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Diagnosing Excess Nitrogen in Rice Using Post-Season Tissue Samples

Kyle Hoegenauer
University of Arkansas, Fayetteville

Diagnosing Excess Nitrogen in Rice Using Post-Season Tissue Samples

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Kyle Hoegenauer
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This thesis is approved for recommendation to the Graduate Council.

_____
Trenton Roberts, Ph.D.
Thesis Director

_____
Mike Daniels, Ph.D.
Committee Member

_____
Jarrod Hardke, Ph.D.
Committee Member

_____
Mike Richardson, Ph.D.
Committee Member

Abstract

Proper nitrogen (N) management in rice production can be difficult to achieve without the aid of tools such as soil and tissue tests. There are no tests currently available to rice producers that determine whether N has been managed properly throughout the season or that detect instances of over fertilization. Rice stalk samples were collected from N response trials within 3 days of harvest from the primary Arkansas rice growing region in 2016-2018. Stalk samples were analyzed for N concentration through a KCl extraction and spectrofluorometric quantification. Samples analyzed with leaf material intact contained statistically different N concentrations than samples without leaf material ($P < 0.0001$). Although a large amount of variability was observed in N concentrations measured in the form of NH_4^+ and amino acids, N measured in the form of NO_3^- were more consistent in response to N rates. Samples collected lower on the rice stalk contained higher concentrations of N under excessive N fertilization conditions ($P < 0.0001$) indicating these samples are more tightly linked to N fertilization rates. These results suggest that rice stalk samples collected at the end of the growing season from the lower portion of the plant and analyzed for NO_3^- could be used to effectively determine if excess N was available to the rice plant. To further examine these results, additional samples were taken, analyzed in the same manner, and used to establish the relationship between stalk-N concentration and grain yield. Two models were used to describe this relationship: the linear plateau model and the quadratic plateau model. The linear plateau model was found to represent the data better than the quadratic plateau model (F-test: $P < 0.0001$, mean absolute error: 8.08 vs 8.27, RMSE: 0.128 vs 0.131, AIC: 3409 vs 3453). The join point of the linear response region and plateau region represents the critical tissue concentration at which no increase in yield is observed from addition N input. To promote fewer false negatives and ensure yield is not limited, the upper limit of the 95% confidence interval (2.1 mg NO_3^--N kg^{-1}) was selected as a practical agronomic threshold for the post-season tissue test. Rice producers can use the results of this tissue test to improve N management in future growing seasons or

this test can also be used to either confirm or eliminate N as the cause of undesirable yields for the current growing season.

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2	Hoegenauer, K.A., Roberts, T.L., Hardke, J.T., Ortel, C.C., & Slaton, N.A. (2020). Developing a post-season tissue test for rice nitrogen management. Agrosystems, Geosciences, and Environment, 3(1), e20134. https://doi.org/10.1002/agg2.20134	Published

Chapter 1: Introduction and Literature Review

Introduction

Arkansas is the leading producer of rice (*Oryza sativa L.*) in the nation accounting for about 49% of the total United States production (USDA-ERS, 2017). In 2015, Arkansas rice production accounted for approximately 1 billion dollars towards the state's economy (USDA-NASS, 2016). Arkansas rice production also has significant global impact as the United States is the twelfth largest rice producing country in the world (FAO, 2016). Nitrogen fertilizers are an important part of crop management and are one of the most expensive inputs in rice production systems. Farmers in Arkansas spent about 630 million dollars on fertilizers in 2012, which constitutes a significant portion of their annual input costs (USDA-NASS, 2017). According to USDA-NASS (2017), fertilizers are responsible for about 8 percent of operating expenses in Arkansas production systems. Since N is an essential nutrient and required in large quantities for growth and production, proper N management is crucial for sustainable crop production.

The typical N recommendation in Arkansas is 168 kg N ha^{-1} for rice on a silt loam soil. This base N rate is adjusted for rice cultivar, soil texture, and previous crop to provide a more accurate N rate for producers. Inadequate plant available N causes stunted growth and a reduction in yield. Excessive N applications can cause lodging (Kashiwagi et al., 2010) and increased disease pressure (Cu et al., 1996). Improper management of N can result in negative environmental effects, including the production of greenhouse gasses such as nitrous oxide and contamination of water supplies by leaching or runoff of nitrate (Cai et al., 1997). Due to difficulty in managing N because of various loss pathways, proper timing and rates of N applications are necessary for optimal yield and maximal profitability.

Soil testing can be used to predict N needs for the growing season given a specific yield goal. The Nitrogen Soil Test for Rice (N-STaR) was developed by Roberts et al. (2011) using the direct steam distillation method to quantify N that will be made available throughout the

growing season from N mineralization. It does this by measuring amino acids, amino sugars, and ammonium. N-STaR is able to provide an accurate N recommendation for pre-flood application in silt loam and clay soils in Arkansas. Although N recommendations based on N-STaR are accurate, some of the applied fertilizer can be lost via several N loss pathways resulting in inadequate N availability to the plants. It is important to evaluate the N status of rice to determine if a midseason-N application is necessary at the panicle initiation growth stage. Midseason-N applications can compensate for losses to the pre-flood application or errors in the pre-flood N recommendations. Midseason-N status can be evaluated using several techniques. One technique uses a chlorophyll meter to measure the chlorophyll content and correlates that information to the N status of the plant. From the reading given by the chlorophyll meter, predictions can be made whether the rice crop needs a midseason-N application or has sufficient N to facilitate adequate growth for the remainder of the season (Turner & Jund, 1991).

Another technique that is more cost effective but less accurate, is the use of leaf color charts. Leaf color charts allow a producer to estimate the N status and make midseason-N management decisions. Yang et al. (2003) reported a close relationship between SPAD values and leaf color chart scores. Recently, a technique has been developed to utilize Normalized Difference Vegetative Index (NDVI) to determine if a midseason-N application is needed. The NDVI values are taken from a N rich strip and compared to areas of normal N treatment to create a response index. The response index is then used to determine N requirement of the field to produce maximal yields.

Through the use of soil testing and midseason analysis technologies, accurate N rates can be determined. However, there are currently no post-season analysis techniques for verifying that the rice crop received the proper quantity of N to result in the desired yield goal. Nitrogen is thought of as the nutrient that determines plant growth and yield, therefore N is often the first to be assessed when trying to determine the yield limiting factor in a field with

inadequate yield. Without a method for analyzing N at the end of the season, there is no way to neither confirm nor rule out N as the yield limiting factor.

Literature Review

Arkansas Rice Production

Arkansas produces more rice than any other state and accounts for almost half of all rice produced in the United States (USDA-NASS, 2016). In 2016, Arkansas planted 625,644 hectares to rice which is 49 percent of the total area of rice in the nation. Arkansas has ranked number one in rice production since 1973. Arkansas rice production began in 1902 with a 0.4 hectare rice field. Rice production in Arkansas primarily takes place in the eastern half of the state and is often in rotation with soybean (*Glycine max*), where rice following soybean represents about 71 percent of Arkansas' rice acreage (Hardke & Wilson, 2012). Silt loam is the dominate soil texture for rice production in Arkansas accounting for about 53 percent of all soils produced to rice and clay and clay loam soils are responsible for approximately 43 percent. Planting dates in Arkansas range from late March to early June depending on weather conditions and the chosen cultivar to be planted. Conventional tillage is the primary method of seedbed preparation in Arkansas which encompasses roughly 55 percent of rice production in the state. Conventional tillage of rice in Arkansas consists of fall tillage and tillage again in the spring to prepare the seedbed. Only approximately 10 percent of the state currently practices no-till rice production.

Most rice producers in Arkansas operate in a dry-seeded, delayed-flood system. This system involves drilling or broadcasting seed onto a prepared seed bed and flooding between the 4 and 5 leaf growth stage. Only about 5 percent of the state uses a water seeded system (Hardke & Wilson, 2012). Drill-seeded rice accounted for about 80 percent of Arkansas rice in 2012 and the typical drill row spacing in Arkansas is 19 cm. Broadcast-seeding represents the remaining 20 percent of production, which includes both dry-seeded and water-seeded

approaches. The general seeding recommendation is 323 seeds per square meter for conventional varieties and 108-161 seeds per square meter for hybrid cultivars (Wilson et al., 2013). This recommendation is adjusted based on seeding method, soil texture, quality of seedbed preparation, and seeding date. For those farmers that broadcast seed (both dry-seeded and water-seeded), the seeding rate is increased by 20 and 30 percent respectively. In addition, rice planted on clay soils has a seeding recommendation of 20 percent more than the standard recommendation. Seeding rates are also increased for poor quality seedbeds and both early and late planting dates. The goal of these seeding recommendations is to achieve the optimum stand density of 108 to 215 plants per square meter for conventional cultivars and 65 to 108 plants per square meter for hybrid cultivars. In the case of below optimum stand densities, high yields can be achieved by applying additional N to increase tillering and ensuring proper control of weeds. Stand densities that are above optimum can result in increased disease and lodging.

Ideally, rice should be planted when the average daily soil temperature is 15.5°C taken at 10 cm soil depth (Wilson et al., 2013). Planting in a proper seedbed that provides good seed to soil contact at this temperature promotes good rice emergence and stand establishment. According to soil temperature measurements taken in major rice producing areas of the state, the soil temperature at 10 cm reaches 15.5 degrees between about April 8-16 in Arkansas. Numerous factors can affect the actual planting date. One of the largest factors is weather. Even when the temperatures are adequate for planting, precipitation can stall the process. Wet soil can be difficult to till or seed into, especially on heavier texture soils. Tilling and seeding on wet soils can also create additional compaction of the soil. Soil compaction reduces plant growth, development, and yields by limiting root elongation resulting in a reduction of nutrient and water uptake (Unger & Kaspar, 1994). Rice planted earlier requires longer to germinate and longer to reach the 5 leaf growth stage. Longer duration to the 5 leaf growth stage results in

greater production costs due to flushing and weed control before the flood can be established. Early seeding can also result in seedling stress and stand reduction (Wilson et al., 2013). Disease is also a concern when considering early seeding in rice. Early seeding can reduce the risk of severe damage of diseases such as blast, smuts, and panicle blight while increasing the risk of sheath blight. Late seeded rice should be planted so that the estimated date of 50 percent heading is before mid-September. Rice that reaches 50 percent heading after mid-September is at risk for reduced yield due to cool temperatures.

Groundwater is the most common irrigation source for rice production in Arkansas. Groundwater use for rice irrigation represents approximately 77 percent of rice acreage (Hardke & Wilson, 2012). The remaining 23 percent of irrigation is from surface water sources including reservoirs, streams, and bayous. As a result of growing concerns of irrigation water availability, farmers have begun to implement tailwater recovery systems to help reduce waste by collecting excess water in reservoirs and reusing it. Reservoirs are used on about 10 percent of rice acres in Arkansas (Henry et al., 2013). Multiple inlet irrigation has been growing in popularity in Arkansas. Multiple inlet irrigation is an irrigation technique that uses poly-tubing stretched perpendicular to levees across bays to decrease labor and water use. This system is used to deliver equal amounts of water to each bay across the field simultaneously. Three major advantages of multiple inlet irrigation are the ability to establish the flood quickly, reduced pumping cost, and ability to capture rainfall. Under a multiple inlet irrigation system it is important to limit water from spilling over levee gates, failure to do so results in increased pumping costs and water use. Approximately 39 percent of rice in Arkansas utilizes multiple inlet irrigation (Hardke & Wilson, 2012). Multiple inlet irrigation has been shown to increase irrigation efficiency regardless if straight levees or contour levees are used (Smith, 2007). Only about 0.6 percent of the state uses sprinkler or furrow irrigation for rice (Hardke & Wilson, 2012).

Rice irrigation requirements can exceed 900 mm (Lourence & Pruitt, 1971). Near the 4- or 5-leaf growth stage the permanent flood is established. This flood is usually maintained at 5 to 10 cm unless the presence of blast is detected or the field is at high risk for developing a blast problem. In this case, the flood is increased to a depth of 10 to 15 cm. Irrigation water quality is a growing concern in Arkansas. Irrigation from ground water sources in Eastern Arkansas typically has a high pH and soluble salts (Henry et al., 2013). Some common irrigation quality issues that arise in Arkansas are excessive sodium (Na), zinc (Zn) and phosphorous (P) deficiencies resulting from increasing soil pH with alkaline irrigation water, and accumulation of soluble salts. Excessive Na causes poor physical soil characteristics such as deflocculating soil aggregates resulting in poor soil structure (Frenkel et al., 1978). Zinc and P deficiencies are typically caused by high soil pH. In the case of Arkansas, the high soil pH is a result of irrigation water that is high in calcium (Ca) and magnesium (Mg). These two elements form calcium bicarbonate and magnesium bicarbonate which increase the soil pH when the irrigation water is applied. Accumulation of soluble salts can lead to chloride (Cl) toxicities in soybeans that follow rice. Excess Cl can also damage rice seedlings due to the salt sensitivity of seedling rice (Khan et al., 1997). Managing the salinity and alkalinity of the soil is important to rice production in Arkansas.

As with most crops, plant nutrition is critical to achieving high yields and quality grain in rice production. Arkansas rice production is based on a two-way split N application strategy (Norman et al., 2003). Pre-flood N applications are made just before the permanent flood is established (4- to 5-leaf growth stage). Pre-flood N recommendations are made based on soil testing or the standard recommendation that uses soil texture, rice cultivar, and previous crops. Mid-season N applications are made when the permanent flood has been established for at least three weeks and the rice is at the internode elongation growth stage. Pre-flood N rates typically range from 90 to 170 kg N ha^{-1} and the standard mid-season N rate is 50 kg N ha^{-1}.

Common N fertilizer sources for Arkansas rice production are ammonium sulfate and urea. Urea that is applied pre-flood is often treated with a urease inhibitor to prevent N loss. It is important to use routine soil tests to evaluate the status of available nutrients in the soil. Phosphorous needs can be difficult to determine due to the alternating aerobic-anaerobic environment created by rice production (Xu et al., 2020).

Pest management is another key tool to producing a successful yield. Disease can only occur when a virulent pathogen is present, a susceptible variety is planted, and the environment is conducive (Wamishe et al., 2013). Some common diseases that impact rice production in Arkansas are sheath blight, blast, bacterial panicle blight, and black sheath rot. Sheath blight thrives on short rice varieties with a full, over-lapping canopy. Sheath blight is more prevalent under high N rates and hot (26.7 to 32 °C during the day and >23.3 °C at night) and humid weather conditions. Blast favors a moist environment due to the necessity of free moisture on the plant for infection by the fungal spores. This environment could be caused by a long periods of dew, fog, or frequent light rains. Blast responds to slightly cooler temperatures than sheath blight. Late seeding dates in fields where dew or fog occurs promotes the infection and spread of blast. Bacterial panicle blight can be hard to manage because although the disease may be present all season, symptoms do not appear until heading and there are currently no labeled chemicals to treat bacterial panicle blight. High night time temperatures, high N rates, water stress, high seeding rate, and late seeding can increase the risk of bacterial panicle blight developing. Black sheath rot can develop under dense rice stands and high N rates. Yield loss caused by black sheath rot on the commercial scale appears to be negligible although yield losses of 20 percent have been observed in research plots. In 2012, 46.2 percent of rice land area received foliar fungicide applications (Hardke & Wilson, 2012). Three major insect pests in Arkansas rice production are rice water weevil, rice stink bug, and grape colaspis (lespedeza worm). Planting date, rice stand, fertilization, weed control, and water management all play

significant roles in insect populations in rice fields. Foliar insecticides are recommended to be used only when significant yield or quality losses are expected (Lorenz & Hardke, 2013). Producers used foliar insecticide on 29 percent of rice in 2012, while seed treatment insecticide was used on approximately 58 percent (Hardke & Wilson, 2012).

Nitrogen in rice

Nitrogen represents the largest nutrient requirement in rice production. Sufficient Nis crucial for producing a rice crop of high quality and yield. Insufficient plant available N can be caused by inadequate N application rates or improper N management. Insufficient N causes stunted plant growth and decreased chlorophyll concentration which results in less photosynthetic activity. Nitrogen is used to form amino acids, is a major constituent of chlorophyll, and is critical in the production of biomass.

Over-application of N can decrease profits by lowering yield and increasing production costs. When excess N is present, rice is more susceptible to lodging (Berry et al., 2002) and diseases (Savary et al., 1995) than optimally fertilized rice. Environmental concerns arise when N fertilizer is over-applied and can leach out of the root zone and permeate ground water supplies (Ju et al., 2006). The leaching of N is rarely a concern in rice production. Rice fields create specific and unique environmental challenges concerning the application and management of N fertilizers. Due to the anaerobic bacteria that operate in the flooded environment of a rice field, N fertilizers can be converted into the greenhouse gas nitrous oxide (Smith et al., 1982) and influence methane release into the atmosphere (Cai et al., 1997). Linquist et al. (2012a) showed an increase in N_2O production as N fertilizer rate increases; however, did not find any trend between N rate and CH_4 in rice.

Proper N fertilization is important for yield, lodging, disease, environmental concerns, and farmer profitability. Site Specific N Management (SSNM) is a N management system designed to increase N use efficiency by prescribing N recommendations on a field-to-field

basis. This method gives a custom N management plan for each field that is specific to the conditions and needs of that particular field. There are four key steps in estimating total N rate under SSNM: (1) set a realistic yield goal, (2) estimate quantity of N that will be supplied by the soil throughout the growing season, (3) estimate N response of the rice, (4) develop recommended N application rate (Peng et al., 2010). Site-specific N management can be summarized as a technique that creates N recommendations based on indigenous N supply and the upward or downward adjustment of mid-season N applications based on leaf N status. Surveys and field studies in China have shown that SSNM and other improved N management techniques increase grain yield and fertilizer use efficiency when compared to conventional farmer practices (Hu et al., 2007).

Arkansas currently uses two methods of N recommendations in rice production. One of these methods is a blanket application that assumes that each field with the same soil texture, cultivar, and previous crop will require the same N application. Using this method, the standard recommendation – unmodified for cultivar and previous crop - for a silt loam soil is 168 kg N ha^{-1}. The second method for making N recommendations is using the Nitrogen Soil Test for Rice (N-STaR) developed by Roberts et al. (2011). The N-STaR program provides an estimate of N that will be made available to the rice during the growing season. From this estimate, N application rates can be adjusted to improve yield and farmer profitability.

Soil Nitrogen Interactions

As discussed earlier, accounting for soil-supplied N is important for proper implementation and success of SSNM. Soil-supplied N is not accounted for in the blanket application method. Depending on the soil characteristics, this can represent a large pool of N that should be taken into consideration when making recommendations. Using the standard N fertilizer rates can result in either over or under application of N depending on the amount of soil-supplied N and the yield goal (Roberts et al., 2013). Quantity of soil-supplied N varies

greatly from location to location and is influenced by factors such as: soil organic matter, soil texture, tillage practices, pH, and cropping history (Franzluebbers et al., 1995; Sollins et al., 1984; Cabrera et al., 2005).

Mineralization and immobilization are the two primary processes responsible for the availability of N. Mineralization is the process of transforming organic-N to inorganic-N which is plant available. Immobilization is the opposite process of mineralization, transforming inorganic-N to organic-N. Net mineralization is responsible for the amount of N supplied by the soil. Mineralization is a microbially mediated process, therefore is subject to the environmental and soil conditions (Cabrera et al., 2005). In a rice field, this environment is stabilized by the flood, making it easier to predict the amount of net mineralization that will occur during the growing season.

Standard two-way split and optimum pre-flood (single pre-flood) are the two application methods used in Arkansas rice production. The optimum pre-flood method requires only one N application made immediately before the flood is established. This method should be used only on fields that are small enough and have the irrigation capacity to flood the field in a timely manner directly after the N application is made. In cases where the flood is not established in a timely manner, significant N can be lost. Optimum pre-flood is not the recommended method for hybrid cultivars or lodging susceptible pure-line cultivars due to lodging concerns. The standard two-way split method requires a pre-flood and mid-season N application. Mid-season applications are made between the panicle initiation and panicle differentiation growth stages. In this method pre-flood applications are 65 to 100 percent of the total N recommendation with the remaining percentage being applied at midseason. Properly managed pre-flood applications are essential to yield. Whether the optimum pre-flood or two-way split application method is used, when the pre-flood N is not managed properly, the mid-season application cannot recover all of the lost yield potential. Fertilizer uptake for mid-season applications is greater than pre-flood

applications (Wilson et al., 1989; Moore et al., 1981). This is likely due to the rice plant having a larger, more developed root system than when the pre-flood application is made, as well as fewer N loss mechanisms. Data from Wilson et al. (1989) shows uptake of the pre-flood application taking 21 days to complete while complete uptake of the mid-season application taking less than 7-10 days. Reddy and Patrick (1980) showed a relationship between N uptake and grain yield in rice, indicating the importance of N management in rice production systems.

Ammonium or ammonium-forming fertilizers are recommended to reduce N losses in rice production systems. Common N sources for rice production are urea (46% N) and ammonium sulfate (21% N). Urea is a popular choice for having a high N content and relatively low cost. However, urea is prone to ammonia volatilization losses. Norman et al. (2009) reported that 15 to 25 percent of applied urea was lost to ammonia volatilization within 10 days of application. Establishing the flood helps to incorporate the urea into the soil profile, therefore reducing losses caused by ammonia volatilization and denitrification. This indicates the importance of establishing the flood in a timely manner. Urease enzyme inhibitors can be used to reduce the amount of urea lost to ammonia volatilization. Urease inhibitors work by competing with the urease enzyme for the active site therefore slowing the rate of urea hydrolysis. The urease inhibitor N-(n-butyl) thiophosphoric triamide (NBPT) is commonly used in crop production. Rawluk et al. (2001) showed significant reductions in ammonia volatilization when urea was treated with NBPT. Norman et al. (2009) found that urea treated with NBPT delayed most ammonia volatilization until 5 to 10 days after application. Although total ammonia volatilization losses were still significantly less for the NBPT treated urea compared to other N sources, establishing the flood in a timely manner is still very important when using a urease inhibitor. Ammonium sulfate is comparatively lower in N content and higher in price. Ammonium sulfate is at a lowered risk of N loss due to the acidic environment created by sulfate reactions. Ammonia volatilization losses of ammonium sulfate are minimal in comparison to urea. Norman

et al. (2009, 2004) showed ammonia volatilization loss of less than 5 percent in ammonium sulfate fertilizers.

Proper N management can be difficult due to various loss pathways and multiple forms of N in the soil. Ammonia volatilization and denitrification are two of the major N loss pathways of concern in rice production. Ammonia volatilization is the loss of ammonia gas from the system. Soil moisture, high temperatures, high pH, low cation exchange capacity, and wind contribute to an environment that increases risk of ammonia volatilization. Nitrification is the aerobic process of converting of ammonium to nitrate which requires the enzymes nitrobacter and nitrate reductase.

Denitrification is the loss of nitrate from the system in the form of N_2 or N_2O gasses. Denitrification can only occur under anaerobic conditions such as a flooded rice field. Significant loss can occur under the following conditions (i) if the flood is not applied in a timely manner, (ii) if the flood is lost and reestablished, (iii) if a fertilizer containing nitrate is used. In the absence of a permanent flood, ammonium is converted to nitrate. Once the flood is applied or reapplied, any nitrate in the system is lost to the atmosphere via denitrification. Under flooded conditions, Wilson et al. (1989) did not detect any nitrate in soil samples and from this concluded there was not sufficient oxygen present for nitrification to occur and any nitrate present had been quickly taken up or lost via denitrification. Studies have shown insignificant amounts of N_2O captured from midseason fertilizer applications made to flooded rice fields (Simpson et al., 1984; Smith et al., 1982). However, Linquist et al. (2012b) conducted a meta-analysis and concluded that drained fields produce higher N_2O emissions than continuously flooded fields. Nitrate is the N form associated with leaching due to its negative charge. Since nitrate is rarely found in flooded rice fields, leaching is rarely a N loss mechanism of concern in rice production. Simpson et al. (1984) recorded a negligible amount of leached fertilizer N below the top 100 mm of soil. Products of denitrification can have a significant impact on the environment. Nitrous oxide is

known to be a large contributor to the depletion of the ozone (Ravishankara et al., 2009). Flood management is critical to limiting the effects of these N loss mechanisms.

Plant Nitrogen Interactions

The first step in nutrient uptake into plant roots is the diffusion of nutrient ions into the apparent free space of the root from the soil solution. Ions can diffuse freely into the apparent free space without crossing a membrane. The apparent free space has been empirically measured to be approximately the same volume as the apoplastic space outside of the Casparian strip. Hydrophobic compounds make up the Casparian strip which blocks ion movement to the xylem through the apoplast. Two primary functions of the Casparian strip are to (i) prevent unwanted ions from entering the xylem and (ii) restrict movement of desired ions back out of the root. Rice roots have been shown to contain two Casparian strips (Tabuchi et al., 2007). With the Casparian strip blocking the apoplastic pathway in the endodermis, the ions must rely on symplastic movement. This forces the ions to cross a membrane and enter a root cell either in the epidermis or endodermis. Ion movement into the symplast is typically carried out by ion specific channels and pumps against a concentration gradient. This process usually requires the expenditure of energy and leads to the accumulation of ions in the symplast. Ion movement between cells is almost always carried out by symplastic connections, specifically plasmodesmata. Next, the ions are actively secreted into the xylem for long distance transport to other portions of the plant. Depositing ions into the xylem is done against a concentration gradient and is sensitive to metabolic inhibitors and the plant hormones abscisic acid and cytokinin.

Uptake of nitrate is insignificant during periods when the field is flooded. However, nitrate is available to the plant prior to the establishment of the flood when the soil is aerobic. Nitrate uptake is made possible by carrier proteins that are inducible, meaning that the presence of external nitrate causes the root to synthesize new carrier proteins. In rice, the low-affinity

transport system (LATS) is mediated by the OsNRT1 gene and is expressed in the epidermis and root hairs (Lin et al., 2000). Two gene families interact to make up the high affinity transport system (HATS) (Masclaux-Daubresse et al., 2010). OsNRT2 and OsNAR2 gene families are responsible for this system in rice. Three out of the four genes in the OsNRT2 family are nitrate inducible. OsNAR2 is composed of two genes (Araki & Hasegawa, 2006). Once nitrate has made it into the root, it can be stored in a vacuole or translocated in the xylem. However, nitrate must be reduced to ammonium before it can be assimilated into the plant (Masclaux-Daubresse et al., 2010). The first step in this reaction is the reduction of nitrate to nitrite by the enzyme nitrate reductase. Nitrite is toxic to plants so it is rapidly reduced to ammonium by the enzyme nitrite reductase.

Ammonium is the dominant form of N taken up by rice in a flooded system. It is hypothesized that uptake of ammonium could be through a channel. Evidence has even been found that potassium channels can be used for ammonium ions to enter plant cells (Loqué & von Wirén, 2004). It has also been suggested that aquaporins could be used for transport across plasma membranes and for accumulation in the vacuole (Miller & Cramer, 2004). Ammonium movement in rice is controlled by four gene families OsAMT1, OsAMT2, OsAMT3, and OsAMT4. OsAMT1 genes are responsible for the high-affinity transport system, while the other three gene families are responsible for the low-affinity transport system (Sonoda et al., 2003a; Loqué & von Wirén, 2004).

The xylem contains small quantities of ammonium. Approximately 11 percent of N in the xylem is in the form of ammonium. It is common for ammonium to be transformed into amino acids for long distance transport in the xylem and phloem. Mechanisms for the loading of ammonium into xylem are not currently defined. Total plant amino acid concentrations have been shown to be higher in ammonium fed plants when compared to nitrate fed plants (Britto &

Kronzucker, 2002). Glutamine and asparagine are the amino acids found in the highest concentrations in plant tissue.

Accumulation of ammonium at high concentrations can be toxic to plants and results in chlorotic and necrotic leaf tissue, stunted growth, and plant death. The mechanisms of ammonium toxicity are unknown. It has been hypothesized that ammonium toxicity is caused by a decrease in the uptake of essential cations like K^+, Ca^{2+}, and Mg^{2+}. Other factors contributing to ammonium toxicity include issues regulating pH and excessive consumption of sugars during the ammonium assimilation process. Highly speculative studies have linked ammonium content with altered hormone production. These studies conclude that ammonium accumulation induces ethylene production and subsequent stress responses (Barker, 1999). Rice has been described as ammonium tolerant when compared to an ammonium sensitive crop such as barley (*Hordeum vulgare*). Influx of ammonium into rice is much lower than influx into barley and other ammonium sensitive plants. These ammonium sensitive plants release ammonium back into the soil whereas ammonium tolerant plants regulate the influx of ammonium (Britto & Kronzucker, 2002). It is thought that plants have a mechanism to sense ammonium concentrations and respond by transporting excess ammonium out of the plant or storing in the vacuole, but these mechanisms have not currently been defined.

Whether N is taken up as ammonium or as nitrate and reduced to ammonium, the assimilation process is the same. Ammonium assimilation takes place as a two-step process. First, ammonium is combined with glutamate to form glutamine in the following reaction:

$$\text{glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + P_i$$

Expenditure of one ATP molecule is necessary for the assimilation of every ammonium molecule. This reaction is catalyzed by the enzyme glutamine synthetase (GS). Next, two

glutamate molecules are formed by transferring the amide group to α-ketoglutarate (2-oxoglutarate) as seen in the following reaction:

$$\text{glutamine} + \alpha\text{-ketoglutarate} + \text{NADH} \rightarrow 2\text{glutamate} + \text{NAD}^{+}$$

This reaction requires the reducing potential of NADH and is catalyzed by the enzyme glutamate synthase (GOGAT). One of the glutamate molecules produced in this reaction is recycled to be used in the first reaction. The second glutamate molecule can be exported, stored, or used for biosynthesis of proteins. The enzymes GS, GOGAT, and asparagine synthetase (AS) also have valuable functions during senescence. These enzymes are responsible for the remobilization of N from senescing tissue to new sinks. Amino acids, proteins, and other N structures are broken down and transformed into transportable forms by the previously mentioned enzymes (Tabuchi et al., 2007; Masclaux-Daubresse et al., 2010). Mae (1986) reported that 70% of the N in the grain originates from remobilization of N during senescence. Glutamine dehydrogenase (GDH) also has functions in leaf senescence as well as N assimilation during dark conditions (Masclaux-Daubresse et al., 2010)

There are two forms of glutamine synthetase in plants. The first form is cytosolic GS1 and is used for leaf senescence and the synthesis of glutamine for transport in the phloem. The second form is chloroplastic GS2 and is responsible for synthesizing glutamine from ammonium produced during photorespiration. The gene families GLN1 and GLN2 code for the GS1 and GS2 isoforms respectively.

Plants also contain two forms of glutamate synthase. Ferredoxin is used by Fd-GOGAT as an electron donor. Similarly, NADH is used as a donor by NADH-GOGAT. Fd-GOGAT can be found primarily in the leaf chloroplast. NADH-GOGAT is typically located in non-photosynthetic tissue such as the roots and seems to be responsible for the synthesis of glutamine during periods of normal growth (Masclaux-Daubresse et al., 2010). Asparagine

synthetase can act as an ammonium assimilation compliment to the GS/GOGAT cycle under certain situations. It is thought that asparagine could be used for long-range transport or storage due to asparagine having a higher N/C ratio than glutamine (Masclaux-Daubresse et al., 2006).

Sonoda et al., (2003b) has shown that glutamine acts as a signaling molecule for the up and down regulation of OsAMT genes. Similar effects were seen when using asparagine instead of glutamine. This suggests that amino acids, particularly glutamine and asparagine, are mostly responsible for regulating the uptake of ammonium. Wang et al., (1993) have evidence to suggest that LATS may not be subject to the negative feedback of ammonium supply. One possible explanation for this is that when more N is taken up plants are larger and have more photosynthetic tissue that produces more carbon skeletons for the assimilation of N.

Glutamine is also thought to be responsible for the regulation of ammonium assimilation. The actual mechanisms for amino acid sensing and regulation of GS and GOGAT are unknown. Bacteria have been shown to contain PII proteins that have functions in N sensing and regulation through uridylyation (Ninfa & Jiang, 2005). It has been hypothesized that similar PII proteins are present in plants and have similar functions. Plant-like PII proteins have been found in *Arabidopsis,* Castor bean (*Ricinus communis)* (Hseih et al., 1998), and rice (Sugiyama et al., 2004). GLB1 is the gene that encodes these plant-like PII proteins. GLB1 expression is modified by light exposure and plant metabolites. Hsieh et al. (1998) further concluded that the plant-like PII proteins found in *Arabidopsis* and Castor bean may be a component involved in the signaling of carbon and N status through phosphorylation. Tabuchi et al., (2007) believe that plants have a different N sensing system from bacteria due to the fact that the plant-like proteins found in *Arabidopsis,* Castor bean, and rice lack a uridylyation site. Further research is needed to describe the N sensing mechanisms as they pertain to the GS/GOGAT cycle.

Light has also been shown to alter the levels of GS at the transcriptional and post-transcritptional levels (Cren & Hirel, 1999). Experiments conducted using differing wavelengths

of light show the involvement of phytochrome and blue-light photoreceptors in the regulation of GS. A response was also seen to sucrose suggesting that sugars could have a role in regulating N assimilation. It is thought that light has an indirect effect on GS levels and that it is more likely to be photosynthetic activity or sugars that are actually used to regulate GS function. Availability of carbon skeletons is another factor in regulating N assimilation. Thus, anything that limits the photosynthetic process, such as temperature, can have an effect on GS levels in the plant.

In general, plants tend to accumulate N in the vegetative tissue when plant available N is not limiting to plant growth (Millard, 1987). This luxury consumption of N results in accumulation of different N forms including amino acids (Näsholm et al., 1994), ammonium (Ludewig et al., 2007), nitrate (Hanway & Englehorn, 1958), and proteins (Warren et al., 2003). Grasses have been shown to contain increasing concentrations of N in response to increased N supply (Murphy & Smith, 1967; Zhen & Leigh, 1990; Ntanos & Koutroubas, 2002). In rice, lower leaves and stems accumulated N until approximately 55 days after emergence then decreased slightly until reaching maturity while upper leaves and stems increased internal N concentration until 55 days after emergence then remained at a constant concentration (Norman et al., 1992). This suggests that rice translocates N from the lower portion of the plant during the reproductive stage and that soil N supply was limiting to growth. Similarly, Guindo et al., (1994) reported that no additional N was accumulated between 21 days after heading and maturity when applied as a single pre-flood application. Wang et al., (2006) showed differences in N concentration for protein-N and non-protein-N for individual upper leaves across increasing N rate and multiple cultivars. The general trends described by the data are (i) plant N concentrations increase with increasing N rate (ii) upper leaves accumulate more N than leaves lower on the plant (iii) there are slight differences in the quantity of N accumulated between cultivars.

Corn Stalk Nitrate Test

The Corn Stalk Nitrate Test (CSNT) is a post-season test created in Iowa to determine when insufficient or excess N was applied to corn (*Zea mays*). Binford et al. (1990) determined nitrate concentrations in a segment from the lower portion of the corn stalk by shaking 0.25 g of tissue in 100 mL of 2 M KCl. These procedures produced relationships that could best be described by the linear-response-and-plateau and the quadratic-response-and-plateau models. From these models, critical concentrations of nitrate accumulation were determined. The critical concentration represents a nitrate concentration where little to no additional yield is expected with increasing N fertilizer application. Variability in the slopes of yield over concentration were observed, but were determined to not invalidate CSNT. However, this variability in slopes means the quantity of excess N in the plant cannot be determined.

Procedures for the CSNT are to remove a 20 cm section of the corn stalk between 15 and 35 cm above the ground. Fifteen of these sections are required to make up one sample. Areas that are more variable require more samples. Samples should be taken between one and three weeks after black layer formation on about 80 percent of the kernels (Blackmer & Mallarino, 1997). Isla & Blackmer (2007) describes the process of developing sampling procedures by Binford et al. (1990) as the result of arbitrary decisions made during the exploratory studies and goes on to evaluate the effects of changing the number and length of stalks collected per sample. From the data collected, it was concluded that when less than 10 stalks are used per sample, the size of the confidence interval increases dramatically. Nitrate concentrations in 4 cm segments were shown to be correlated to nitrate concentrations in full 20 cm segments (R^2=0.99). Therefore, 4 cm segments could be used in place of 20 cm segments under the condition that the segment is taken in the middle (18-22 cm) of the original sampling location (Isla & Blackmer, 2007). The use of 4 cm segments instead of 20 cm segments needs to be further tested for accuracy in field experiments.

Wilhelm et al. (2005) also examined the CSNT sampling procedures and their data showed no difference in nitrate concentration between the node and internode of corn stalks. Samples collected 5 cm above or below the standard CSNT sample location (15-35 cm above soil) contained only a 10 to 15% difference in nitrate concentration when compared to the standard sample. It was further proposed that critical concentration values could be adjusted by 15% to accommodate sampling error of up to 5 cm. The new critical concentrations would be 640 to 860 and 1700 to 2300 mg NO_3-N kg^{-1}. This is supported by the idea that critical concentrations should not be defined too precisely as proposed by Binford et al. (1992). This evidence further supports the claim of Isla and Blackmer (2007) that the sampling height on the corn stalk was determined based on arbitrary decisions.

Procedures for CSNT dictate that stalk samples should be taken in the three weeks following blacklayer. Formation of the blacklayer in corn grain represents physiological maturity and the end of N movement to the grain. Brouder et al., (2000) evaluated the effects of premature stalk sample collection. Samples were taken weekly from mid R5 to post-blacklayer. Most site years showed a higher nitrate concentration before maturity which is consistent with the idea that N is still being translocated to the grain. Results from linear regression analysis showed high correlation ($r > 0.79$), but highly variable coefficients. Data showed that for site years with lower soil N availability, nitrate concentrations declined more rapidly during the five weeks.

Objectives

There is a growing concern for the environment as well as food production around the world. These concerns have provoked a movement to more accurately determine fertilizer requirements for crops to limit the amount of excess fertilizer used while increasing yield and profits for producers. Although rice and corn have different production practices, similarities in N uptake, assimilation, and accumulation provide evidence to suggest that a test similar to the CSNT could be developed to determine N status at the end of the growing season in rice. The research objectives are:

1. Examine correlations in N concentration
    a. Determine an appropriate sampling position on rice stem.
2. Diagnostic test development
    a. Use a model to describe relative yield as a function of plant N concentration.
    b. Determine critical concentration levels based on yield response.
    c. Develop management categories based on critical values to describe expected yield response.

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Chapter 2: Developing a Post-Season Tissue Test for Rice Nitrogen Management

Abstract

No post-season analysis method currently exists for rice (*Oryza sativa* L.) to determine whether N was managed properly during the growing season. Our primary research objective was to determine whether post-season, rice stem N concentrations can be used to predict if sufficient or excessive N was available. Aboveground biomass samples were taken from N response trials at harvest. Stalks were separated into 5 cm segments starting at the soil surface and extending upwards to 45 cm. One-half of the samples had all of the leaf material (leaves and leaf sheaths) removed, while the other half remained intact. All samples were analyzed for N concentration in the form of amino acids (AA-N), ammonium (NH_4^+-N), and nitrate (NO_3^--N). Samples analyzed with leaf material were found to be statistically different from samples analyzed without leaf material for all three forms of N ($P < 0.0001$). Due to significant variability in response, AA-N and NH_4^+-N were discovered to be incompatible for a post-season tissue test in rice. However, NO_3^--N concentrations showed strong consistency in response to N fertilization rates. Upon further analysis, NO_3^--N concentrations in the lower portion of the rice stem were found to be more responsive to excessive N fertilization than the upper sections of the rice stem ($P < 0.0001$). These results suggest that rice stalks sampled from the lower portion of the plant near maturity and analyzed for NO_3^--N could be used to predict over-fertilization and should be investigated further.

Introduction

The anaerobic environment created by the continuous flood in direct-seeded, delayed-flood rice (*Orzyza sativa L.*) production creates specific and unique challenges in the application and management of fertilizer N. Improper application timing, the wrong fertilizer source, excessive application rate, and improper management of N fertilizer can negatively affect the yield and profitability of a rice production system as well as contribute to environmental issues such as greenhouse gas emissions. These impacts can be broken down into two main categories: environmental and economical. In rice production, excess N fertilizer can influence the production of greenhouse gasses such as methane and nitrous oxide (Linquist, et al., 2012; Pittelkow et al., 2013). Leaching of N is rarely of concern in direct-seeded, delayed-flood rice production due to ammonium being the dominant form of N added as fertilizer and the primary inorganic form found in anaerobic soils. Negative economic impacts associated with improper N management are concerning as they influence producer profitability. Over-application of N can result in increased disease pressure and higher instances of lodging of rice plants which contributes to a difficult harvest as well as reduced yield (Walters & Bingham, 2007). Applying excessive N also represents an unnecessary added input expense regardless of its effect on rice yield and lodging. Reductions in yield and grain quality in combination with the cost of excess N can result in overall decreased profit for producers, which underscores the importance of proper N management.

Applying N fertilizer at rates greater than what is needed to maximize yield results in the accumulation of excess N in the vegetative tissue of plants (Millard, 1987). This accumulated N has been found in the form of amino acids (AA-N) (Näsholm et al., 1994), ammonium (NH_4^+-N) (Ludewig et al., 2007), nitrate (NO_3^--N) (Hanway & Englehorn, 1958), and proteins (Warren et al., 2003). A positive correlation between N concentrations in the vegetative tissues and N supply in grasses has been observed in many research trials (Murphy & Smith, 1967; Zhen &

Leigh, 1990; Ntanos & Koutroubas, 2002). In a study conducted by Norman et al. (1992), N accumulation in the lower leaves and stems increased until approximately 55 d after emergence then decreased slightly until maturity while N accumulation in the upper leaves and stems increased until 55 d after emergence and then remained constant until maturity. These results demonstrate the remobilization of N from the lower portion of the plant to the upper portion during reproductive growth. In a similar study by Guindo et al. (1994), results showed no additional N accumulated in aboveground biomass between 21 d after heading and maturity. In addition, upper leaves have been shown to accumulate more N than lower leaves and slight differences have been observed in the amount of N accumulated among different cultivars (Wang et al., 2006).

Binford et al. (1990) outlined the procedures for a post-season NO_3^--N test in corn (*Zea mays* L.), named the Corn Stalk Nitrate Test (CSNT) that is used to help manage N in corn production systems across the U.S. Stalk samples are removed from the lower portion of the corn plant at physiological maturity and analyzed for NO_3^--N concentration. Nitrate concentrations compared to relative yield produced relationships that were described as linear-response-and-plateau. Stalk NO_3^--N concentrations remained low until yield was maximized and then NO_3^--N began to accumulate rapidly without an associated increase in yield. From this response curve, a critical stalk NO_3^--N concentration was established to represent the NO_3^--N concentration in which little to no increase to yield is expected from the addition of N fertilizer.

Wilhelm et al. (2005) attempted to further investigate the NO_3^--N concentration as it changed vertically in the corn stalk as well as determining if NO_3^--N differences could be detected for node and internode tissues. They determined that NO_3^--N concentration decreased with increasing height above the soil surface. Nitrate concentrations in the nodes were shown to not be significantly different from the segment closest to the node nor the average internode concentration. However, a difference in the NO_3^--N concentration was observed between the

internode segments closest to the node and the internode segments furthest from the node, indicating that NO_3^--N concentration can vary within an internode. Data from samples collected 5 cm closer to (10 to 30 cm) and further from (20 to 40 cm) the soil surface than the recommended sampling height of 15 to 35 cm by Binford et al. (1990) showed a difference of about 10 to 15 % in the NO_3^--N concentration. From data collected by Willhelm et al. (2005), it was suggested that samples could be taken at heights other than that established by Binford et al. (1990) and still accurately predict when N was limiting or excessive provided that the critical concentrations were adjusted to reflect the change in sampling height. Isla and Blackmer (2007) concluded that the sampling height selected by Binford et al. (1990) was made as the result of arbitrary decisions while exploring the potential of a stalk NO_3^--N test. Similar to results from Wilhelm et al. (2005), no difference was found between the NO_3^--N concentrations of the node and internode (Isla & Blackmer, 2007). Data from Isla and Blackmer (2007) showed that within the 15 to 35 cm sample, the upper third contained approximately 55% of the NO_3^--N concentration when compared to the lower third of the sample. Isla and Blackmer (2007) also observed that no appreciable difference was detected in a 4 cm sample taken near the middle of the 15 to 35 cm range when compared to the concentration of the entire 20 cm sample. Additionally, they determined that sampling a stalk 1 cm different from the recommended 15 to 35 cm results in approximately a 4% error when the recommended sampling height concentration is equal to 1000 mg N kg^{-1}. The ability to buffer a slight error in sampling height and differences in hybrids add to the practical robustness of this test (Wilhelm et al., 2005; Isla & Blackmer, 2007).

The primary objective of this study was to evaluate the potential use of N concentrations analyzed as AA-N, NH_4^+-N, and NO_3^--N in rice stems at the end of the season for determining if deficient, adequate, or excessive N was made available to the plant to optimize rice grain yield. To accomplish this, we developed secondary objectives which include: (i) determine if

differences exist in the N concentration of samples with all leaf material intact compared to samples in which the leaf material had been removed, (ii) explore the concentration differences between forms of N measured in the rice stem including NH_4^+-N, NO_3^--N, and AA-N, and (iii) identify if N concentrations in the aboveground biomass vary as a function of height above the soil surface.

Materials and Methods

Site Description and Plot Management

This research was conducted at two locations in 2016, the Pine Tree Research Station (PTRS) near Colt, Arkansas and the Rice Research and Extension Center (RREC) near Stuttgart, Arkansas. The soil at PTRS is a Calhoun silt loam (fine-silty, mixed, active, thermic Typic Glossaqualf). Soil test values for this site were 31 mg P kg^{-1}, 76 mg K kg^{-1}, 1.3 mg Zn kg^{-1}, and a pH of 7.7. While the soil at RREC is a Dewitt silt loam (fine, smectitic, thermic, Typic Albaqualf). Soil test values for this site were 21 mg P kg^{-1}, 105 mg K kg^{-1}, 5.1 mg Zn kg^{-1}, and a pH of 6.4. Plots were arranged in a randomized complete block design with four replications. Plot dimensions were 1.7-m wide by 4.9-m long and 1.7-m wide by 5.2-m long for PTRS and RREC, respectively, with plots at each location containing nine rows of rice spaced 19 cm apart. 'LaKast', a pureline cultivar, was drill seeded at a rate of 84 kg ha^{-1} at PTRS and 67 kg ha^{-1} at RREC. The second cultivar planted was the hybrid 'RiceTec XL 753', which was drill seeded at 28 kg ha^{-1} and 22 kg ha^{-1} for PTRS and RREC, respectively. All the planted rice seed was treated with Clothianidin 478 g a.i. kg^{-1} as NipsIt INSIDE (Valent, Walnut Creek, CA) insecticide seed treatment at a rate of 1.25 mL kg^{-1} seed prior to planting to ensure adequate emergence and growth.

Plots were managed under common direct-seeded, delayed-flood rice production practices for silt loam soils in Arkansas as recommended by the University of Arkansas Cooperative Extension Service (Hardke, 2019). Six N rate treatments (0, 50, 101, 151, 202, 252

kg N ha^{-1}) were applied at PTRS while plots at RREC only received five N rate treatments (0, 50, 101, 151, 202 kg N ha^{-1}) applied as a single pre-flood application at the four to five leaf stage. The N source used in this study was urea (460 g N kg^{-1}) treated with Agrotain 267 g a.i. kg^{-1} (Koch Industries, Wichita, KS), a N-n-butyl thiophosphoric triamide (NBPT) urease inhibitor at a rate of 3.1 mL of Agrotain kg^{-1} urea. The flood was established within 3 d after pre-flood N application and was maintained at a depth of 10 to 12.5 cm until maturity. In addition to N, preplant applications of P, K, and Zn were made at rates of 29 kg P ha^{-1}, 84 kg K ha^{-1}, and 11 kg Zn ha^{-1} at both locations to ensure nutrients other than N were not yield limiting.

Sampling Methods and Statistical Analysis

Total aboveground biomass samples were taken from each plot within 3 d of harvest, which occurred at an average grain moisture content of 14%, to ensure all translocation of N within the plant had been completed. A 1 m sample was collected from an interior, bordered row of rice to represent the aboveground biomass. The biomass samples were oven dried at 60°C to a constant weight. The biomass for each plot was divided in half for separate analysis. The stems of each biomass subsample were cut into 5 cm segments starting at the soil surface and extending up the stalk to 45 cm to produce a total of nine segments. Once cut, each stalk segment was treated as an individual sample. One-half of the biomass from the plot (nine segments) was stripped of all leaves and leaf sheaths from each segment where only the stem remained, while the leaf material remained intact for the other half of the samples. This resulted in a stem only sample and an intact stalk sample (stem + leaves) for each 5 cm increment from the soil surface to 45 cm for each plot. Average plant heights for optimally fertilized rice plants according to Hardke (2018) are 91.44 cm for both XL 753 and LaKast.

Each sample was ground with a Wiley mill (Thomas Scientific, Swedesboro, New Jersey) to pass a 2 mm sieve. From the ground plant tissue a 0.5 g subsample was combined with 30 mL of 2 mol L^{-1} KCl and shaken for 30 min. This solution was then filtered using No. 4

Whatman filter paper. The extract was then analyzed for NO_3^--N, NH_4^+-N, and AA-N concentrations using the Skalar (Skalar Analytical B.V., Breda, The Netherlands) automated segmented flow spectrofluorometric analyzer. Yield was measured for each plot using a small plot combine by harvesting the center seven rows, recording grain weight and moisture content, then adjusting grain weight to 120 g kg^{-1} moisture.

All data were analyzed using the statistical software R (The R Foundation, Vienna, Austria). Analysis of variance was used to detect differences between several factors for each form of N analyzed including the effect of cultivar, N rate, and segment height. When appropriate, Tukey's HSD was used for post-hoc analysis to separate means within factors that were significantly different. To explore the differences among samples containing leaf material and stem only samples, a paired t-test was used. Due to the vastly different scales of magnitude measured among analytes in this study, variability was measured as the mean-scaled parameter of coefficient of variation in percent. Graphical representations were used to explore and visualize trends and differences in the data. Non-linear regression techniques were used to fit segmented models onto graphs depicting a plateau of N accumulation under deficient N rates and linear, quadratic, or similar curves with increasing N application rates above optimum. Analyzing the variability observed in response variables under specific treatments, exploring the graphical trends, and assessing regression parameters were all used to generate conclusions regarding the suitability of all parameters examined in this study for an end of season N sufficiency test as laid out by the research objectives.

Nitrogen rates were divided into deficient or excessive categories based on the N rate needed to achieve 95% relative grain yield. The N rate at which yield was maximized for each site was determined by fitting a quadratic curve with grain yield as the response variable and applied N rate as the explanatory variable. The yield at the vertex was multiplied by 0.95 to represent 95% of the maximum yield. Using the regression equation, the N rate at which 95% of

the maximum yield occurred was determined for each site. This value will be referred to in the remainder of this paper as the yield maximizing N rate. Within each site, applied N rates that fell below the associated yield maximizing N rate were classified as deficient, while those that fell above were classified as excessive.

Results and Discussion

Upon sample analysis, it was evident that the N analytes were in concentrations that were on very different orders of magnitude from each other. Overall average concentrations in mg N kg^{-1} were 148.2, 86.9, and 7.6 for AA-N, NH_4^+-N, and NO_3^--N, respectively. Due to these large differences in concentration between the analytes, variability was expressed as a percentage of the mean (coefficient of variation) to provide a more accurate representation of the variability when observing trends between the analytes. As expected, little NO_3^--N was found in the rice plant due to the denitrification potential of NO_3^--N in the anaerobic rhizosphere of a flooded rice production system.

Influence of Leaf Material

A paired t-test was used to determine if differences existed between the N concentration of samples taken with leaf material intact compared to samples in which the leaf material was removed. For each analyte, there was a statistically significant difference between the two sample types in which the samples analyzed with all leaf material intact contained a greater N concentration. The paired t-test produced a p-value of < 0.0001 for each analyte consisting of 696 observations. Based on the graphs in Figure 2-1, it can be observed that the two sample types show very similar trends of N accumulation across N rates for NH_4^+-N and NO_3^--N. However, the trends observed for AA-N between samples analyzed with and without leaf material were not as closely related. Furthermore, the magnitude of N accumulation that was observed across excessive N rates was compared between both sampling methods using the Student's t-test. The slopes between samples analyzed with leaves and with leaf material

removed were similar when analyzed for AA-N and NO_3^--N (*P* = 0.4371 and *P* = 0.9153 respectively). However, the slopes were statistically different when samples were analyzed for NH_4^+-N (*P* = 0.0018). In addition, the amount of variability was very similar for the two sampling methods within each analyte as seen in Table 2-1. There were significant differences in the N concentrations between samples containing leaf material and samples with leaf material removed. Since the amount of N accumulated across instances of over-fertilization and amount of variability do not differ much between the two sample types, it was concluded that either sampling method could be utilized. Due to the similar variability and laborious, time-consuming task of removing leaf material from rice stems, it was decided that samples be taken with leaf material remaining attached to the stem for the end of season N analysis. Since it was determined that sampling rice stems and leaving leaf material intact was more appropriate, data discussed in the remainder of this paper will primarily be from the samples in which leaf material was not removed.

Amino Acids

Based on the fact that glutamine is the first product in N assimilation into the plant (Masclaux-Daubresse et al., 2010) it was hypothesized that AA-N would be the most responsive to excessive fertilizer N rate and therefore be the best candidate for a post-season tissue test. Amino acids did show the greatest N concentrations of the three analytes with a mean of 148.2 mg N kg^{-1} across all segments. There was a wide range of AA-N concentrations observed within this study with a minimum concentration of 9.0 mg N kg^{-1} and a maximum of 888.1 mg N kg^{-1}. However, there was a large amount of variability in the concentration of AA-N ranging from about 32 to 50%. This suggests that AA-N concentrations are not as sensitive to changes in N availability or the rice plant's overall N status which results in an inability of AA-N to accurately predict whether N fertilization was adequate or excessive.

According to the ANOVA results presented in Table 2-3, there is a significant Cultivar x N rate interaction ($P = 0.0237$) indicating the two cultivars accumulate differing amounts of AA-N at different applied N rates. Since hybrid rice has the ability to perform better under low N conditions due to their increased ability to scavenge for nutrients (Norman et al., 2013), the presence of this interaction in a study examining N accumulation in a hybrid cultivar and a pureline cultivar is logical. There was also a significant main effect of segment ($P < 0.0001$) on the concentration of AA-N, but differences in N concentrations were expected between the segments due to the translocation and remobilization of N throughout the plant. Similar results were reported for NO_3^--N that showed differences in N concentration with increasing plant height ($P < 0.001$) (Wilhelm et al., 2005). Additionally, Mae (1986) reported that 70% of N in the grain originates from remobilization of N during senescence.

Figure 2-2A shows the erratic nature in which AA-N accumulates in the rice stem across different N application rates. Under N limiting conditions, it is expected that little to no N will accumulate in the stem of rice because it is being translocated to sinks elsewhere in the plant, especially during grain fill. However, the opposite is true under conditions of excessive N availability. Due to the nature of luxury consumption of N, rice will take up N even when it is not needed. This results in the accumulation of N when excessive amounts are available to the plant through soil supply or N fertilizers. Sites 1 and 2 show an unstable trend of rapid N accumulation as the rate of N applied increases. However, sites three and four show no logical trend in the way N is accumulated in relation to rate of N applied. This leads to the conclusion that the use of AA-N as a measurement of N to determine N sufficiency would likely lead to recommendations that lack precision in some locations and complete inaccuracy in other locations.

Ammonium

Due to the anaerobic nature of the flooded environment in which rice is grown, NH_4^+-N is the primary form of N taken up by rice plants (Wang et al., 1993) and therefore should be evaluated as a potential analyte for any tissue-N test for flooded rice. Concentrations of NH_4^+-N were found to be on a similar scale to those of AA-N with a mean NH_4^+-N concentration of 101.0 mg N kg^{-1} across all segments. Ammonium concentrations ranged from 11.1 to 438.8 mg N kg^{-1} which is similar to the minimum of AA-N; however, the maximum is substantially lower than that of AA-N (roughly half). Overall, the NH_4^+-N concentrations were less variable than the AA-N concentrations with a coefficient of variation of 20% and 44%, respectively, under deficient N rates and 26% and 46%, respectively, under excessive N rates (Table 2-1).

From the ANOVA results (Table 2-3), the only significant interaction was the Cultivar x N Rate interaction (P = 0.0001). This indicates that the two varieties accumulated NH_4^+-N at different amounts across the N rates similarly to AA-N analysis. The insignificant Segment x N Rate interaction (P = 0.9406) signifies that the amount of NH_4^+-N accumulation does not vary for each segment across different amounts of N applied. Ammonium concentrations had little variability across location and cultivar (Table 2-1). Table 2-2 shows a consistent coefficient of variation for NH_4^+-N concentration across treatments. In sites 1 and 2, NH_4^+-N accumulated in the rice stem as expected, with very low concentrations for lower N rates and increasing accumulation of NH_4^+-N after N rates neared and exceeded the yield-maximizing N rate. Sites 3 and 4 showed a much less logical accumulation with increasing N rate. Some insufficient N rates accumulated approximately as much NH_4^+-N as sufficient and excessive N rates (Figure 2-2B). Due to this unpredictability, NH_4^+-N does not seem to be a good candidate for a post-season N tissue test in rice. Prior to this research, it was expected that NH_4^+-N would be the leading candidate for such a tissue test based on the current production system being primarily

composed of a flooded system creating an anaerobic environment in which NH_4^+-N is the dominant form of N due to the lack of nitrification.

Nitrate

Contrary to NH_4^+-N, the anaerobic root zone is detrimental to soil-N in the form of NO_3^--N. Nitrate subjected to anaerobic conditions is reduced and lost to the atmosphere in the process of denitrification. Due to this loss pathway, it was expected that very little NO_3^--N would be present in the soil; therefore, very little, if any, NO_3^--N would be taken up after the flood was established. This expectation held true as NO_3^--N concentrations were found in considerably lower quantities than the previous two forms of N. Average NO_3^--N concentrations across all treatments was 7.8 mg N kg^{-1} measuring 4.4% and 7.7% of the concentrations of AA-N and NH_4^+-N, respectively. Even considering the low concentrations, NO_3^--N was less variable between treatments across all sites than the previous two forms of N (Table 2-2). Variability in NO_3^--N concentration for leaf treatment, location, and cultivar were comparable to NH_4^+-N and considerably less than AA-N.

Similar to AA-N and NH_4^+-N, NO_3^--N showed a significant Cultivar x N rate interaction (Table 2-3). Again the hybrid and pureline cultivars accumulated different amounts of N across N rates once yield was maximized (Figure 2-3C). The three-way interaction was insignificant for NO_3^--N just as it was for the previous two N forms tested. In addition, there was no significant Cultivar x N rate interaction which indicates that the relative slopes and amount of NO_3^--N accumulation did not vary across N rates (Table 2-3) and Figure 2-3C illustrates this response. Nitrate concentrations in the stem remain low under N rates which are considered insufficient for yield maximization. Once N rates transition from insufficient to adequate and excessive, concentrations of NO_3^--N begin to increase rapidly. Although NO_3^--N concentrations in the rice stem at the end of the season were very low compared to AA-N and NH_4^+-N, relatively low

variability and consistent response to N rates across sites and substantial accumulation under excessive N application make NO_3^--N a strong candidate for a post-season N test in rice.

Sampling Height

The final characterization of sampling procedures for a post-season N tissue test is the height or section of stalk to be sampled. From the scientific perspective, the sampling height needs to have a consistent response across sites and conditions. From the practical perspective, the sampling procedures need to be easily reproducible and simple to follow. These were the primary concerns kept in mind when selecting the most plausible rice stalk sampling height. Since NO_3^--N was selected as the N analyte with the most likelihood of accurately predicting instances of excessive N application, the discussion of sampling height will focus on NO_3^--N.

As shown in Figure 2-4, higher concentrations of NO_3^--N can be found in the lower portion of the rice stem. Concentration of NO_3^--N decreases with increasing height and begin to stabilize to a consistent concentration ~ 25 cm above the soil surface. Hanway and Englehorn (1958) reported that corn accumulated NO_3^--N at the base of its stalk under conditions of excess N. They further hypothesized that the accumulation in the lower part of the stem would occur in most monocots. The data resulting from this study seem to support this claim, at least in the case of rice. Others have since documented data in more depth to support the claim that NO_3^--N accumulates in the lower portion of the corn stalk (Wilhelm et al., 2005, Isla & Blackmer, 2007, Ketterings et al., 2017). Contrary to the trend seen in NO_3^--N, the concentration of AA-N and NH_4^+-N increased as plant height increased (data not shown).

The lowest three segments (0-15 cm) were more responsive to excessive N applications than the upper six segments (15-45 cm) in terms of NO_3^--N concentration (Figure 2-5). This relationship is desirable because it allows for more accurate detection of excessive N conditions when a specific portion of the stalk is sampled. As shown in Figure 2-6, all sections of rice stem

tested in this study contained a constant concentration of NO_3^--N across N rates classified as yield limiting or optimal. No significant difference was observed in NO_3^--N concentration across plant height when fertilizer N applications were considered insufficient (P = 0.9825) or optimal (P = 0.9047). Once the N rate is increased to a level that is considered excessive, NO_3^--N concentration begins to increase in all sections of the rice stalk analyzed. When fertilizer N rates are considered excessive, NO_3^--N concentrations were significantly different across stem segments (P < 0.0001). Sections analyzed from the lower portion of the rice stem were statistically higher than sections analyzed from the middle and upper portion of the rice stem (Figure 2-6). Although it appears all segments analyzed in this study could be utilized for a post-season N tissue test, it is suggested that the lowest four segments (0-20 cm) be used. This section was selected as the best candidate for several reasons. Sampling the entire above ground biomass would result in an excessive amount of plant material that would be difficult to handle and cause unnecessary wear to plant grinding equipment and increase the amount of time from plant sampling to results being delivered. As discussed earlier, the concentrations of NO_3^--N in the lower segments were more responsive to over-fertilization of N. In addition, starting at the soil surface would allow for more consistent sampling, leading to more accurate results. Four segments were chosen instead of a lower quantity to ensure that a large enough sample was taken to capture the variability and provide an accurate estimation of the N status of the rice plant. As shown in Table 2-4, variability in NO_3^--N concentration decreases with an increase in the number of segments that make up a section. A section comprised of four segments will reduce the variability while keeping sample processing times and equipment wear low while also achieving high sampling accuracy and reproducibility.

Conclusion

Prior to the novel research discussed in this paper, no experiments have been conducted to evaluate the plausibility of developing a post-season tissue test in rice for

diagnosing conditions of excessive N applications. Currently, no test exists for determining whether rice has been subjected to over-application or insufficient application of N. Such a test could be used to identify N management issues or fine tune N recommendations in subsequent growing seasons by limiting over-application of N and thereby maintaining high yields while reducing input costs and potentially harmful environmental impacts by rice production systems. A post-season test of this nature could be used in combination with pre-season and in-season evaluation methods, such as the Nitrogen Soil Test for Arkansas Rice (N-STaR) (Roberts et al., 2013) and Normalized Difference Vegetative Index (NDVI) based measurements, to accurately predict and monitor N. Additionally, the results of a post-season test could be used to adjust N rate recommendations for future rice crops. Based on the findings presented in this paper, a stem sample taken with all leaf material left intact, from 0 to 20 cm above the soil surface, and analyzed for NO_3^--N could be utilized to accurately determine when rice has been subjected to excessive N fertilization. Further research still needs to be conducted for this test to be implemented in rice production systems.

Research should be conducted to determine whether sampling time has an effect on the NO_3^--N concentration found in the rice stem. It is not always practical for these samples to be taken at the time of harvest. Samples which are taken either before or after harvest could result in different concentrations and therefore alter the results and interpretation of the test. It is important to outline the sampling time with specificity for tissue tests. Another important sampling parameter that needs to be evaluated is the number of plants that need to be sampled to capture the variability in a given area or field. Sample storage and transport should also be investigated to determine if certain sample storage practices, such as freezing, room temperature storage, or extending storage of samples, will introduce error into the process. The use of other analysis methods to determine NO_3^--N concentrations such as a water-based extraction or ion specific electrode should be investigated for better accuracy or simplified lab

procedures. Finally, NO_3^--N concentrations should be subjected to correlation and calibration across a multitude of cultivars and sites to quantify the crop response associated with the NO_3^--N concentrations determined from the tissue test.

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Tables and Figures

Table 2-1. Coefficient of variation of nitrogen (N) concentration in percent for all three analytes measured: amino acid (AA-N), ammonium (NH_4^+-N), and nitrate (NO_3^--N) separated by leaf material, location: Pine Tree Research Station (PTRS) and the Rice Research and Extension Center (RREC), and cultivar expressed for each of the N rate categories: deficient and excessive.

	Coefficient of Variation (%)					
	AA-N		NH_4^+-N		NO_3^--N	
	Deficient	Excessive	Deficient	Excessive	Deficient	Excessive
Leaves						
Without	49.31	61.26	19.33	29.81	18.40	31.53
With	44.60	46.22	20.07	25.75	15.30	32.81
Location						
PTRS	57.36	51.67	22.33	25.18	27.63	36.16
RREC	38.92	58.18	17.76	35.09	8.60	20.35
Cultivar						
LaKast	44.07	55.70	20.20	30.65	16.6	39.92
XL 753	49.57	50.89	19.24	24.66	16.91	24.49

Table 2-2. Coefficient of variation in percent for each analyte: amino acid (AA-N), ammonium (NH_4^+-N), nitrate (NO_3^--N) across the six nitrogen (N) rate (kg N ha^{-1}) treatments.

N rate (kg N ha^{-1})	Coefficient of Variation (%)		
	AA-N	NH_4^+-N	NO_3^--N
0	50.23	21.70	16.53
50	32.06	15.42	19.19
101	53.28	22.72	14.57
151	38.84	21.51	13.32
202	50.20	28.56	23.79
252	47.88	23.62	63.51

Table 2-3. Analysis of variance results for the main effects of cultivar, nitrogen (N) rate, Segment, and their interactions for each analyte: amino acid (AA-N), ammonium (NH_4^+-N), nitrate (NO_3^--N). Results shown for samples analyzed with and without leaf material.

	With Leaf Material				Without Leaf Material			
Source	DF	AA-N	NH_4^+-N	NO_3^--N	DF	AA-N	NH_4^+-N	NO_3^--N
Cultivar (C)	1	<0.0001	<0.0001	<0.0001	1	0.0001	<0.0001	<0.0001
Nitrogen Rate (NR)	5	<0.0001	<0.0001	<0.0001	5	<0.0001	<0.0001	<0.0001
Segment (S)	8	<0.0001	<0.0001	<0.0001	7	0.0001	<0.0001	<0.0001
C x S	8	0.9922	0.2237	0.0651	7	0.2167	0.6617	0.7487
C x NR	5	0.0237	0.0001	0.0001	5	0.0035	<0.0001	0.0011
S x NR	40	0.6937	0.9406	0.1612	35	0.9943	0.4349	<0.0001
C x NR x S	40	0.4399	0.9898	1.0000	35	0.9125	0.9691	0.8495

Table 2-4. Coefficient of variation in percent of the mean nitrate concentration for segments composed of consecutive sections.

Segment Length (cm)	Coefficient of Variation (%)	
	Deficient	Excessive
5	5.74	12.35
10	5.80	10.45
15	5.90	9.98
20	5.82	8.89
25	5.72	8.76
30	5.76	10.09
35	5.77	10.18
40	5.81	10.45
45	5.86	10.31

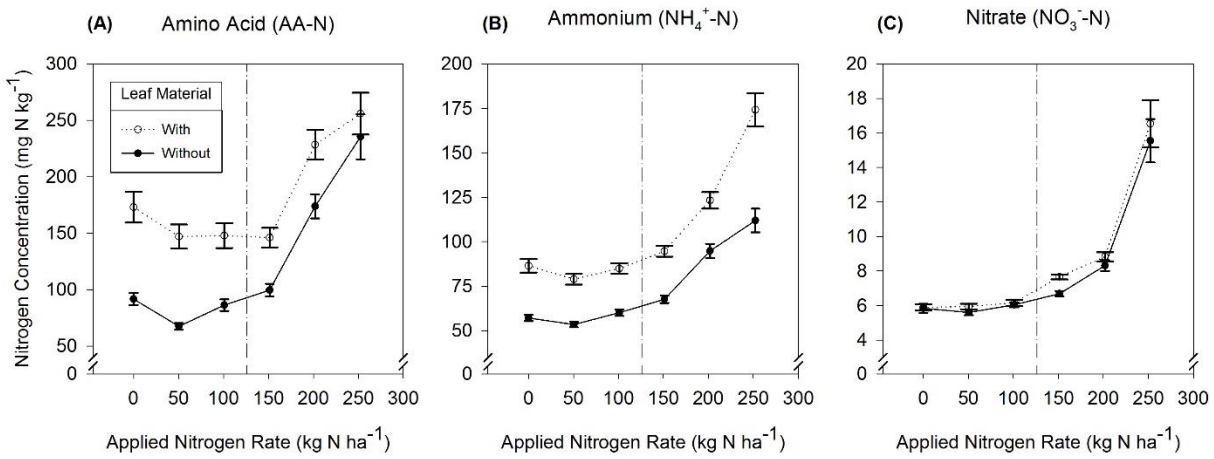

Figure 2-1. Nitrogen (N) concentration measured as amino acid (A), ammonium (B), and nitrate (C) across N application rates comparing samples analyzed with leaf material and without leaf material averaged over all sites. The vertical line represents the N rate needed to achieve 95% of maximal yield (126 kg N ha^{-1}). Error bars are a representation of the standard error. Wide error bars represent samples with leaf material and narrow error bars represent samples without leaf material.

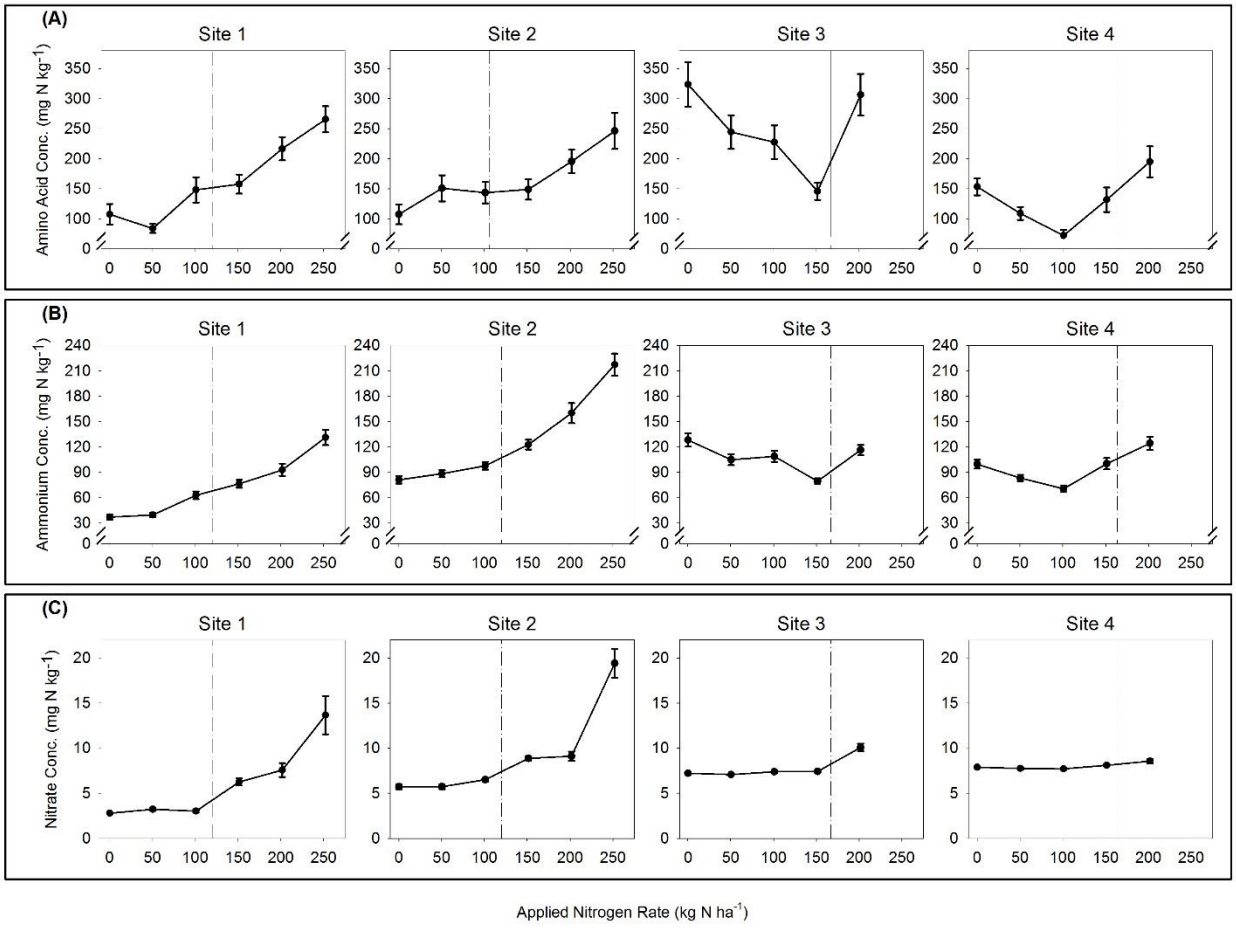

Figure 2-2. Average nitrogen (N) concentration measured as amino acid (A), ammonium (B), and nitrate (C) across N rate for each location (Pine Tree Research Station (PTRS) and Rice Research and Extension Center (RREC)) cultivar combination. The vertical line represents the N rate required to achieve 95% of maximum yield (Site 1 (PTRS – LaKast) = 120 kg N ha^{-1}, Site 2 (PTRS – XL 753) = 106 kg N ha^{-1}, Site 3 (RREC – LaKast) = 167 kg N ha^{-1}, Site 4 (PTRS – XL 753) = 164 kg N ha^{-1}).

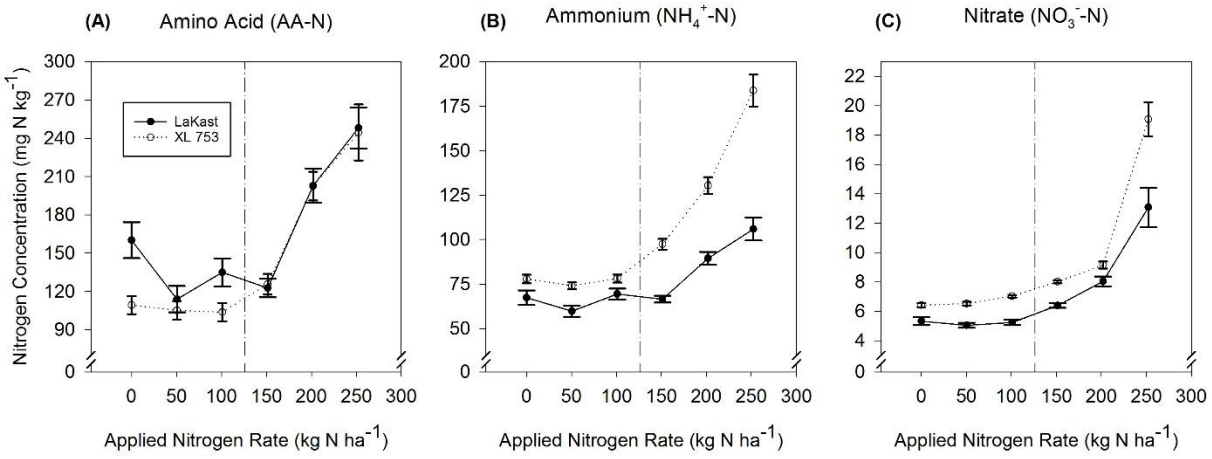

Figure 2-3. Average nitrogen (N) concentrations across all sites compared to N rates applied for each analyte and cultivar in the study. Vertical line represents the N rate needed to achieve 95% of maximum yield (126 kg N ha^{-1}). Error bars are a representation of the standard error. Wide error bars represent LaKast and narrow error bars represent XL 753.

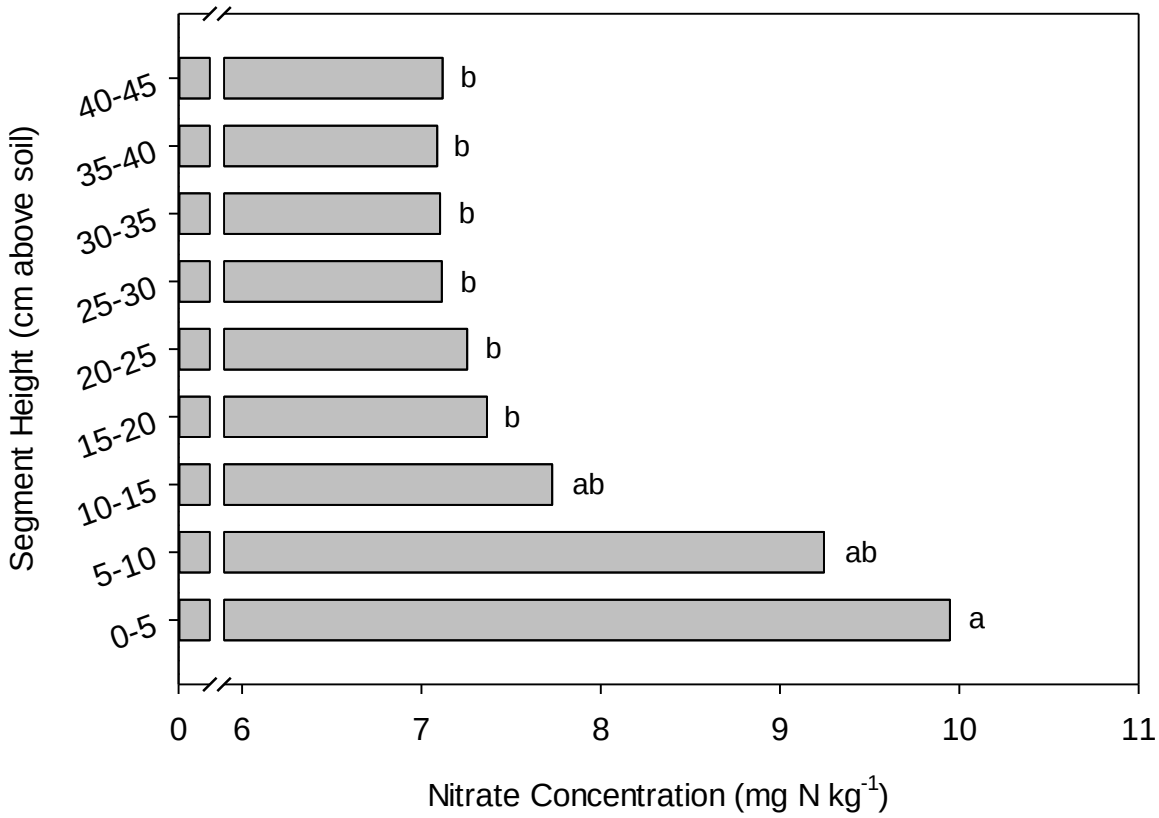

Figure 2-4. Nitrate concentration averaged across location and nitrogen (N) rate as it changes with segment height from the soil surface. Letter separation performed by Tukey's HSD (P < 0.0001).

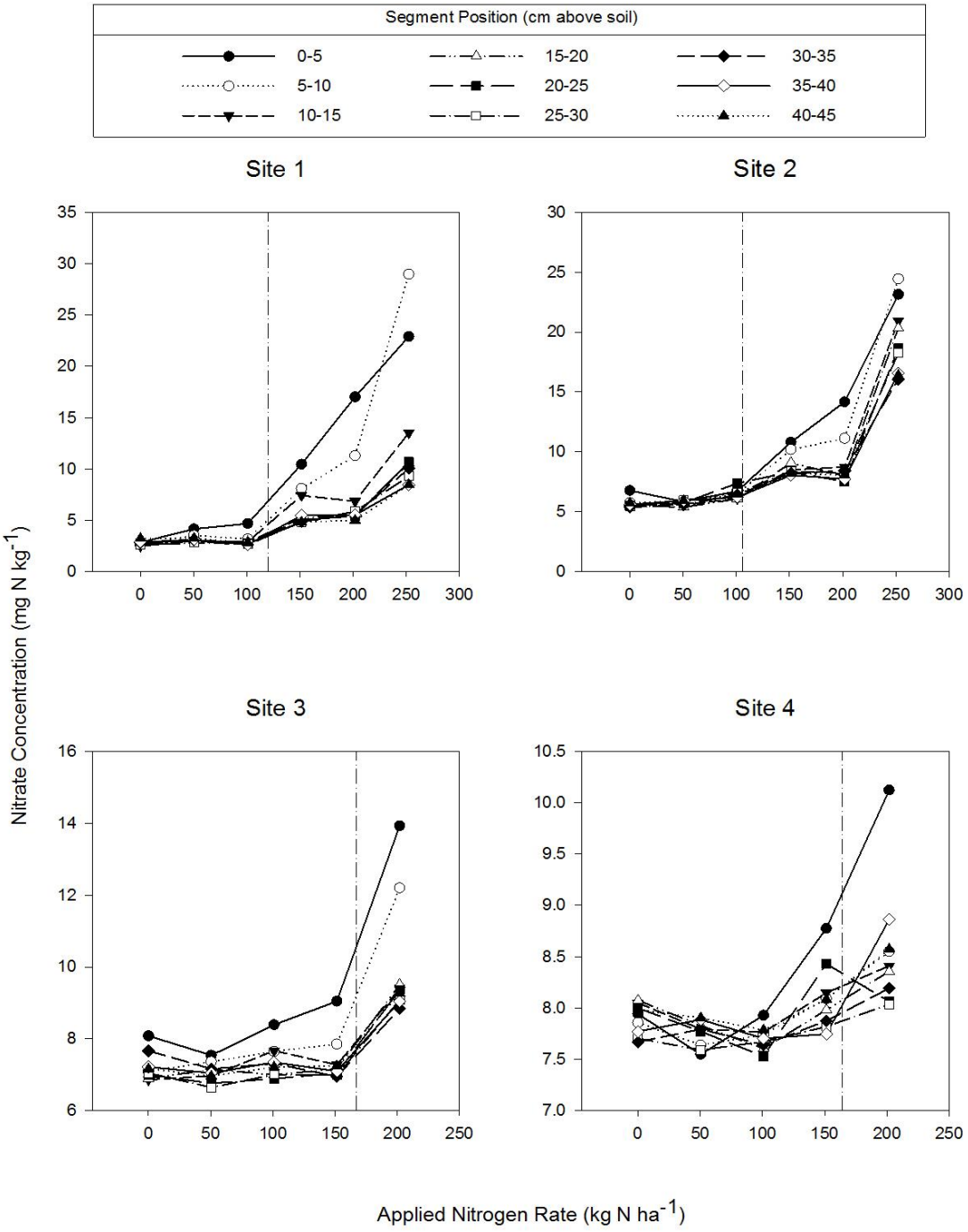

Figure 2-5. Average nitrate (NO_3^-) concentration across nitrogen (N) rates for each location (Pine Tree Research Station (PTRS) and Rice Research and Extension Center (RREC)) cultivar combination separated by segment. Vertical line represents the N rate required to achieve 95% of maximum yield (Site 1 (PTRS – LaKast) = 120 kg N ha^{-1}, Site 2 (PTRS – XL 753) = 106 kg N ha^{-1}, Site 3 (RREC – LaKast) = 167 kg N ha^{-1}, Site 4 (RREC – XL 753) = 164 kg N ha^{-1}).

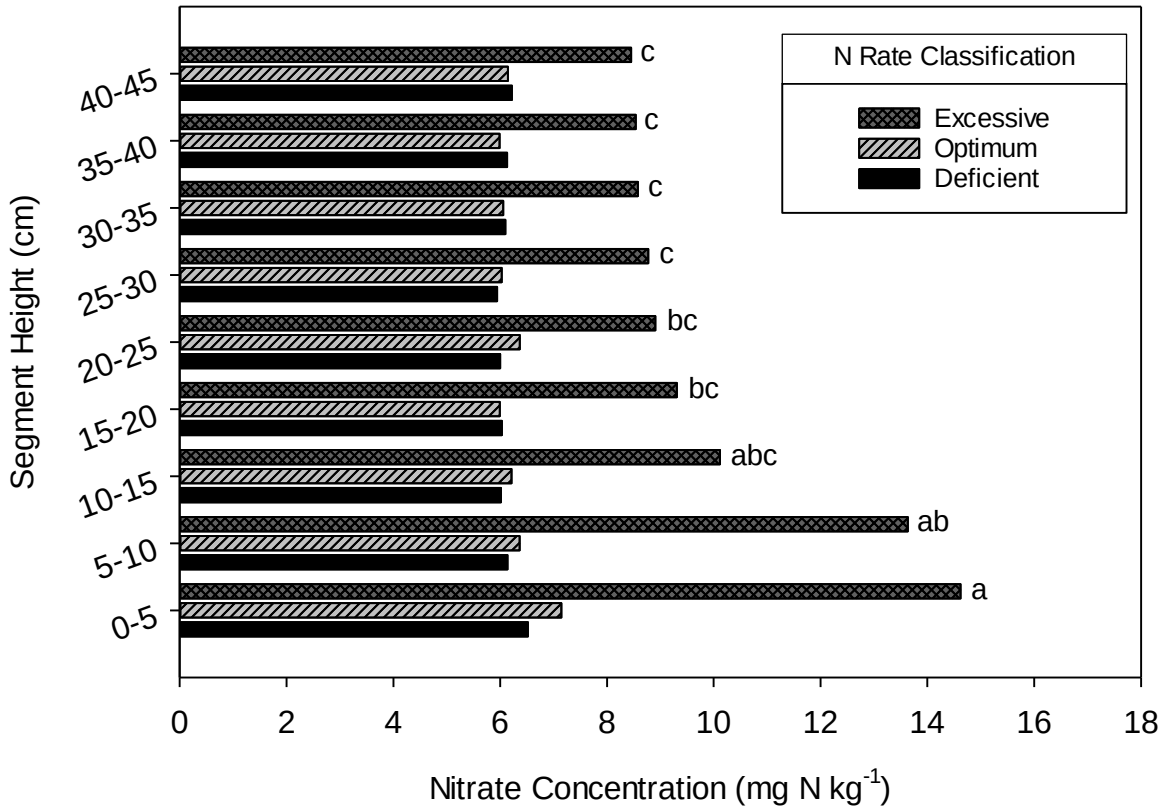

Figure 2-6. Distribution of nitrate (NO_3^-) concentration across plant height separated by the nitrogen (N) rate classification. Letter separation performed by Tukey's HSD for the excessive category ($P < 0.0001$). Optimum and deficient classifications were not significant ($P = 0.9825$, $P = 0.9047$, respectively).

Chapter 3: Using Post-Season Tissue Nitrogen Concentrations to Predict Adequacy of In-season Nitrogen Management

Abstract

High yielding mechanized rice production requires large amounts of N fertilizer, which can be particularly costly to producers when applied in excess. The development of a tissue test to detect over application of N to rice is needed. A trial was designed to identify a critical concentration threshold for a tissue test to detect excess N availability. Rice stalk samples were collected from 0-20 cm above the soil within 5 days of harvest and analyzed for NO_3^--N concentration. Two models were fit to the data to represent rice grain yield as a function of N concentration: the linear plateau model and the quadratic plateau model. The data was well represented by both models; however, the linear plateau model more accurately described the data with a normalized root mean square error of 0.128 for linear plateau and 0.131 for quadratic plateau and a significant F-test ($P < 0.0001$). The join point of the linear response region and the plateau region of the model serves as a critical value that separates the responsive (N deficient) and non-responsive (N optimum or excessive) regions. The 95% confidence interval of the join point (2.1 mg NO_3^--N kg^{-1}) was selected as a practical, agronomic critical concentration threshold to reduce false positive errors. The data presented here further supports the use of a post-season tissue test in rice to detect over fertilization and established a critical concentration threshold for this tissue test.

Introduction

Nitrogen fertilization is an important component of mechanized rice (*Oryza sativa*) production systems for maximizing grain yield and profitability. Predicting the optimum N rate and managing N properly throughout the season can be difficult to achieve using generic approaches where N rates are based on cultural practices and soil texture. Improper N fertilization rates or management strategies can create many issues associated with both over- and under-fertilization with N, including reduced yield, increased incidence of disease (Slaton et al., 2004), adverse environmental impacts (Pittelkow et al., 2013), and reduced profits for producers (Salassi et al., 2013). Conditions of severe N deficiency are often obvious and characterized by visual N deficiency symptoms and reduced grain yield; however, subtle N deficiencies are not as easy to detect and may reduce yield with no obvious visual deficiency symptoms. There is a pronounced relationship between N and yield with most producers assuming the relationship is linear. Due to this perception of N and yield improvement, producers often apply additional N to ensure the crop's N needs are satisfied with the hope of achieving maximal yield. Unfortunately, these additions to standard N rates or the fact that the standard N rates do not account for variations in N availability across soils, can lead to the over application of N fertilizer. Currently, no post-season test exists to determine whether rice was supplied with more N than was required to maximize yield.

The consequences of excess N fertilization are not always apparent as in the case of reduced profits and negative environmental impacts. Reduced profits are primarily a result of the unnecessary cost of purchasing and applying additional N beyond the amount required to maximize yield. Excess N applications can further reduce profits through a reduction in grain yield or quality (Salassi et al., 2013). Negative environmental impacts, primarily the increased production of greenhouse gases or degradation of water quality, are not immediately visible to the human eye and go largely undetected.

The most noticeable consequence of excess N fertilization in rice is lodging; however, lodging does not always occur in the presence of excess N and is largely dependent on other factors such as environmental conditions (Weng et al., 2017). Similarly, increased incidence of disease can be associated with over fertilization (Slaton et al., 2004), but is not necessarily a definite predictor of excessive N conditions. Regardless of whether the consequences of excess N fertilization are obvious or go unnoticed, there is a need for a way to quantitatively determine whether N was available in sufficient or excessive quantities.

Milard (1988) found that crops tend to accumulate N in the tissue when N is not limiting growth. Luxury consumption of N provides the foundation for detecting excessive N conditions by measuring the N content of the plant tissue. To further support this idea, an increase in tissue-N concentration was observed with increasing N supply (Murphy & Smith, 1967; Zhen & Leigh, 1990; Ntanos & Koutroubas, 2002). Other considerations for an accurate test of excess N conditions are determining what tissue or plant parts to sample and how to measure the N concentration within that tissue. Wang et al. (2006) presented data to suggest that N concentrations are stratified with plant height and that the largest concentrations of N accumulate in the upper leaves when compared to the lower leaves of rice plants. In previous work conducted by Hoegenauer et al. (2020), NH_4^+-N concentrations were determined to accumulate in the upper portion of the rice plants stalk and leaves; however, concentrations of NO_3^--N were found to accumulate more in the lower portion of the stalk and leaves and were found on a much lower magnitude than NH_4^+-N concentrations. From this research, it was proposed that NO_3^--N concentrations measured from the rice stalk samples collected 0-20 cm above the soil could be used in a test to detect excess N fertilization in rice.

Similar efforts have been made in corn (*Zea Mays*) production through the development and testing of the Corn Stalk Nitrate Test (CSNT) (Binford et al., 1990). The principle that N, in particular NO_3^--N, accumulates in the lower portion of grass crops when N is available in excess

(Hanway & Englehorn, 1958) was used as the basis for the CSNT. The methods for selecting the parameters of CSNT are unclear and have been described as the result of seemingly arbitrary decisions during exploratory studies (Isla & Blackmer, 2007). However, the CSNT has been shown to accurately predict excess N fertilization in corn across many production systems (Binford et al., 1990; Binford et al., 1992; Greub et al., 2018; Isla & Blackmer, 2007).

The CSNT procedure consists of collecting stalk samples, 20 cm in length (15-35 cm above the soil), after physiological maturity. The samples are then analyzed for NO_3^--N concentration and compared to a threshold concentration to determine whether N fertilization was deficient, optimal, or excessive (Binford et al., 1990). The critical concentration threshold was determined by fitting a linear plateau model and interpreting the join point as the NO_3^--N concentration at which there was no further yield response. Since the origin of CSNT, several studies have been conducted to evaluate many of its parameters. Isla and Blackmer (2007) found a strong correlation between standard CSNT samples (15-25 cm above soil) collected as described by Binford et al. (1990) and 4 cm samples collected in the middle of the standard sample (18-22 cm above soil). Wilhelm et al. (2005) examined samples collected 5 cm higher or lower than the standard sample. Samples collected at the alternate height deviated from the standard sample by 10-15%. The authors suggested that the critical concentration threshold established by Binford et al. (1990) could be adjusted by 15% and successfully used to determine if N was supplied in excess. Similarly, Ketterings et al. (2017) concluded that the sampling height, location in the field, and sample processing could be altered without compromising the accuracy of the test and possibly increase producer adoption.

The results of these studies exploring CSNT methodology indicate that many techniques and procedures can be used to detect excess N fertilization in corn with the proper research and data collection. Therefore, the objective of this study was to develop a post-season tissue test to quantitatively determine whether N fertilization applied to a rice crop was excessive. To

accomplish this, rice stalk samples collected at the end of the season from 0-20 cm above the soil were analyzed for NO_3^--N concentration, as proposed by Hoegenauer et al. (2020), and used to establish a critical concentration threshold that can be used to identify rice that has received excess N fertilization.

Materials and Methods

Site Description and Plot Management

Research was conducted at 21 sites across three locations during the 2016, 2017, and 2018 growing season. The locations are as follows: (1) Pine Tree Research Station (PTRS) near Colt, Arkansas, (2) Rice Research and Extension Center (RREC) near Stuttgart, Arkansas, and (3) Rohwer Research Station (RRS) near Rohwer, Arkansas. Soils data for all sites are listed in Table 3-1. All plots were arranged in a randomized complete block design with four replications. Plot dimensions at RREC were 1.7 m wide by 5.2 m long while all other locations had plot dimensions of 1.7 m wide by 4.9 m long. All plots contained 9 rows of rice spaced at 19 cm. Plots were seeded at rates appropriate for both pureline cultivars and hybrids as recommended by the University of Arkansas Cooperative Extension Service (Hardke et al., 2020). Seeds were treated with the insecticide seed treatment NipsIt INSIDE (Valent) (Clothianidin 478 g a.i. kg^{-1}) at a rate of 1.25 ml kg $seed^{-1}$ to ensure adequate emergence rates and early season growth. Further details for each site including cultivar selection can be found in Table 3-1.

Plots were managed according to the recommendations for direct-seeded, delayed-flood rice by the University of Arkansas Cooperative Extension Service (Hardke et al., 2020). Nutrients, particularly P, K, and Zn, were managed to ensure proper growth and to eliminate the potential of those nutrients as yield limiting factors. Nitrogen applications were made based on a N response treatment structure. Studies at RREC received six N treatments (0, 67, 101, 135, 168, 202 kg N ha^{-1}). Studies conducted at all other locations also received six N treatments (0,

50, 101, 146, 202, 252 kg N ha^{-1}). All N treatments were applied as urea (460 g N kg^{-1}) at approximately the V-4 or V-5 growth stage (Counce et al., 2000) and were coated with a urease inhibitor to reduce ammonia volatilization loss potential. Agrotain (Koch Industries, Wichita, KS) was used as the *N-n*-butyl thiophosphoric triamide (NBPT) urease inhibitor and was applied at a rate of 3.1 ml of Agrotain kg^{-1} urea. Shortly after N applications were made, a flood was established and maintained at a depth of 10 to 12.5 cm until maturity.

Tissue Sampling

Based on previous research conducted by Hoegenauer et al. (2020), tissue samples were collected from the base of the plant that included the stalk and leaf material. These samples were collected from 0-20 cm above the soil surface within 5 d of rice harvest to ensure that all N required for adequate grain fill had been translocated to the panicle, potentially depleting N from the lower portion of the plant and leaving only excess N which was not needed for grain fill. The tissue samples were taken from a linear 0.5 m section of an interior row of rice from each plot.

The tissue samples were oven-dried at 60°C to a constant weight. The dried samples were then ground with a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass a 2 mm sieve. One-half of a g of the ground tissue was added to 30 ml of 2 mol L^{-1} KCl and shaken for 30 min. This solution was passed through Whatman no.4 (Cytiva, Malborough, MA) filter paper to filter out the extract. A Skalar (Skalar Analytical B.V., Breda, Netherlands) automated segmented flow spectrofluorometric analyzer was used to determine the NO_3^--N concentration of the tissue sample on a dry weight basis (Hoegenauer et al., 2020; Roberts et al., 2016; Rogers et al., 2018). Plots were harvested when the grain moisture, averaged across treatments, was between 150 – 180 g H_2O kg^{-1} with a small plot combine. Grain weights were adjusted to 120 g H_2O kg^{-1} moisture and extrapolated to a kg ha^{-1} basis.

Statistical Analysis

All statistical analysis was performed using the R statistical program (The R Foundation, Vienna, Austria). Visualizations were made using the "ggplot2" package (Wickham, 2016) within R. The yield maximizing N rate (YMNR) represents the N rate needed to achieve 95% of the maximum yield within a site. The YMNR was calculated by regressing applied N rate by relative grain yield. The vertex of the quadratic equation was found and multiplied by 0.95 to find the value that corresponds to 95% of the maximum yield. The N rate associated with this value was then designated as the YMNR for that independent site and this procedure was used to find the YMNR for each of the 21 sites discussed in this paper. The difference between the applied N rates and YMNR for each site was calculated so that the YMNR is equal to zero, applied N rates less than YMNR (deficient) are negative, and applied N rates greater than YMNR (excessive) are positive. These values will be referred to as the differential N rates.

The linear plateau model was applied to the combined data from all sites in this study. The linear plateau model equation:

$$f(x) = \begin{cases} \beta_0 + \beta_1 x & if\ x < x_0 \\ Y_m & if\ x \geq x_0 \end{cases}$$

contains two regions for the data to fit into. The first region is linear where β_0 is the linear regression intercept and β_1 is the linear coefficient. The second region is a horizontal plateau (maximal yield), Y_m, that is equal to $\beta_0 + \beta_1 \times x_0$. The two regions meet at a join point, x_0. In the context of the data presented in this paper, the linear region represents rapid increase in grain yield resulting from the increase of NO_3^--N in the lower portion of the rice stalk and the plateau represents a region where no additional yield is added from an increase in NO_3^--N in the stalk. Non-linear regression techniques were implemented to apply the linear plateau model to the data. Regression outliers were identified as standardized residuals greater than 2 or less than -2 with the help of the "nlstools" package (Baty et al., 2015).

The quadratic plateau model is also commonly applied to data exhibiting this type of response (Greub et al., 2018). The equation for the quadratic model:

$$f(x) = \begin{cases} \alpha + \beta x + \gamma x^2 & if\ x < x_0 \\ Y_m & if\ x \geq x_0 \end{cases}$$

shares similarities to the linear plateau model, except the alpha, beta, and gamma are coefficients of the quadratic region. In the quadratic model Y_m is still the maximal yield and the plateau of the model, but is calculated as $Y_m = \alpha + \beta x_0 + \gamma {x_0}^2$ and x_0 remains the contiguous join point between the two regions. The two regions of the quadratic plateau model are described by a quadratic response that represents increases in yield caused by increases in NO_3^--N concentration followed by a plateau region that is characterized by no additional increase in yield under the presence of increasing NO_3^--N concentrations. The quadratic plateau model was fit to the data using the same methods as the linear plateau model and the two models were compared using a F-test.

Results and Discussion

The sites presented in this paper represent a wide variety of location and cultivar combinations that are representative of Mid-south rice production. The sites also provide a wide range of yield responses with check plot grain yield ranging from 3253 to 7578 kg ha^{-1} and a range in YMNR of 81 to 157 kg N ha^{-1}. Within the tissue samples collected across all trial locations there was also a wide range of NO_3^--N concentrations in the tissue samples ranging from 0.12 to 239 mg NO_3^- kg^{-1}. Median tissue NO_3^--N concentrations for plots that received excess N in relation to the YMNR and plots that received less N than the YMNR were 6.24 and 1.07 mg NO_3^- kg^{-1}, respectively. Indicating that under N limiting conditions (deficient), the N in the base of the plant is translocated for grain-fill and depleted, while N remains in the base of the plant when N applications exceed what is required for grain-fill. Similar results showing accumulation of NO_3^--N in the lower portion of the plant in the presence of excess N has been

noted in several studies examining the relationship between NO_3^--N and yield in corn (Binford et al., 1990, Greub et al., 2018).

Relationship of NO_3^--N and YMNR

The relationship between N rate and NO_3^--N can be examined in Figure 3-1 and Figure 3-2. In all sites, NO_3^--N concentrations were generally low for N rates less than the YMNR. Conversely, NO_3^--N concentrations dramatically increased in a linear fashion when N rates were above the YMNR. However, the rate at which the NO_3^--N concentration increased varies by site which results in a large amount of noise within the excessive N region when the data are combined from all sites (Figure 3-3). The difference in the rate of NO_3^--N concentration increase among sites could be the result of several factors including sample timing, soil moisture, and differences in nitrification rates amongst soils. These factors require further investigation and future research to tease out these relationships and effects.

Quadratic Plateau Model

The quadratic plateau model was fit to NO_3^--N concentration by relative grain yield data using non-linear least squares techniques (Figure 3-4). The fitted model contained the equation $64.5 + 17.6x - 2.3x^2$ for the responsive region and plateaued at 98.4% relative grain yield. The join point between the two regions was 3.9 (±0.8) mg NO_3^--N kg^{-1}. Metrics related to the quality of fit can be found in Table 3-2. Tissue NO_3^--N concentrations less than the join point of 3.9 mg kg^{-1} are indicative of unrealized yield potential from a N standpoint. While NO_3^--N concentrations greater than 3.9 mg kg^{-1} indicate optimal or excessive N fertilization. Greub et al. (2018) used the quadratic plateau model to describe the relationship between tissue-NO_3^--N and yield in 24 site years of corn trials. The join point of the quadratic plateau model used for irrigated corn in Arkansas was found to be 170 mg NO_3^--N kg^{-1} and a plateau of 97% relative grain yield. Nitrate concentrations in the corn tissue (and model join point) are greater than those found in the rice tissue collected in this study. The aerobic nature of corn production allows for greater

nitrification of ammonium and results in greater uptake of nitrate when compared to a rice crop grown primarily in flooded conditions.

Linear Plateau Model

The linear plateau model was also used to describe the relationship between NO_3^--N concentrations in the lower portion of the rice plant and relative grain yield (Figure 3-5). The linear response region of the model was characterized by the equation $19.4x + 60.5$ and the model plateaued at 97.1% relative grain yield. The join point separating the responsive and plateau regions was 1.9 (±0.2) mg NO_3^--N kg^{-1}. Like the quadratic plateau model, NO_3^--N concentrations less than 1.9 mg kg^{-1} indicate a condition of under-fertilization and values greater than 1.9 mg kg^{-1} fall into the plateau region and indicate optimal or excessive fertilization. Data presented by Binford et al. (1992) shows an average join point of 463 mg NO_3^--N as determined by the linear plateau model across 23 site years of corn trials. Similar to the results of quadratic plateau, the join point of the linear plateau model for rice is much less due to the anaerobic environment of rice and significantly reduced availability of nitrate in the soil profile under conventionally flooded rice conditions.

Model Comparison

Although the interpretation of these two models are similar, the resulting join points for the quadratic plateau and linear plateau models differ and the models must be assessed to determine which model is most appropriate for the data. Based on the metrics describing the quality of fit for both models listed in Table 3-2, both the quadratic plateau and the linear plateau models fit the data equally well. However, the linear plateau model has a lower normalized root mean square error than the quadratic plateau model (0.128 and 0.131, respectively). Additionally, the linear plateau model performed better than the quadratic plateau model in terms of Akaike's information criterion (3409 and 3453, respectively), Bayesian information criterion (3426 and 3470, respectively), mean absolute error (8.08 and 8.27, respectively), and

log-likelihood (-1701 and -1723, respectively). As a final comparison of the models to determine which model suits the data best, a F-test was performed. Once again, the linear plateau model was shown to be a more accurate fit than the quadratic plateau model ($P < 0.0001$).

Based on this information, the linear plateau model was determined to fit the data better and was selected to represent this data for further interpretation. The most important parameter in the model is the join point because it can be interpreted as the threshold between deficient N conditions and optimal or excessive N conditions. To reduce the number of false-positives when identifying situations of excess N fertilization, the upper bound of the 95% confidence interval for the join point, 2.1 mg NO_3^--N kg^{-1}, was selected as a practical threshold for distinguishing instances of over fertilization and under fertilization (Table 3-2).

Conclusion

The data presented in this paper support the hypothesis that NO_3^--N concentrations in the lower portion of the rice plant can be used as an indicator of over fertilization. Comparing the relationship between NO_3^--N concentrations and the applied N rate in relation to the YMNR revealed a clear trend in which NO_3^--N concentrations remained low when applied N rates were lower than the YMNR and NO_3^--N concentrations quickly began to increase when applied N rates were greater than the YMNR for most sites. There is some variability in the magnitude and rate at which NO_3^--N concentrations increase after exceeding the YMNR. The variability in this region of the data is likely due to differences in nitrification rate or environmental conditions for each site (flood removal timing, soil saturation, ambient air temperature and ET) and requires further research to fully understand. However, the variability in this region has no effect on evaluating whether the rice was exposed to excessive N fertilization or not. Samples over the NO_3^--N concentration threshold were likely over fertilized, regardless of how much higher the NO_3^--N concentrations are than threshold. Further research could be conducted to explore the

causes of the variability and use the magnitude of excess NO_3^--N above the threshold to estimate the amount of N that was applied in excess.

Although the quadratic plateau model adequately described the relationship between NO_3^--N concentrations in the rice stalk and relative grain yield, the linear plateau model was determined to better represent this relationship through the use of goodness of fit metrics and practicality considerations. The linear plateau model identified the join point of the responsive and plateau regions to be 1.9 mg NO_3^--N kg^{-1}. The authors suggest that the upper bound of the 95% confidence interval of the join point (2.1 mg NO_3^--N kg^{-1}) be used as the threshold to distinguish under and over fertilized rice.

Using these findings to implement a post-season N test would be the first tissue test in rice to accurately identify likelihood of over fertilization with N. The utilization of such a tissue test would result in more accurate N management, increased profits to producers, and reduced environmental implications. Based on the results of a post-season N test, future management decisions can be altered to optimize N efficiency including field specific N rates, use of N stabilizers, application timing and splits, flood establishment and maintenance, and other cultural management decisions. A post-season tissue test can easily be combined with existing N management tools to reduce or correct errors in N management. The use of soil tests such as the Nitrogen Soil Test for Rice (N-STaR) (Roberts et al., 2013) can be used to establish accurate site-specific N rate recommendations. Mid-season monitoring techniques such as normalized difference vegetation index measurements can be used to assess the in-season N status and provide corrective action. Utilizing this post-season tissue test can provide information about the availability of N through harvest to ensure N was managed properly throughout the season. Implementation of a post-season test can be useful on its own or used in combination with other management tools to improve the decision-making process for improving N management in rice.

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Tables and Figures

Table 3-1. Selected cultivar and site information for each location in this study from 2016 to 2017. Yield Maximizing nitrogen (N) Rate (YMNR) is the N rate (kg N ha^{-1}) needed to achieve 95% relative grain yield for each site.

Site	Year	Location[a]	Cultivar	Soil Series	Soil Classification[b]	YMNR (kg N ha^{-1})
1	2016	PTRS	CL 153	Calloway SiL	Fine-silty, mixed, active, thermic aquic fraglossudalfs	119
2	2016	PTRS	CL 172	Calloway SiL	Fine-silty, mixed, active, thermic aquic fraglossudalfs	144
3	2016	PTRS	Diamond	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	142
4	2017	PTRS	CL 111	Henry SiL	Coarse-silty, mixed, active, thermic typic fragiaqualfs	96
5	2017	PTRS	CL 153	Henry SiL	Coarse-silty, mixed, active, thermic typic fragiaqualfs	93
6	2017	PTRS	Gemini	Henry SiL	Coarse-silty, mixed, active, thermic typic fragiaqualfs	131
7	2017	PTRS	LaKast	Henry SiL	Coarse-silty, mixed, active, thermic typic fragiaqualfs	81
8	2017	PTRS	XL 753	Henry SiL	Coarse-silty, mixed, active, thermic typic fragiaqualfs	125
9	2017	RRS	CL 111	Hebert SiL	Fine-silty, mixed, active, thermic aeric epiaqualfs	140
10	2017	RRS	CL 153	Hebert SiL	Fine-silty, mixed, active, thermic aeric epiaqualfs	116
11	2017	RRS	LaKast	Hebert SiL	Fine-silty, mixed, active, thermic aeric epiaqualfs	123

[a] Pinetree Research Station (PTRS), Rohwer Research Station (RRS), Rice Research and Extension Center (RREC)
[b] Source: Soil Survey Staff, USDA-NRCS

Table 3-2. Selected cultivar and site information for each location in this study for the year of 2018. Yield Maximizing nitrogen (N) Rate (YMNR) is the N rate (kg N ha^{-1}) needed to achieve 95% relative grain yield for each site.

Site	Year	Location[a]	Cultivar	Soil Series	Soil Classification[b]	YMNR (kg N ha^{-1})
12	2018	PTRS	CL 111	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	138
13	2018	PTRS	CL 153	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	146
14	2018	PTRS	Diamond	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	119
15	2018	PTRS	Gemini	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	144
16	2018	PTRS	LaKast	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	138
17	2018	PTRS	XL 753	Calhoun SiL	Fine-silty, mixed, active, thermic typic glossaqualfs	144
18	2018	RREC	CL 153	Dewitt SiL	Fine, smectitic, thermic typic albaqualfs	124
19	2018	RREC	Diamond	Dewitt SiL	Fine, smectitic, thermic typic albaqualfs	157
20	2018	RREC	CL 272	Dewitt SiL	Fine, smectitic, thermic typic albaqualfs	122
21	2018	RREC	PVL01	Dewitt SiL	Fine, smectitic, thermic typic albaqualfs	135

[a] Pinetree Research Station (PTRS), Rohwer Research Station (RRS), Rice Research and Extension Center (RREC)
[b] Source: Soil Survey Staff, USDA-NRCS

Table 3-3. Regression parameters and quality of fit metrics for linear plateau and quadratic plateau models fit to NO_3^--N concentration by relative grain yield for all sites.

	Quadratic Plateau	Linear Plateau
	Coefficient	
Join point	3.9 ± 0.8[a]	1.9 ± 0.2[a]
Plateau	98.4	97.1
	Fit Metrics	
Mean Absolute Error	8.27	8.08
Mean Absolute Error Percent	0.110	0.107
Mean Square Error	135	129
Root Mean Square Error	11.6	11.3
Normalized Root Mean Square Error	0.131	0.128
AIC[b]	3453.2	3409.4
BIC[c]	3469.6	3425.8
Log-likelihood	-1722.6	-1700.7

[a] 95% confidence interval for the regression parameter
[b] Akaike Information Criterion
[c] Bayesian Information Criterion

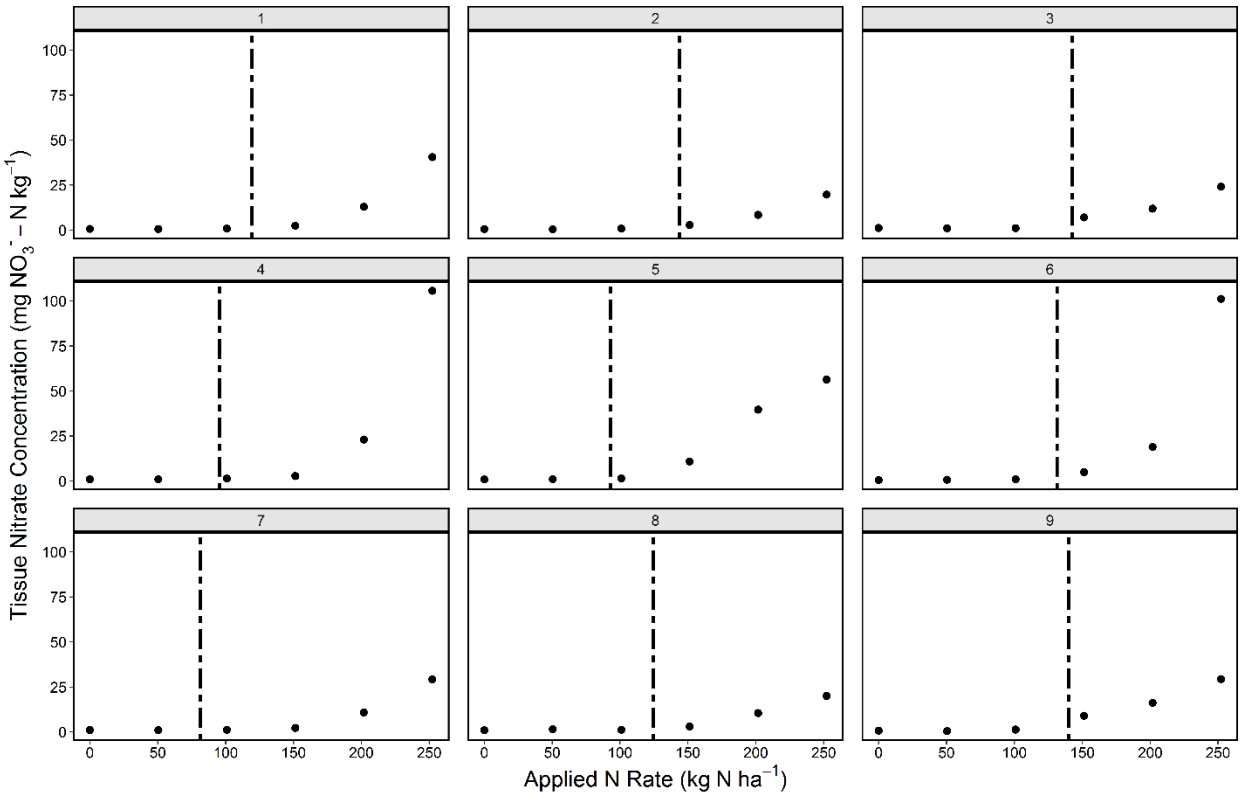

Figure 3-1. Mean tissue NO_3^--N concentration from rice stalk tissue (0-20 cm above the soil) as it responds to nitrogen (N) application rate for sites 1 through 9. The dashed vertical line represents the N rate required to achieve 95% relative grain yield for each site or the yield maximizing N rate (YMNR).

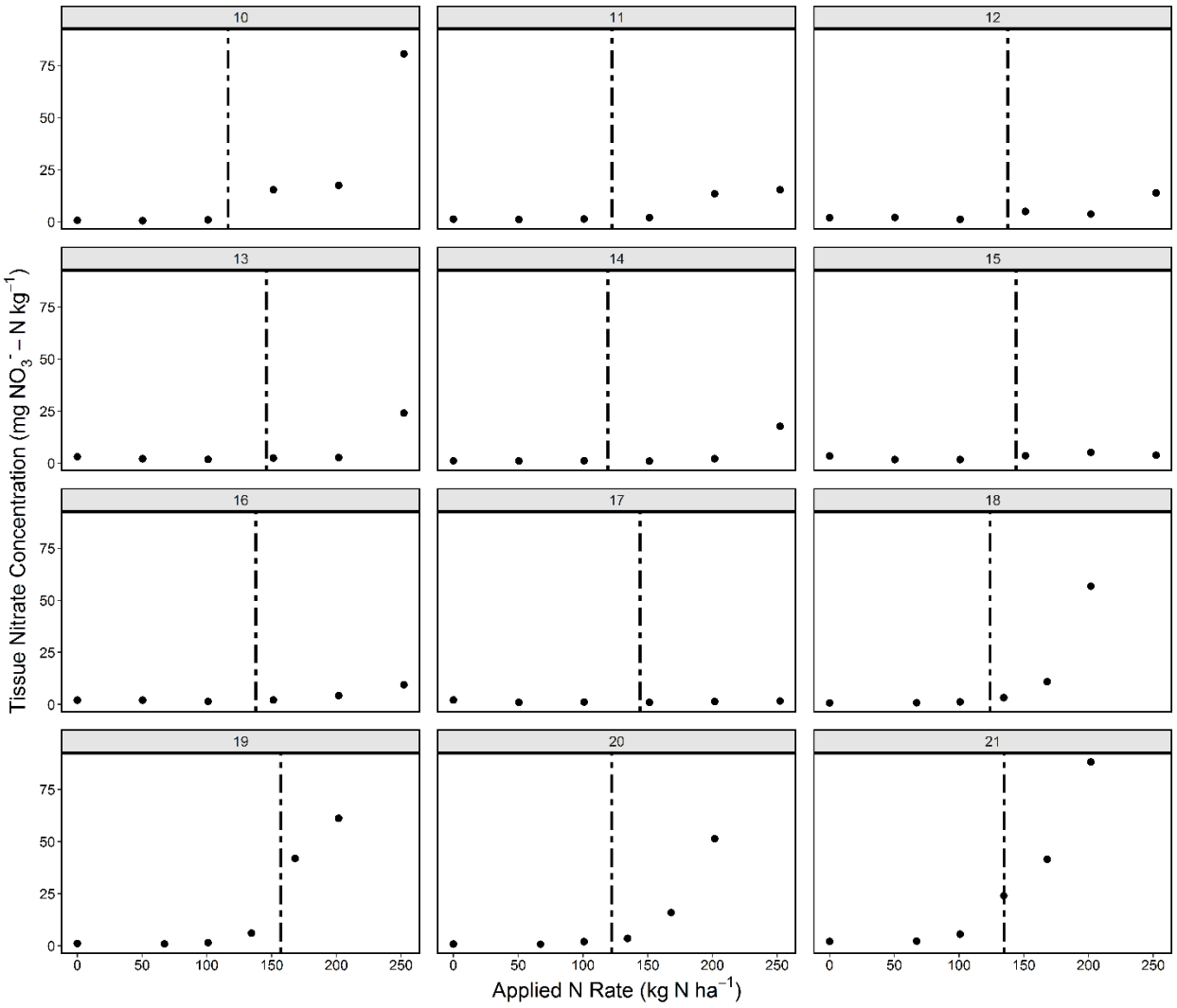

Figure 3-2. Mean tissue NO_3^--N concentration from rice stalk tissue (0-20 cm above the soil) as it responds to nitrogen (N) application rate for sites 10 through 21. The dashed vertical line represents the N rate required to achieve 95% relative grain yield for each site or the yield maximizing N rate (YMNR).

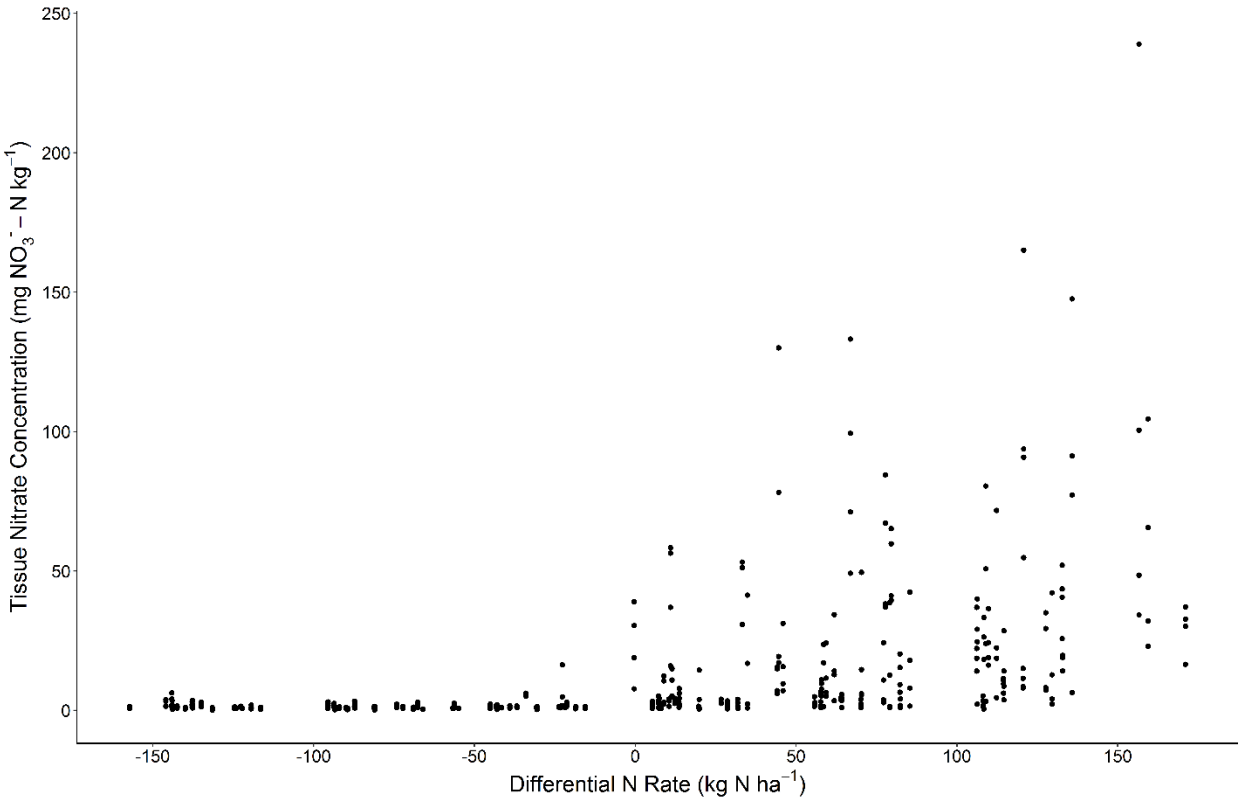

Figure 3-3. The relationship between rice stalk tissue NO_3^--N concentration (0-20 cm above the soil) and the differential nitrogen (N) rate. Differential N rate is the difference between the applied N rate and the N rate required to achieve 95% relative grain yield or the yield maximizing N rate (YMNR). Positive values indicate over fertilization and negative values are indicative of under fertilization.

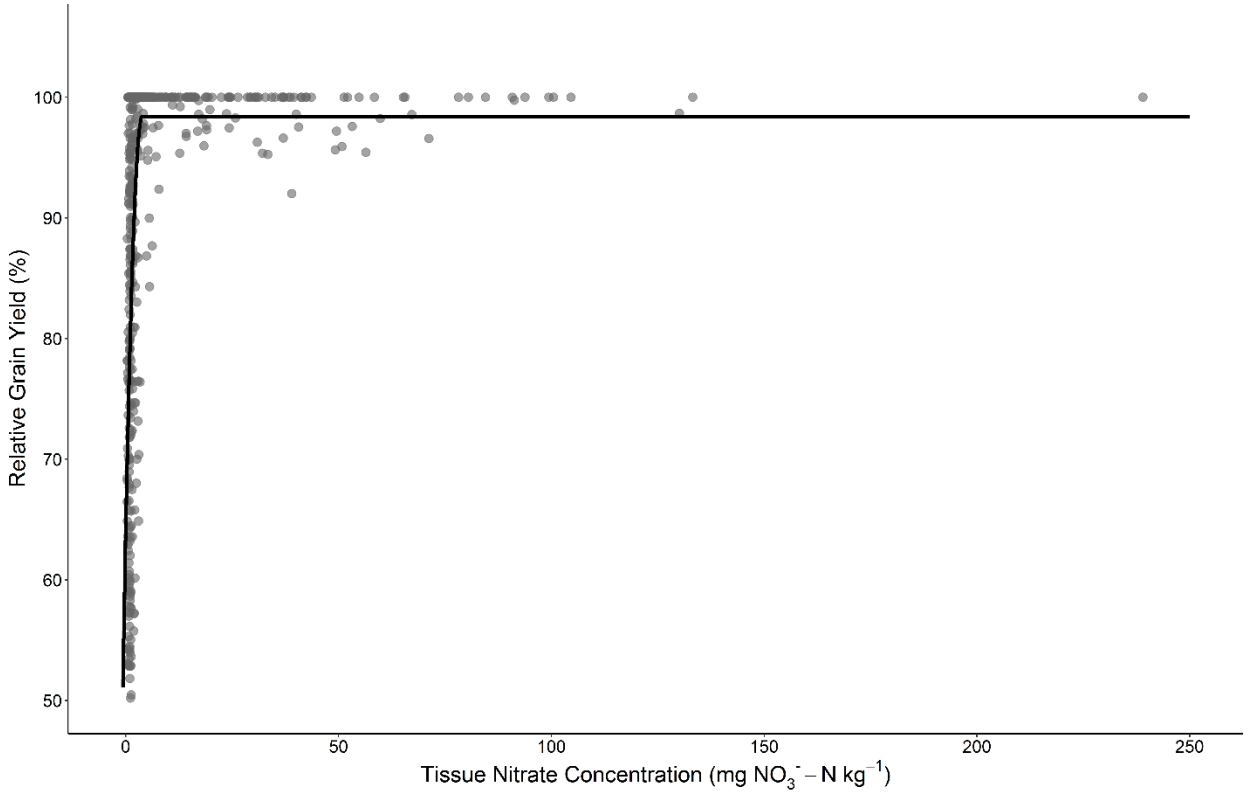

Figure 3-4. The relationship between rice stalk tissue NO_3^--N concentration (0-20 cm above the soil) and relative grain yield as modeled by the quadratic plateau model for all sites.

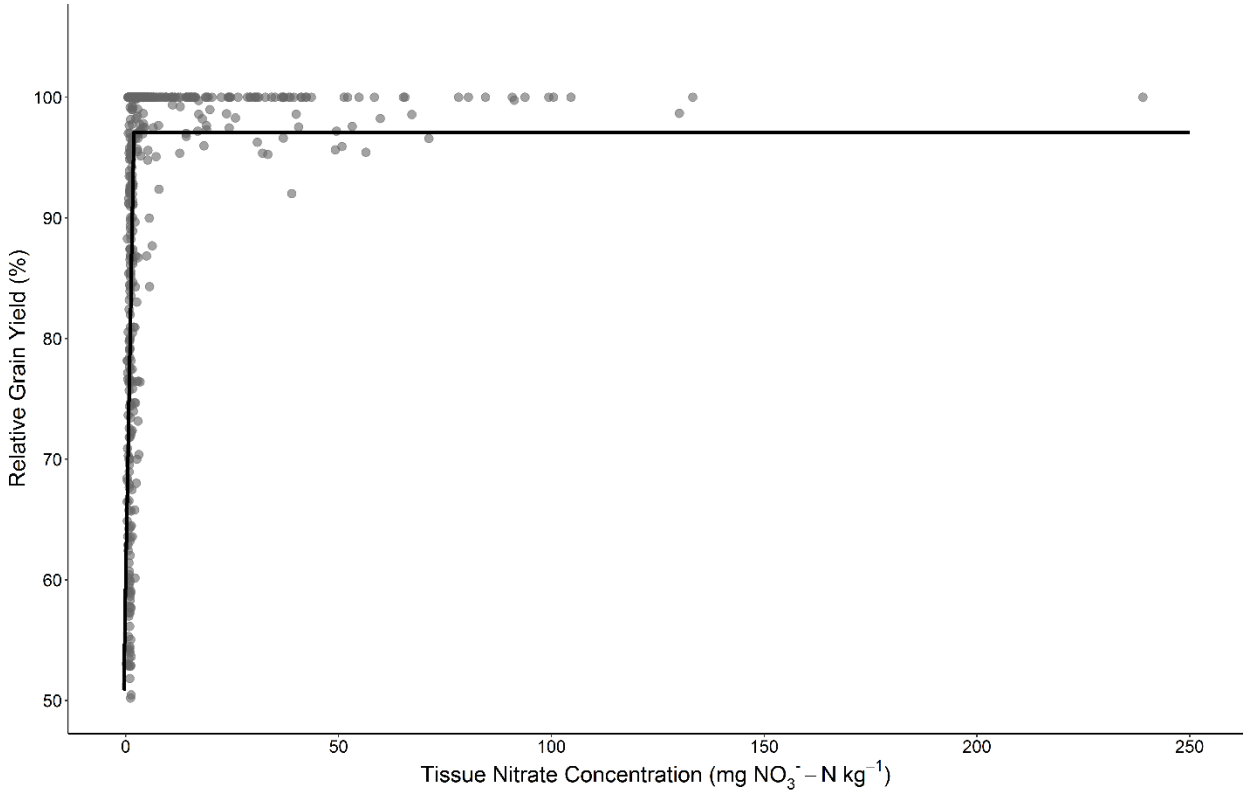

Figure 3-5. The relationship between rice stalk tissue NO_3^--N concentration (0-20 cm above the soil) and relative grain yield as modeled by the linear plateau model for all sites.

Chapter 4: Conclusions

The studies outlined in this thesis were designed to determine if the concentration of N in tissue samples collected from rice stalks at the end of the growing season could be utilized for evaluating and correcting improper N fertilization in rice. Initial results showed an accumulation of N in rice stalks because of over fertilization. Further analysis was conducted to examine this relationship for NO_3^--N, NH_4^+-N, and Amino Acid concentrations. All three of these analytes exhibited a similar trend, but large differences in the variability of each analyte were observed. Ultimately, NO_3^--N concentrations showed more consistency throughout the locations, cultivars, and years evaluated in this research than the other two forms of N measured.

Next, the NO_3^--N concentrations of the rice stalk were examined in relationship to the height at which the samples were taken. The NO_3^--N concentrations decreased with increasing plant sample height. Combining congruent 5 cm segments reduced variability of NO_3^-N concentration. Taking all of these findings and combining them with practical considerations, it was concluded that the best sampling protocols are to collect rice stalks from 0-20 cm above the soil at the end of the season. Once the samples are collected, they can be processed and analyzed for NO_3^--N concentration.

Additional data was collected using the sampling protocols established in chapter 2. This data was used to further explore the relationship between late season NO_3^--N concentrations to yield and applied N rate. Two models were used to describe the data, linear plateau and quadratic plateau models. The two models both separate the data into a region that is responsive to the independent variable (N rate) and a region that does not respond to the independent variable. The intersection of these two regions is the join point.

Although the quadratic plateau model fit the data well, the linear plateau model fit the data better according to various metrics. The join point of the linear plateau model was 1.9 mg

NO_3^--N kg^{-1}. Practically, this value represents the critical concentration that separates sufficient from deficient N rates. To further account for variability in samples and reduce false diagnosis, the upper bound of the 95% confidence interval (2.1 mg NO_3^--N kg^{-1}) was selected as the critical concentration for this diagnostic tissue test.

Results from this thesis support the hypothesis that a post-season N test can diagnose N deficiency in rice. Based on observations and conclusions made from this data, protocols were established as follows: Rice stalks should be sampled at 0-20 cm above the soil surface within one week of harvest. Samples should then be analyzed for NO_3^--N concentrations and compared to the established critical concentration. Samples that fall below this threshold are likely N deficient and would have benefitted from additional N during the growing season. While samples with a concentration above this threshold were likely supplied adequate or excessive N to maximize yield.