to ammonia.