INVESTIGATION OF NITROGEN-SOURCE PREFERENCE IN SOUTHERN HIGHBUSH BLUEBERRY

by

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(Under the Direction of Anish Malladi)

ABSTRACT

Blueberry production has increased rapidly resulting in cultivation in soils not optimal for blueberry growth and development. One important aspect of plant growth and development is nutrition. Nitrogen (N) is one of the most essential nutrients for plants. This study investigated the uptake kinetics for both ammonium (NH_4^+) and nitrate (NO_3^-) at low and high external concentrations of N, as well as N-source preference when providing both N forms simultaneously to a split root system. Investigation of uptake kinetics displayed saturable uptake systems at low N concentrations and non-saturable uptake at high N concentrations for both NH_4^+ and NO_3^-. Uptake kinetics evaluation indicated a greater capacity in blueberry to acquire NH_4^+ compared to NO_3^- at low and high concentrations. N-source preference evaluation also indicated greater capacity to acquire NH_4^+ and that uptake was not influenced by the additional N-source in the split root system.

INDEX WORDS: Blueberry, Nitrogen, Nitrogen-Source preference, Nitrogen uptake kinetics

INVESTIGATION OF NITROGEN-SOURCE PREFERENCE IN SOUTHERN HIGHBUSH AND RABITEYE BLUEBERRY CULTIVARS

by

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DEDICATION

This work is dedicated to my family that is no longer with me, but instilled the drive to pursue a Master of Science at the University of Georgia. Dr. George Hugh Boyd (Pop), Mrs. George Hugh Boyd (Mama Boo), Mrs. Jean Boyd Palmer (Nana), Dr. Jack Roles Palmer (Datch), Mr. Jack Roles Palmer Jr. (Uncle Jay) and Betty Palmer Doyle (Mom), thank you for always being here with me. This work is also dedicated to my wife and father, Katie and John Doyle. This work would not have been accomplished without your constant support. I am forever grateful for all that you have done for me in helping achieve this goal.

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CHAPTER 1

Introduction and Literature Review

Many horticultural crops have long and extensive histories of cultivation and production and the same can be said about blueberries. It is thought that blueberries were first utilized by Native Americans when encountered in the wild. Wild blueberry management began with European settlers in the early 1800s and this style of management continued utilizing native lowbush and highbush blueberries [1]. In 1905, Frederick V. Coville purchased a farm in New Hampshire and the fields were occupied with native highbush and lowbush blueberries. He believed that the plants could be cultivated, and berries not just harvested from native plants. Coville worked with George W. Oliver to conduct the first experiment with blueberry cultivation and diversity [2, 3]. Frederick Coville is also known for determining that blueberries needed moist soils, low pH, required chilling for production and had a low nutrient requirement [2].

Since the early 1900s, blueberries (*Vaccinium* spp.) have become a major fruit crop in the United States [1]. Increased interest in blueberries and how they positively influence human health has also led to an increase in popularity and likewise production [4]. Studies have investigated the role of blueberry and how they influence aspects of human health such as cardiovascular health and aging [5]. Many of these approaches involve *in vitro* studies and can be used to identify processes, such as oxidative stresses, and the roles that antioxidants can play in combating reactive oxygen species [6]. Interaction between blueberries and human health continues to be studied. One study investigated if wild blueberry (*Vaccinium angustifolium*)

would increase the level of "postprandial serum antioxidant status" in the bodies of middle-aged males and it was determined that consumption of wild blueberry did increase antioxidant status in the test subjects and this increase may reduce risks of other degenerative diseases [7]. Follow-up studies also investigated the availability of these antioxidants that are acquired through the consumption of blueberries. It was identified that polyphenols from blueberry were absorbed poorly in the human digestive system and determined that many polyphenols were absorbed differently and therefore led to different availabilities within the human body. The researchers determined that more data with respect to polyphenols is needed to fully understand the importance of blueberries, antioxidants, and the human health impacts [8].

Increased understanding of the relationship between human health and blueberries has led to an increase in blueberry production. In 2019, global blueberry production exceeded 294,000 acres, almost doubling production area from the early 2000s [9]. Blueberry fruit production exceeded 820,00 tons in 2019, quadrupling fruit production from the early 2000s. Currently the United States is the leading blueberry producer world-wide, followed by Canada (176,127 tons) and Peru (142,427 tons) [9]. In the United States there are four types of blueberries grown: lowbush (*Vaccinium angustifolium*), northern highbush (*V. corymbosum*), southern highbush (*V. corymbosum;* hybrids) and rabbiteye (*V. virgatum*). Lowbush blueberry grows in colder climates and is not produced commercially in Georgia. Highbush blueberries can be split into two categories: northern and southern highbush. In the United States, northern highbush blueberry is the most widely produced. This variety is not grown in Georgia due to its high chilling hour requirement of approximately 800 – 1000 hours [10]. In Georgia, southern highbush and rabbiteye blueberries are the two types produced commercially. Southern highbush and rabbiteye blueberries require fewer chilling hours than lowbush and northern highbush [10, 11]. Depending

on the variety, rabbiteye and southern highbush blueberries need approximately 400 – 600 hours of chilling [11].

Blueberry is a member of the *Ericaceae* family, which are adapted to acidic soils and considered 'calcifuge' or lime-avoiding plants [12, 13]. Blueberry growth and development are usually optimal under low pH conditions (4 – 5.5), where nutrient availability is limited, leading to describing blueberry as having low nutrient requirements and displaying slower growth [12-15]. Blueberry plants generally contain less than 2% nitrogen (N; dry-weight), however there may be variation in N concentrations across different *Vaccinium* cultivars and would be directly related to N-fertilization [12, 13, 16-18].

Improving production of blueberries will require a better understanding of blueberry growth and development due to increased production and the likelihood that plants will be established on soils that are not optimal. One important aspect of growth and development is the plant's nutritional needs and understanding these needs can lead to increases in production and yield [19]. Nitrogen is a major component of nutrition and contributes greatly to plant growth and development.

Nitrogen Physiology in Blueberry

Nitrogen (N) is one of the most important macronutrients and is essential for plant growth and development [20]. Nitrogen, like many macronutrients, has multiple fates in plant systems including acquisition, transport, storage, assimilation, and remobilization, which all influence N homeostasis and availability to plants [21]. Nitrogen is present in multiple forms in the soil; organic forms of N consist of amino acids, peptides, and proteins; while inorganic forms consist

mainly of ammonium (NH_4^+) and nitrate (NO_3^-). Inorganic forms are the most abundant and plant-available [22].

Acquisition of Nitrogen: Organic Sources

Nutrient acquisition has been investigated on many crops from identifying pathways involved in sulfate uptake in *Arabidopsis thaliana* [23] to ammonium (NH_4^+) and nitrate (NO_3^-) uptake in White Spruce [24, 25]. Nutrient acquisition can be facilitated in many ways and is dependent on the form (organic vs. inorganic) being acquired. Organic sources plants utilize consist of proteins, peptides and amino acids and plants are thought to utilize these organic sources of N through excretion of proteases by plants or symbiotic relationships with soil fungi and microorganisms present in the soil [26, 27].

Utilization of free amino acids can occur either through mycorrhizal relationships or directly from the soil [28]. Recent evidence for direct utilization of free amino acids has indicated at least 3 sub-families of amino acid transporter genes. These sub-families are a part of the larger *AMINO ACID/AUXIN PERMEASE (AAAP)* family that encode proteins directly involved with root amino acid uptake and transport [29, 30]. Further details of these amino acid transports have been identified and evaluated in *Arabidopsis thaliana* [31, 32]. In blueberry, Paya-Milans et. al. (2017) conducted a transcriptome analysis of *V. arboretum* and *V. corymbosum* and identified that at high pH (6.5), six *AAAPs* were upregulated, indicating an ability to utilize organic sources of N under these conditions [33]. These results indicate that blueberry can utilize organic sources of N, but also identify an area of research needed to better understand organic N use under adverse soil conditions, which arise more frequently with increased production on soils not ideal for blueberry cultivation.

Mycorrhizal relationships with plant roots are known to greatly increase N acquisition [34]. Members of the Ericaceae family form these relationships with specific fungi and these relationships are called ericoid mycorrhizal associations (ERM) [35]. Stribley and Read (1974) used ^{15}N labeled ammonium (NH_4^+) supplied to cranberry plants with established and non-established ERM associations. They identified that N concentration was higher in plants with ERM associations, but the labeled ^{15}N was lower in these plants than plants without ERM associations, indicating that the presence of ERM increased organic N (non-labeled) acquisition [36]. Ericoid mycorrhizal associations are also established with blueberry and may lead to an increase in NO_3^- uptake, as was identified in cranberry plants. Plants with ERM relationships had greater NO_3^- uptake than plants without ERM associations and may aid in fulfilling N requirements when soil conditions are optimal [37]. As mentioned, ERM associations are present in blueberry and a survey of commercial blueberry fields indicated approximately 44% of blueberry root growth was attributed to ERM relationships [38].

Better understanding of ERM relationships with blueberry needs to be established. Scagel (2005) investigated if blueberries grown in ERM-inoculated peat moss had increased N acquisition. It was suggested that some cultivars may benefit from these relationships with respect to N acquisition, however, other studies yielded minimal improvement in blueberry growth and development in the presence of ERM associations [35, 39]. These studies indicate more research is necessary to truly understand the influence ERM associations have with respect to organic N acquisition. Although much has been learned with respect to blueberry utilization of organic N, there are still aspects of this utilization that need to be further investigated.

Acquisition of Nitrogen: Inorganic Sources

Another form of N that plants utilize is inorganic N. Inorganic is present in the soil as either NH_4^+ or NO_3^-. Due to the presence of two inorganic forms of N, plants can in-turn have a preference for which form they utilize more efficiently and this is known as N-source preference [40, 41]. Plants, such as White Spruce (*Picea glauca*), exhibit a preference for NH_4^+ as its primary inorganic source of N [42]. The fact that members of the Ericaceae family, blueberry included, prefer acidic soils with lower pH indicates a preference for NH_4^+ as their inorganic source of N [43]. To further investigate the phenomenon of N-source preference in blueberry, other studies have investigated plant growth and development with different inorganic N sources. It has been shown that blueberry has greater N accumulation and increased growth when provided NH_4^+ compared to NO_3^- [44, 45]. In rabbiteye cultivars, it has been shown that plants had greater shoot growth and increased foliar N concentrations when provided NH_4^+ compared to NO_3^- [46]. Hydroponic approaches to understanding N-source preference in blueberry have also indicated optimized growth and development when supplied NH_4^+ instead of NO_3^- as the inorganic N source [47].

Nitrogen-source influences blueberry growth and N accumulation within the plant, but source preference can also manifest as a preferential uptake of one form over the other. The rates at which these ions cross the plasma membrane and enter root cells have been investigated with respect to NH_4^+ and NO_3^-. Uptake of both NH_4^+ and NO_3^- has been investigated in many plant species and has been used to indicate N-source preference in White Spruce [24, 25, 42]. Along with investigating the rates of uptake, identification of different systems active during uptake has been accomplished. Inorganic N uptake follows a bi-phasic pattern and is extremely dependent on external concentration surrounding the root [24, 25, 48]. Nitrate uptake at low external

concentration (< 0.5 mM) is conducted by the high affinity transport system (HATS). The HATS system consists of 2 HATS systems that function under low concentrations but have different specific functionality under these conditions. The first HATS system is one that is always expressed in the roots (HATSa) and a second HATS system that is expressed only under low external concentrations and is not always expressed (HATSb). With respect to NO_3^-, HATS functionality is saturable (~ 1 mM) and follows the pattern of Michaelis-Menten kinetics [25, 48-51]. Once external concentrations increase and exceed the range of function of NO_3^- HATS, another system is stimulated. At higher external concentrations (1 – 50 mM), the low affinity transport system (LATS) becomes functional and is the predominant system for NO_3^- uptake [25, 49-51]. This system displays a linear uptake pattern, is not saturable and accounts for the majority of NO_3^- uptake under these conditions.

Ammonium uptake also follows a similar pattern to that of NO_3^-, with both HATS and LATS present depending on external concentration. Ammonium HATS is present under low external concentrations of NH_4^+ (< 1 mM) and follows the pattern of Michaelis-Menten kinetics and is considered saturable [52, 53]. When external concentrations increase, the NH_4^+ LATS system becomes functional and much like NO_3^- LATS, it follows a linear pattern of uptake, is not saturable and accounts for the majority of NH_4^+ acquisition under these conditions [24, 53].

Assimilation of Inorganic N

The assimilation of N is dependent of the form acquired from the soil; either NO_3^- or NH_4^+. Nitrate acquisition is facilitated by transport proteins and once acquired, NO_3^- can either be stored in the vacuole, translocated to the shoots, or assimilated in the roots [54]. Nitrate assimilation consists of the reduction of NO_3^- to NH_4^+, which requires large amounts of energy

and is a two-step processes involving nitrate reductase (NR) and nitrite reductase (NiR) and is compartmentalized between the plastids and cytoplasm in the cell [55]. The first step occurs in the cytoplasm and consists of the reduction of NO_3^- to nitrite (NO_2^-) by NR. The reduction of NO_3^- is usually considered the rate limiting step in NO_3^- assimilation and this is because this process requires large amount of NADPH. Nitrite is then rapidly reduced to NH_4^+ due to its negative impacts on plant cells and this secondary reduction is mediated by nitrite reductase in the plastids [56]. Ammonium assimilation is also a rapid process due to potential toxicity when it is accumulated in high amounts and is assimilated either at the point of uptake or after NO_3^- assimilation [57, 58].

Nitrate can be assimilated in the roots, once acquired, or transported to the shoots where photosynthetic products (ATP, NADPH) are readily available [21]. Many herbaceous plant species are known to conduct NO_3^- assimilation in the shoots, but it was initially thought that woody plants conduct most of this reduction in the roots [59]. Smirnoff et. al. (1984) identified that woody plant species displayed NO_3^- reduction capacity in the shoots when evaluating NO_3^- reduction in multiple plant species, including members of the Ericaceae family, but excluding blueberry [60].

Investigation into the ability for blueberry to conduct NO_3^- reduction in the shoots initially concluded that reduced capacity for reduction existed in the shoots [47, 61]. However, many other studies have shown NR activity in the shoots of blueberry and were able to quantify this activity [62-64]. The NR activity detected in blueberry was considerably lower than in other plant species and could limit the plant's use and therefore lead to a manifestation point for N-source preference in blueberry [47]. The final step of NO_3^- reduction is the further reduction of

NO_2^- to NH_4^+. Once reduced to NH_4^+, another process occurs prior to the formation of amino acids and functional proteins.

When NH_4^+ is acquired by plant cells, it is assimilated directly in the roots due to the potential toxicity of ammonia to plants [57]. Ammonium is converted into the amino acid glutamate via the GS-GOGAT pathway, which consists of reactions mediated by glutamine synthetase (GS) and glutamine-2-oxoglutarate-amino transferase (GOGAT) for the overall conversion to amino acids [65]. Ammonium is first converted to glutamine via GS by adding NH_4^+ to glutamate. Glutamine is then converted into two glutamates through the reaction with 2-oxoglutarate and is catalyzed by glutamine 2-oxoglutarate amino transferase. The products of the reaction with GOGAT are two molecules of glutamates, where one glutamate is recycled to repeat the reaction with GS and the second glutamate is then used in amino acid, protein, and N-containing compound syntheses [66-68]. The conversion of NH_4^+ to glutamate requires less energy than the assimilation of NO_3^- and therefore presents another manifestation point for N-source preference in plants. Localization of GS-GOGAT is primarily in the plastids in the shoots to account for the NH_4^+ produced in the final step of NO_3^- reduction. It is also present in the cytoplasm in root cells, where its primary function is the conversion of acquired NH_4^+ into glutamate and eventually amino acids [21].

Transport, Storage and Remobilization of Nitrogen

Transport of N from the roots to the shoots is dependent on the form of N acquired by the plant and initiates upon acquisition. Ammonium acquired from the soil is assimilated in the roots and transported as amino acids, primarily glutamine and asparagine, from the roots to the shoots [69]. Initial transport of acquired NO_3^- from the soil is facilitated by four transporter families:

NITRATE TRANSPORTER 1 (NRT1) / PEPTIDE TRANSPORTER family (NPF), NITRATE TRANSPORTER 2 (NRT 2), CHLORIDE CHANNEL family (CLC) and SLOWLY ACTIVATING ANION CHANNEL family (SLAC) [70, 71]. The NRT1 and NRT2 transport families are associated with root uptake of NO_3^- from the soil, while CLC and SLAC are thought to be involved in transport into the vacuole (CLC) and outflow of NO_3^- from guard cells (SLAC) [70-72]. Members of the NRT2 transporter family have been linked to being the primary transporters of NO_3^- under HATS uptake conditions, while NRT1 have been primarily associated with LATS uptake conditions [70, 73, 74]. Nitrate acquired by the roots can be transported via the phloem as either NO_3^- or in the form of glutamine or asparagine depending on the plant system [21].

Storage and remobilization of N are vital for growth and development in perennial species following dormancy [75-79]. Storage of N occurs in many tissues and significant storage occurs in roots, stems, and leaves. Generally, amino acids and proteins act as long-term storage units of N but are species dependent. The amount of stored N also influences N availability for plants. When plants have large N reserves, this can alter the processes of N acquisition based on plant needs for N and in early spring can manifest as the plant using reserves for growth and development as opposed to actively acquiring N from the soil [77, 79]. Sources that add to N storage pools in plants come from two primary sources: Active uptake during the current growing season and remobilization from stems and leaves. In deciduous plant species, such as *V. vitis-idaea,* N acquired after vegetative growth was allocated primarily to new leaves indicating that late season N acquisition is primarily for stored pools [80]. In blueberry, N remobilization studies have been attempted using labeled ^{15}N sources to trace the movement of these isotopes throughout the plant. Two blueberry cultivars were evaluated with respect to remobilization of

$^{15}NO_3^-$ and it was identified that in early spring, loss of shoot and root stored N occurred. It was noted that a high decrease in stored N occurred in the roots, indicating that they are the primary storage organ in these two blueberry cultivars [81]. The results of this study show the importance of N storage and late season acquisition to provide adequate storage pools for growth and development in the following growing season. The specific tissues for storage, forms of N storage and regeneration of these storage pools are all species dependent and require further investigation to truly understand their mechanics in plant systems.

Nitrogen-Source Preference in Blueberry

Nutrient preferences exhibited by plants manifest under different physiological processes such as acquisition, translocation, and assimilation [64]. Nitrogen-source preference is a phenomenon in which plants exhibit a preference for one inorganic form of nitrogen over the other [41]. It has been shown that plants respond to different forms of inorganic N differently and some plants grow better when only provided NO_3^- compared to NH_4^+. In strawberry, it has been shown that plants grown hydroponically had greater biomass accumulation, leaf area and yield when supplied higher ratio of NO_3^- and plants that received more NO_3^- also exhibited lower levels of Calcium (Ca^{2+}) and reduced storage capacity and concluded that strawberry exhibited a preference for NO_3^- as the inorganic source of N based of growth and developmental results [82].

Blueberry, and other plants in the Ericaceae family, are thought to prefer the inorganic nitrogen source, NH_4^+, and previous research suggested that growth declined when only provided NO_3^- [61]. A second study showed that NH_4^+ is essential for lowbush blueberry growth and development. The goal of this study was to determine if lowbush and highbush blueberries had nitrate-reducing systems. The plants that were supplied only NH_4^+ did not show nitrate-reducing

activity, as was to be expected. However, nitrate-reducing activity was observed at NO_3^- concentrations of 0.1 parts per million (ppm) and higher in treatments receiving NO_3^-. They found that lowbush blueberry performed better when supplied NH_4^+. They also concluded that lowbush and highbush blueberries did in fact have nitrate-reducing systems, but due to plant performance with NH_4^+, they concluded that the plants may have a exhibited a preference for NH_4^+ as the N-source [83]. A more recent study used hydroponics to evaluate nitrate reductase (NR) and ferric chelate reductase (FCR) activity when supplied with NO_3^- or NH_4^+. The researchers tested a wild blueberry (*Vaccinium arboreum*) and a cultivated blueberry (*Vaccinium corymbosum*). They found that both species acquired more NH_4^+ solution than NO_3^-. They concluded that the wild blueberry was able to acquire NO_3^- better than the cultivated blueberry due to increased nitrate-reductase activity observed during the study [47].

These studies have observed and identified enhanced growth characteristics of blueberry when supplied with NH_4^+ as the inorganic source of N. This is indicative of the presence of N-source preference within blueberry and, based on the findings of Poonnachit and Darnell (2004) suggest that cultivars may differ in exhibiting this preference [47]. Due to this, it is important and essential to investigate N-source preference in blueberries widely cultivated in the state of Georgia. Previous research in lowbush and northern highbush blueberries has suggested a preference for NH_4^+ as the inorganic nitrogen source, but little research has been conducted with respect to rabbiteye and southern highbush blueberries. With blueberry production increasing in the southeastern US, new varieties of both rabbiteye and southern highbush blueberries are being developed and used for commercial production, yet their N-source preference is not known. This is an area of research that is currently lacking and deserves more attention in the future to enhance blueberry production in the southeastern US and world-wide.

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CHAPTER 2

Evaluation of Nitrate and Ammonium Uptake Kinetics in Southern Highbush Blueberry[1]

[1] Doyle, John, Malladi, Anish, Cabrera, Miguel, and Coolong, Timothy. To be submitted to Horticulturae.

Abstract

Blueberry (*Vaccinium* sp. L.), has quickly emerged as a major fruit crop in the world and United States. With rapid growth through cultivation and newly emerging cultivars, it is essential to understand blueberry growth and development through nutrient management. Understanding inorganic N uptake kinetics of blueberry is essential to obtaining management goals and reducing fertilizer costs. Naturally, blueberry grows in upland forest soils where ammonium (NH_4^+) is the primary form of inorganic N present in the soil and leads to the hypothesis that blueberry acquires NH_4^+ more readily than NO_3^-. In this study, blueberry uptake kinetics were evaluated in the cultivar 'Suziblue'. Identification of High and Low Affinity Transport Systems (HATS and LATS) with respect to ammonium and nitrate was conducted via hydroponically grown 'Suziblue' plants. External N concentrations ranged from 0 to 500 µM-N (HATS) and 0 to 50 mM-N (LATS) and uptake was evaluated by supplying ^{15}N to plants via the hydroponic solutions. Ammonium and nitrate HATS displayed a saturable uptake pattern and was fit to Michaelis-Menten model with a K_m = 33.7 µM-NH_4^+ and 16.5 µM-NO_3^- and V_{max} = 85.76 µmol-NH_4^+ g^{-1} d^{-1} and 6.57 µmol-NO_3^- g^{-1} d^{-1}. The V_{max} or rate of reactions for NH_4^+ HATS is 13-fold higher than that of NO_3^- HATS, indicating enhanced uptake of NH_4^+. LATS were evaluated by fitting 3 models: linear, Michaelis-Menten and quadratic. Ammonium and nitrate LATS displayed a linear uptake pattern (AICc= 78.38, R^2= 0.95; AICc= 62.57, R^2= 0.97 respectively). LATS uptake of both inorganic forms of N displayed similar slope values, yet a 2 to 3-fold higher uptake rate, indicating that even in mM concentration range, 'Suziblue' has an enhanced ability to acquire NH_4^+ over NO_3^-.

Introduction

Stable isotopes have been used to investigate ecological relationships and physiological processes within plants and animals, among other areas of interest [1]. In animal research, stable isotopes have been used to understand food web dynamics. Cerling et. al., 2004 used stable isotopes to investigate food web dynamics in the Ituri Forest in the Democratic Republic of Congo. Isotopes of carbon and oxygen were used to identify differences in animal feed behaviors in the ecosystem. They found that levels of ^{13}C were found in animals with known dietary differences. Interestingly, they also identified that animals had chosen different water sources due to varying levels of ^{18}O, suggesting that water source was correlated with dietary differences or different feeding behavior in this ecosystem [2].

In human nutrition, stable isotopes have also been used to identify different food sources. Different environmental systems have different levels of stable isotopes present. Marine and terrestrial systems are known to have major differences in carbon and nitrogen isotope ratios. In plant systems, the form of metabolism (C3 vs. C4) can have major impacts on the ratios of carbon present in the plant tissues. Using this information, researchers have used isotope ratios to correlate sources of food for humans between marine and terrestrial ecosystems [3]. In plant science research, the use of stable isotopes has been used for understanding many pathways and processes in plants. One such area of interest is plant metabolism. Carbon (C) and nitrogen (N) can be supplied to plants in their stable isotope forms of ^{13}C and ^{15}N. Using these isotopes allows researchers to identify and track when and where these elements are incorporated in plant systems and help to identify what forms these elements are in, when functional in plants [4].

In blueberry, ^{15}N has been used to investigate the fate of field supplied N. Retamales and Hanson (1989), supplied ^{15}N-labeled urea to understand N-use in field grown ‘Bluecrop’. They

identified that at 2 weeks after supply, ^{15}N was observed in the shoots and observed that approximately 15% of supplied labeled-N was not utilized during the growing season [5]. This approach allowed for understanding of how 'Bluecrop' blueberry utilizes urea in the field. Additional research into blueberry N-use was conducted by Throop and Hanson (1997), to evaluate when 'Bluecrop' blueberry utilized N most during its growing season. They reported that 'Bluecrop' blueberry utilized more ^{15}N from May to July. Indicating the 'Bluecrop' blueberry actively acquired more N during the active growing season and leading up to fruit maturity [6]. In 1995, Merhaut and Darnell utilized ^{15}N sources of NO_3^- and NH_4^+ to investigate which inorganic form blueberry utilized most efficiently. Containerized 'Sharpblue' blueberry was provided ^{15}N in the form of NO_3^- or NH_4^+. They observed that 'Sharpblue' was able to acquire both forms of inorganic N, however, they identified that NH_4^+ was acquired in greater amounts than NO_3^-. Nitrogen translocation from roots to shoots was greater in plants that received NH_4^+ compared to NO_3^-. However, this study could not determine if these results were due to limited ability to reduce NO_3^- in the roots or due to greater capacity to transport NH_4^+ [7]. This study indicates that more research needed to be conducted to evaluate the reason why 'Sharpblue' blueberry displayed enhanced ability to acquire NH_4^+ compared to NO_3^-.

Many plants utilize both NO_3^- and NH_4^+, and it has been identified that plants uptake these inorganic forms of N in biphasic patterns based on external concentration of N [8, 9]. Nitrate has been shown to have two major systems that aid in N-acquisition depending on external N concentration, high and low affinity transport systems (HATS/LATS). High affinity systems generally function at lower concentrations (< 1 mM), are often saturable, and follow an uptake pattern similar to Michaelis-Menten kinetics. Low affinity systems function at higher concentrations (> 1 mM) and often display a linear uptake pattern and may aid in luxury

consumption of N [10-12]. Research into these transport systems in blueberry is limited, however NO_3^- uptake kinetics was identified in 'Tifblue' rabbiteye blueberry. A functional and saturable HATS was identified with a V_{max} of 1.8 µmol g^{-1} h^{-1} and a K_m of 23 µM [13, 14]. Even though NO_3^- HATS have been identified in blueberry, LATS characteristics have not yet been reported even though most N uptake and utilization studies in blueberry have utilized concentrations within the LATS range (> 1 mM). With respect to ammonium, both HATS and LATS have been identified as functional [14-16]. Ammonium HATS follow a similar pattern and is saturable and functions below 1 mM NH_4^+, much like NO_3^- HATS. Sugiyama and Hirooka (1993) identified functional NH_4^+ HATS in blueberry, however NH_4^+ LATS were not evaluated in their study [14, 17].

In this study, the presence and function of HATS and LATS with respect to both NH_4^+ and NO_3^- were characterized in the southern highbush cultivar, 'Suziblue'. To allow for greater accuracy in measurement of uptake kinetics, the stable isotope (^{15}N) was used in this study.

Methods and Materials

Plant Material

'Suziblue' blueberry cuttings were purchased from Cornelius Blueberry Farms, Manor, GA and transported to the Riverbend Greenhouse Complex in Athens, GA in early 2021. The plants were then transplanted into 3.78-L containers filled with a 1:1 mixture of pine bark mulch and peat moss. Plants were fertigated weekly with J.R. Peter's Acid Special (21-7-7) at 50 mg L^{-1} N. Cuttings were grown for 1 month to allow for proper root development. Three days prior to transfer to hydroponics, plants were not watered to allow for optimal root washing and removal of substrate.

Hydroponic System and Modified Hoagland's Solution

The hydroponic system consisted of 1-L plastic paint buckets with lids, purchased from a local hardware store. Each paint bucket was covered in tinfoil to reduce light transmission into the container. The paint bucket lids had a "X" cut into them to allow for insertion of the plant roots into the vessel. This cap also aided in reducing solution losses due to evaporation in the greenhouse. One plant was placed into each cup and suspended from a trellis system in the greenhouse. The root collar of the plant was suspended 1" above the solution in the paint bucket. Aeration was supplied to each paint bucket *via* an air pump and airline tubing with an air-stone.

Four individual studies were conducted to investigate LATS (NH_4^+/NO_3^-) and HATS (NH_4^+/NO_3^-) uptake kinetics in blueberry. Plants used in all studies were first exposed to an acclimation solution for 5 d, followed by a N-starvation solution for 2 d, then followed by treatment solutions for 6 h (LATS) and 24 h (HATS). The foundation of the hydroponic solution was a modified version of Hoagland's hydroponic solution and consisted of 0.5 mM potassium phosphate, 1 mM magnesium sulfate, 0.5 mM calcium chloride, 0.08 mM Fe-EDTA, 0.045 mM boric acid, 0.01 mM manganese sulfate, 0.01 mM zinc sulfate, and 0.02 µM sodium molybdate. Nitrogen-source was supplied as either ammonium sulfate or potassium nitrate [18, 19]. The acclimation solution consisted of non-labelled N-sources and the treatment solution consisted of labelled ^{15}N-sources. The acclimation solution for HATS had a concentration of 250 µM N and the starvation solution had 0 µM N. There were seven treatment solutions for HATS with N concentrations of 10.15, 20.3, 50.75, 101.5, 150.25, 203 and 507.48 µM. ^{15}N was provided as $^{15}NH_4^+$ at 3.14 at% enrichment within this solution. Nitrate HATS treatments had N concentrations of 5, 10, 25, 50, 75, 100 and 250 µM. ^{15}N was provided as $^{15}NO_3^-$, at 5 at%

enrichment. The acclimation solution for LATS studies consisted of 1 mM N and the starvation solution had N concentration of 0 mM. Seven treatment solutions were supplied to investigate LATS uptake kinetics with concentrations of 0.204, 1.016, 2.04, 5.08, 10.16, 20.30, 50.74 mM for NH_4. ^{15}N was provided as $^{15}NH_4^+$ at 3.14 at% enrichment within this solution. Nitrate LATS was evaluated by providing N at 0.1, 0.5, 1, 2.5, 5, 10 and 25 mM concentrations. ^{15}N was provided as $^{15}NO_3^-$, at 5 at% enrichment. The slight differences in treatment concentrations between NH_4^+ and NO_3^- were due to a calculation error while preparing dilutions of $^{15}NH_4^+$ stock for the initial studies. The NH_4^+ and NO_3^- HATS studies were performed for a duration of 24 h, while the LATS studies were performed for 6 h. These times were chosen based on a preliminary study which indicated quantifiable ^{15}N uptake into roots and some translocation into shoots within the 24 h time period.

^{15}N Stock Solution Preparation and Calculations

Cabrera and Kissel (1989) released a simplification of the calculations needed to properly develop ^{15}N solutions for different applications of tracer studies [20]. For this study, atom % enrichment was generated from the ^{15}N stocks following Cabrera and Kissel, 1989. Labeled N material was purchased from Cambridge Isotope Laboratories in the form of 50 g $K^{15}NO_3$ with 10.4 at% enrichment or $(^{15}NH_4)_2SO_4$ with 5 at% enrichment.

Sample Collection and Preparation

Root and shoot samples were collected. Root samples were washed three times; the first and second wash consisted of the modified Hoagland's solution with nonlabelled N-sources, and the final wash consisted of the modified Hoagland's solution without N. Root wash timeframes

were kept consistent between washes and among root samples. Washes lasted < 1 minute and were conducted to remove any labeled N-sources adhering to the surface of the root tissue. Once roots were washed, they were blotted dry and placed into 50 mL tubes. Shoot samples were collected and entire sample placed in 50 mL tubes. All sample tubes were pre-frozen in liquid Nitrogen with care taken not to contaminate the tubes and then re-frozen once sample was in place. Samples were stored at -80 °C until freeze drying for > 24 h. After freeze drying, samples were submitted to the Stable Isotope Ecology Laboratory at the Center for Applied Isotope Studies at the University of Georgia, Athens, GA (SIEL-UGA) where they were prepared and processed for analysis.

Isotope Ratio Mass Spectrometry Analysis

After drying, the sample material was finely ground and 2-3 mg (root tissue) was added to encapsulation tins after weighing on a semi-microbalance accurate to 0.01 mg. These tins were then placed in a 96-well microtiter plate and submitted for analysis. Analysis consisted of combusting the encapsulated samples at 1100 °C and the gases produced were delivered via continuous-flow for analysis of $\delta^{15}N$ using isotope ratio mass spectrometry (IRMS) where enrichment of samples was compared to natural abundance of ^{15}N in the atmosphere (0.37 atom %). Results were presented as Atom % ^{15}N and this was used to calculate to Atom % Excess (APE) for each sample. The APE was then used to calculate ^{15}N incorporated (during acquisition) into the plant root tissue. The analysis was conducted by using the samples with the least amount of enrichment first and moving to samples with the highest enrichment to reduce contamination within the lab setting. All materials used for the analysis were thoroughly cleaned with ethanol between each sample to further reduce chances of contamination [21].

Statistical analyses

These studies were conducted as randomized complete block designs with four replicates ($^{15}NH_4^+$) and five replicates ($^{15}NO_3^-$). Uptake rates were calculated based on Atom Percent Excess (APE) compared to control plants not supplied ^{15}N and standardized based on total root dry mass (g). Figures were created using SigmaPlot11/JMP software and statistical analyses were conducted using R open source software and JMP software [22, 23].

Results

Uptake Kinetics response to Nitrate supplied in the µM range

Nitrate HATS uptake kinetics were evaluated from 0 to 250 µM. Within this range, N uptake as NO_3^- was saturable and followed (Figure 1). Little to no change in the N uptake rate was observed beyond 100 µM. Parameters estimated from the Michaelis-Menten fit of the uptake data were: V_{max} = 6.57 µmol g^{-1} d^{-1} and a K_m= 16.54 µM.

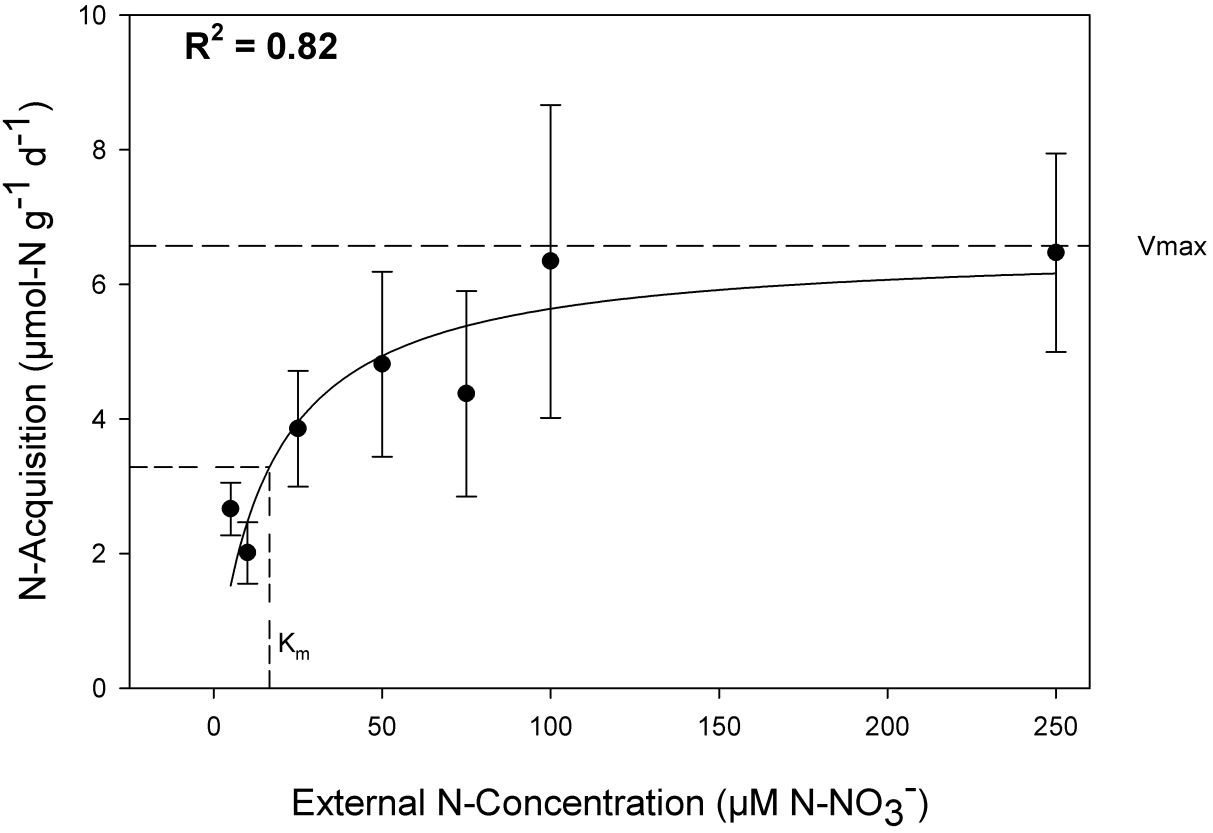

Figure 2.1 – Saturable Nitrate HATS Present in 'Suziblue': Saturable High Affinity Transport System for NO_3^- uptake under external concentrations below 250 µM nitrogen. Data were normalized to the root dry weight (g). Michaelis-Menten equation was fit to the data with an observed K_m**= 16.54 µM and a** V_{max}**= 6.57 µmol N g^{-1} d^{-1}, (p= 0.0001, R^2=0.82).**

Translocation of N from the roots to shoots was also evaluated during the 24 h study period for NO_3^- supplied at a concentration ≤ 250 µM N. During this period, minimal translocation to the shoot occurred with respect to NO_3^-. Only around 3% of the total ^{15}N acquired by the plant was present in the shoots at 24 h after N supply at the highest N concentration treatment (250 µM) in this study (Figure 2).

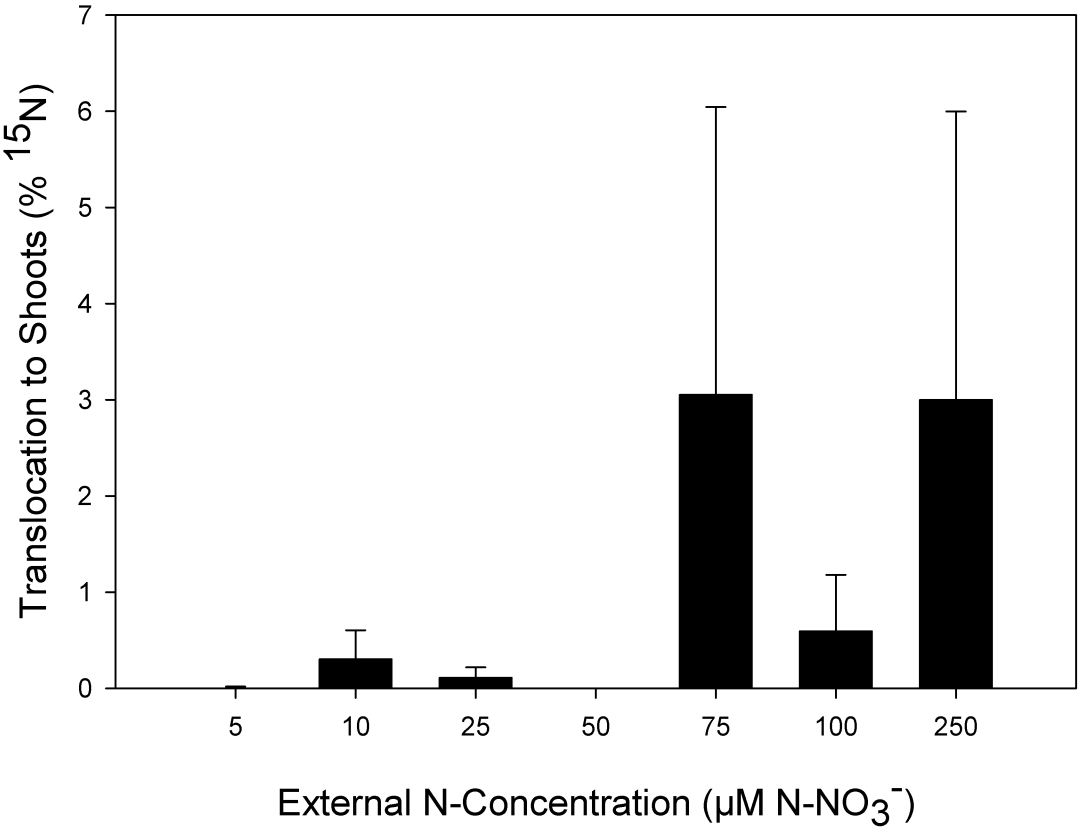

Figure 2.2 – Minimal Translocation of Nitrate to Shoots during 24 h Period: Translocation to shoots during 24 h study period was minimal from 0 – 250 µM N. Maximum translocation occurred where 3% incorporation into the shoot tissues at 75 and 250µM NO_3^-.

Uptake Kinetics in response to Nitrate supplied in the mM range

Evaluation of uptake under millimolar concentrations of NO_3^- indicated the presence of an additional uptake system (Figure 3). Nitrate uptake in response to N supply from 0 to 25 mM-N displayed a linear increase in the uptake rate, indicating the presence of a LATS with respect to NO_3^- uptake. This model fit was selected based on AICc values of three different model fits tested. Quadratic, linear and Michaelis-Menten models were fit to this data. The linear model was considered the best fitting model due to both the low AICc and high R^2 values of 62.57 and

0.97 respectively. The Michaelis-Menten model was the second best fit with AICc and R^2 values of 66.57 and 0.95 respectively.

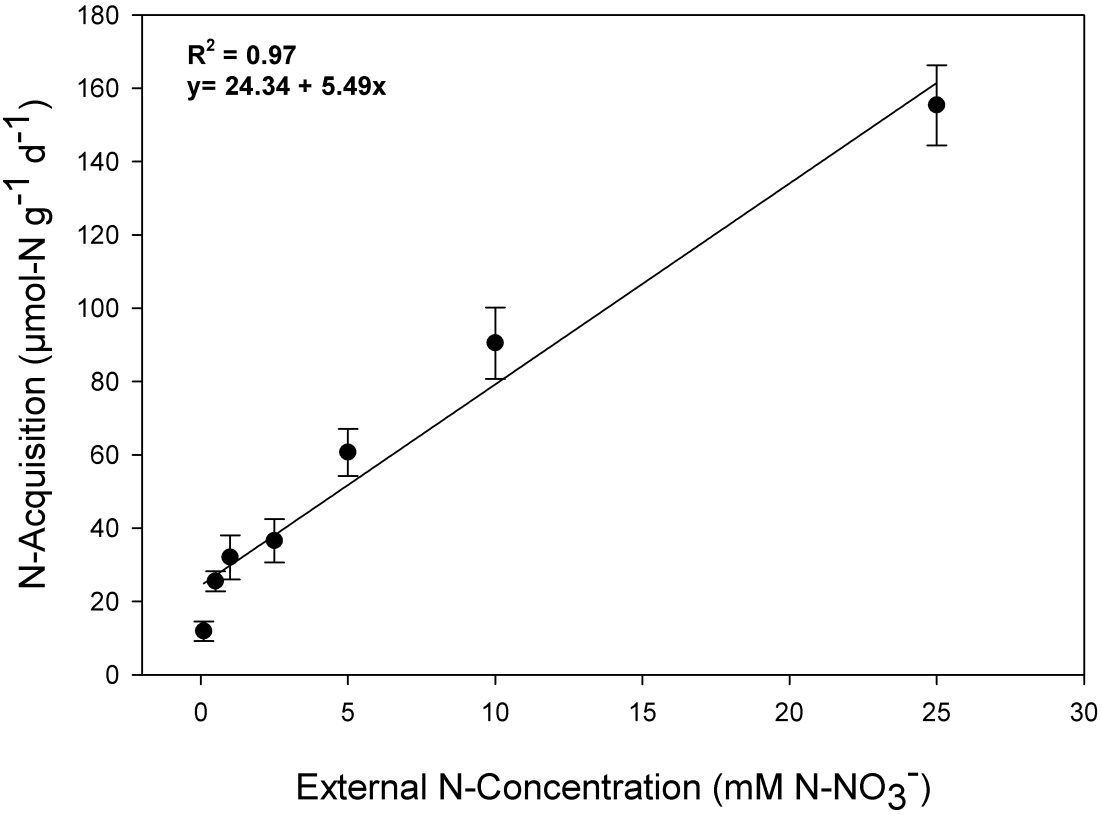

Figure 2.3 – Linear Nitrate LATS Activity in Millimolar Concentration Range: Linear uptake activity observed in 'Suziblue' in mM concentration range. The linear model was the best fit for the NO_3^- LATS data. After evaluating AICc values for linear, quadratic and Michaelis-Menten fits, the linear model fit was most appropriate with an **AIC=62.57 and R^2=0.97**.

Minimal translocation of ^{15}N from the roots to the shoots occurred during this study, except at the highest external N supply concentrations (Figure 4). Supply of N at 25mM concentration resulted in the highest translocation of ^{15}N to the shoots at around 34%. The 0.1

mM and 0.5 mM N treatments resulted in less than 5% of the ^{15}N being translocated to the shoots and were significantly different from that at 25 mM N (P = 0.024 and 0.019, respectively).

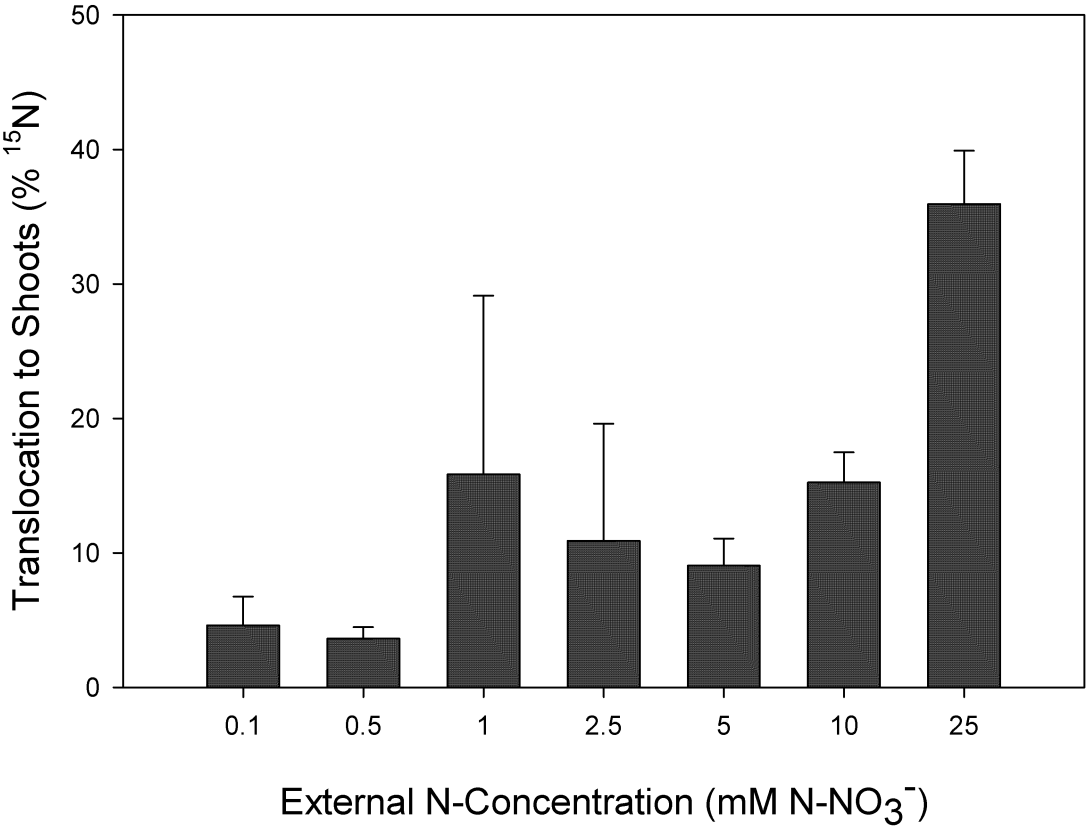

Figure 2.4 – Translocation of Nitrate to Shoots Minimal until 25 mM-N: Translocation of NO_3^- to shoot tissues in the millimolar range. The proportion of ^{15}N present in the shoots to the total ^{15}N acquired by the plants was determined. Minimal translocation occurred until 25 mM NO_3^-, where approximately 35% ^{15}N was translocated to shoot tissues.

Uptake Kinetics in response to Ammonium supplied in the µM range

Ammonium uptake kinetics were evaluated from around 10 to around 500 µM-N. Uptake within this concentration range displayed saturable kinetics indicating a HATS (Figure 5). Little change in the N uptake rate occurred beyond 100 µM N. Michaelis-Menten kinetics fit was

applied to these data. The parameters of this analysis were: V_{max} of 85.76 µmol g^{-1} d^{-1} and a K_m of 33.7 µM.

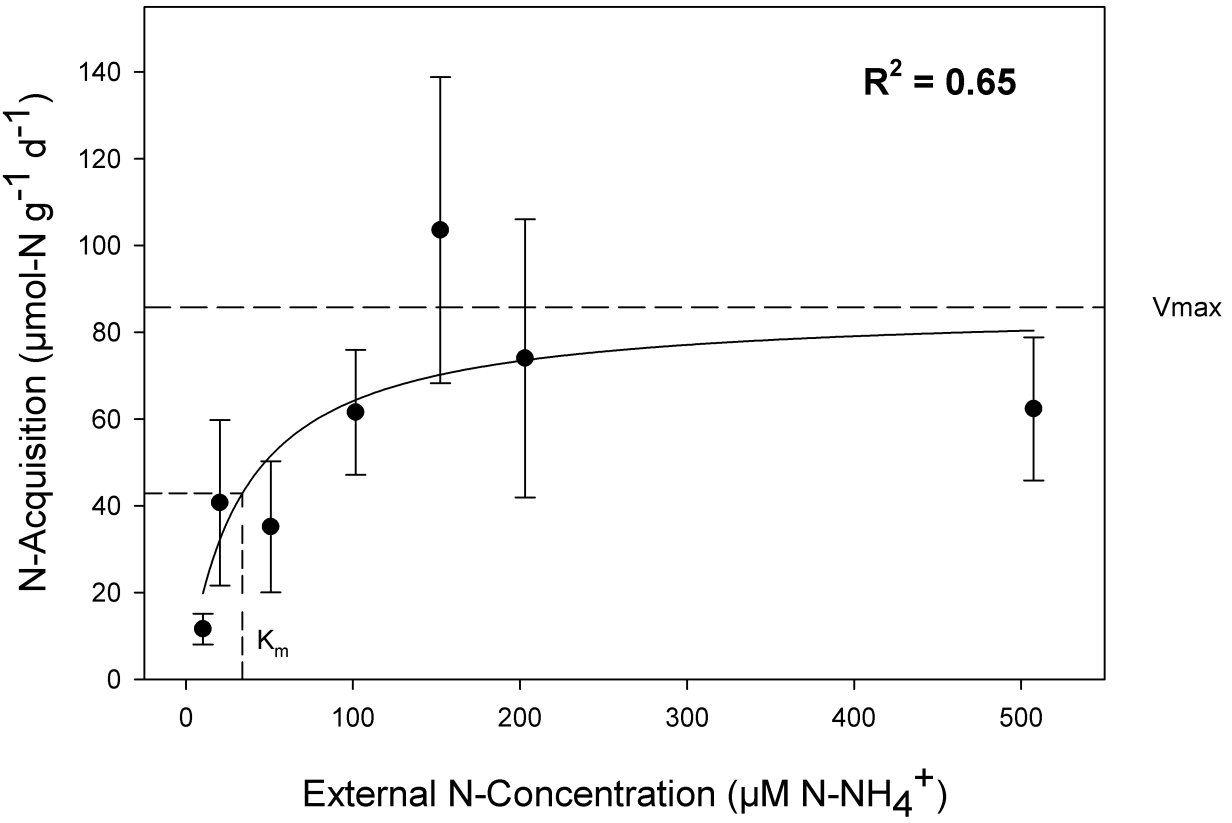

Figure 2.5 – Saturable Ammonium HATS Present in 'Suziblue': Saturable NH_4^+-HATS was observed in 'Suziblue' in the µM range. Saturation of NH_4^+ HATS occurred between 100 and 200 µM NH_4^+. A Michaelis-Menten equation was fit to the data with an observed K_m = 33.7 µM and a V_{max} = 85.76 µmol g^{-1} d^{-1}, (p= 0.2143, R^2=0.65).

Shoot translocation of N when supplied as NH_4^+ in the 10-500 µM range was generally low (Figure 6). The proportion of ^{15}N in the shoots with respect to the total ^{15}N acquired by the plant was generally lesser than 15% (at 152.2 µM N). There were no significant differences in

the shoot proportion of ^{15}N across the different levels of N supplied as NH_4^+ in the 10-500 µM range.

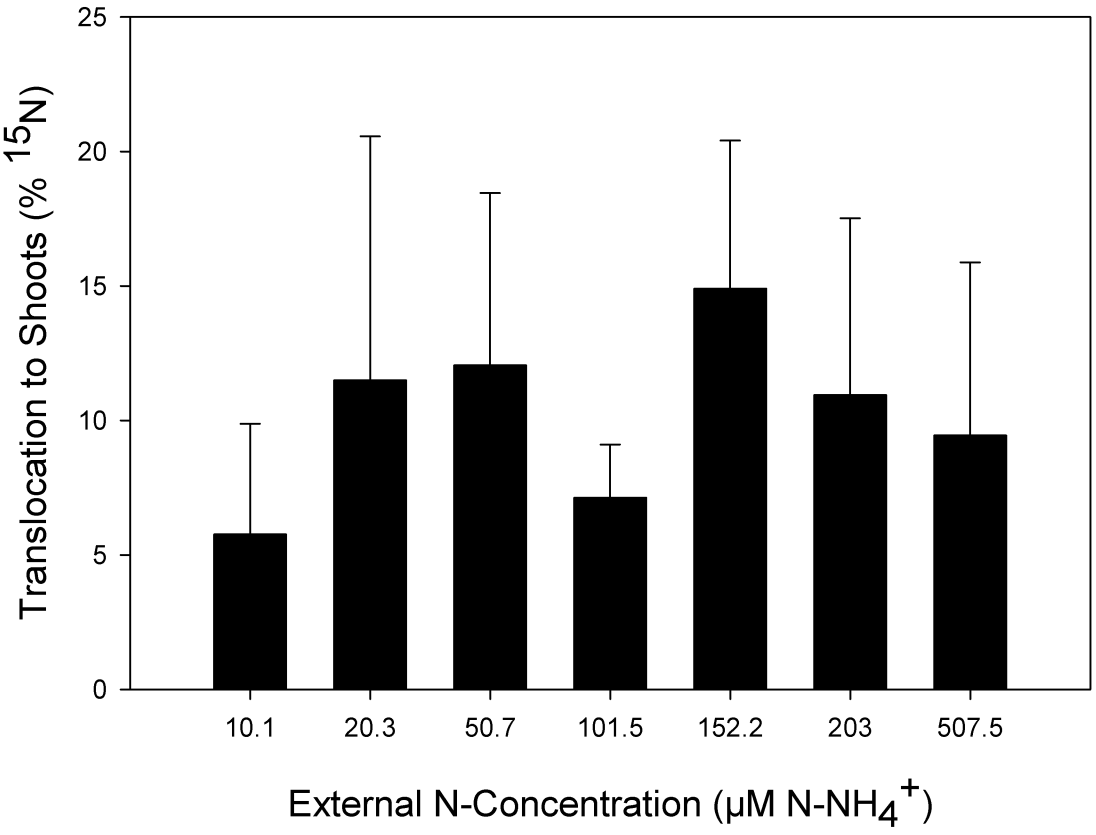

Figure 2.6 – Translocation of Ammonium to Shoot during 24 h Period: Translocation of NH_4^+ to shoots during 24 h study period was minimal with the maximum translocation of 15% occurring at 152.2 µM NH_4^+.

Uptake Kinetics in response to Ammonium supply in the mM range

The uptake rate of N increased with increasing concentration of NH_4^+ supplied in the mM range (0.2 to 51 mM). Linear, quadratic or Michaelis-Menten models were evaluated for goodness of fit to these data. The AICc values were used to determine the best fitting model. The

quadratic model had an AICc value of 87.12, while the Michaelis-Menten model resulted in an AICc value of 88.23. The linear model fit to the data had the lowest AICc value at 78.38, indicating that it was the best fitting model for the data (Figure 7). Further, the linear fit resulted in an R^2 value of 0.95. The data indicate that a functional NH_4^+-LATS was involved in N-acquisition under these conditions (0.2 to 50 mM N).

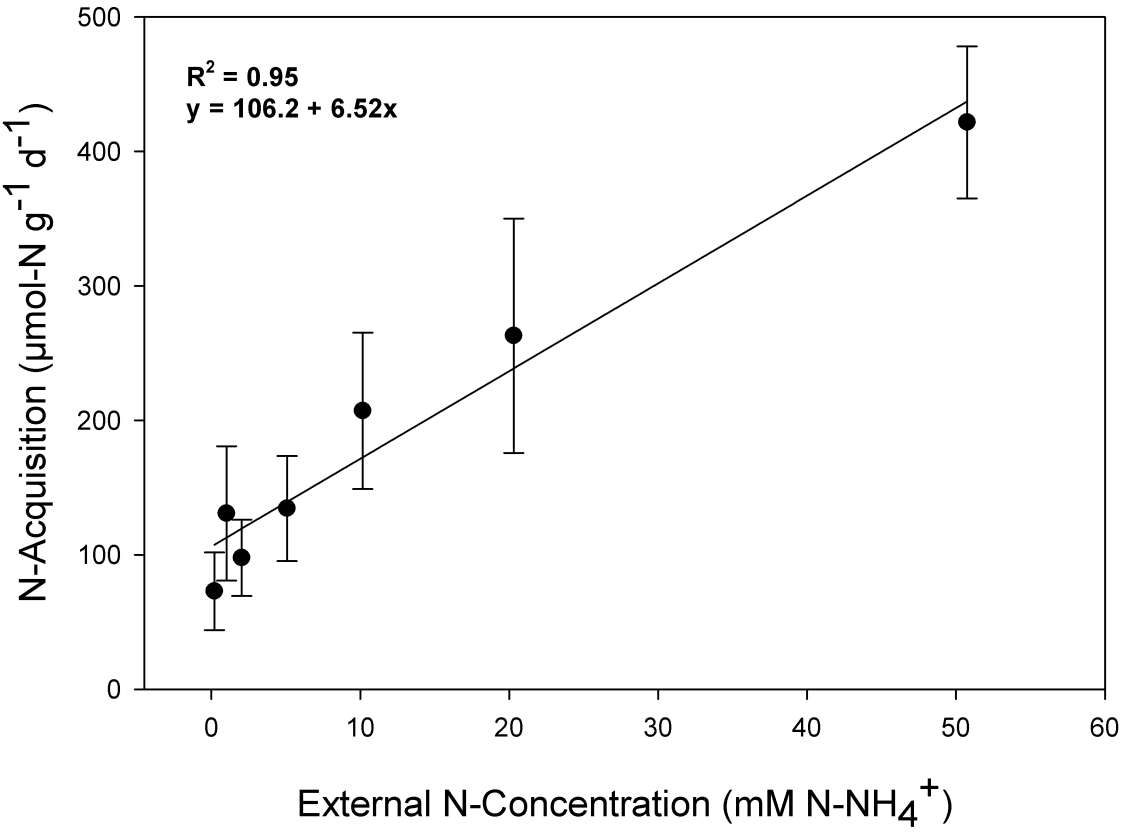

Figure 2.7 – Linear Ammonium LATS activity in 'Suziblue': LATS uptake activity in the mM range displayed a linear uptake pattern. Three models were evaluated for best fit to the data (Linear, Michaelis-Menten and Quadratic) and linear was chosen due to having the lowest AICc value and strong R^2 value (AICc = 78.38, R^2 = 0.95).

Shoot translocation of N was evaluated as the proportion of ^{15}N accumulating in the shoots in relation to the total ^{15}N acquired. This proportion ranged from 10-28% but was not significantly altered with changes in N supplied as NH_4^+ within the mM range (Figure 8).

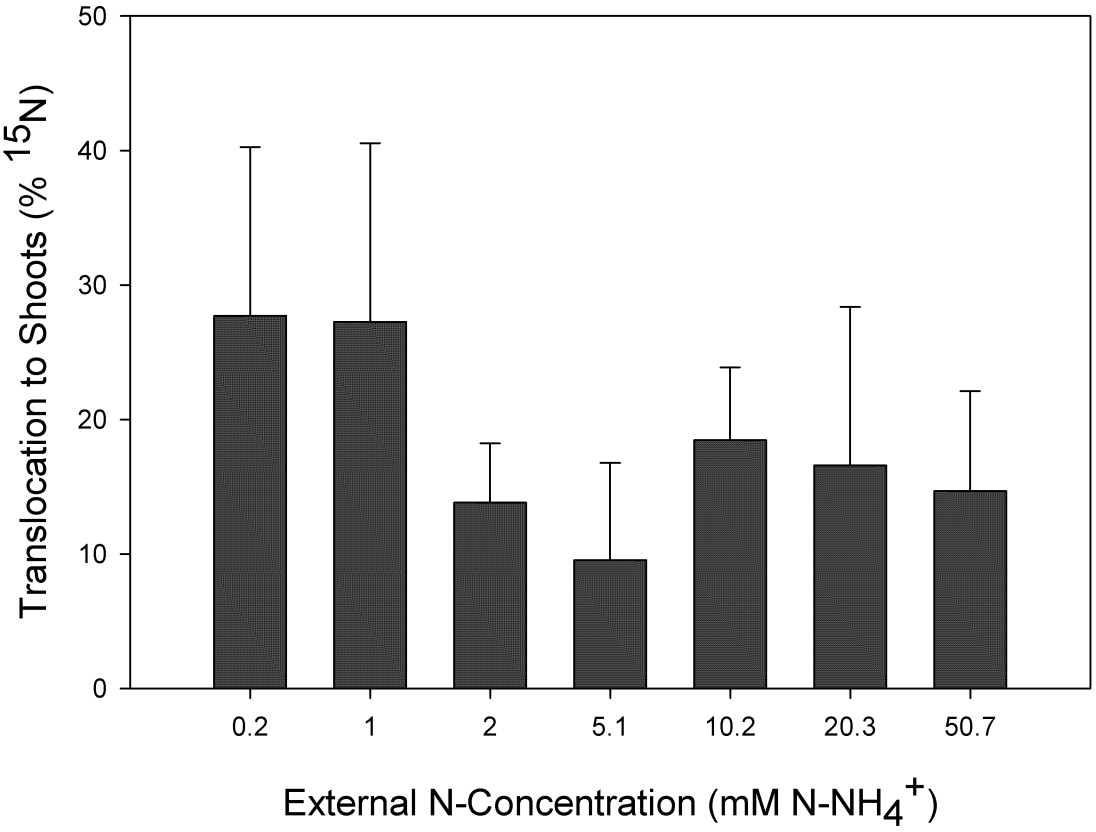

Figure 2.8 – Translocation of NH_4^+ to shoots in mM range: Translocation of NH_4^+ was minimal during the 6 h study period. Maximum translocation occurred at 0.2 and 1 mM NH_4^+ with approximately 30% ^{15}N translocated. Translocation was minimal among all other concentrations during 6 h study period.

The LATS kinetics of NO_3^- and NH_4^+ were plotted together to determine if differences in uptake kinetics occurred between the two N forms when supplied at high concentrations (mM

range). The slopes of the NO_3^- and NH_4^+ LATS responses were 5.5 and 6.5 µmol g^{-1} d^{-1} mM-N^{-1}, respectively, while their intercepts were 24 and 106 µmol g^{-1} d^{-1}, respectively (Figure 9). The N uptake rates at high N supply (mM range) appeared to increase similarly in response to N supplied as NO_3^- or NH_4^+, indicating 'Suziblue' maintains the enhanced ability to acquire ammonium over nitrate at mM concentrations. Interestingly, the value of the intercept for ammonium LATS was similar or comparable to the V_{max} obtained from its respective HATS model.

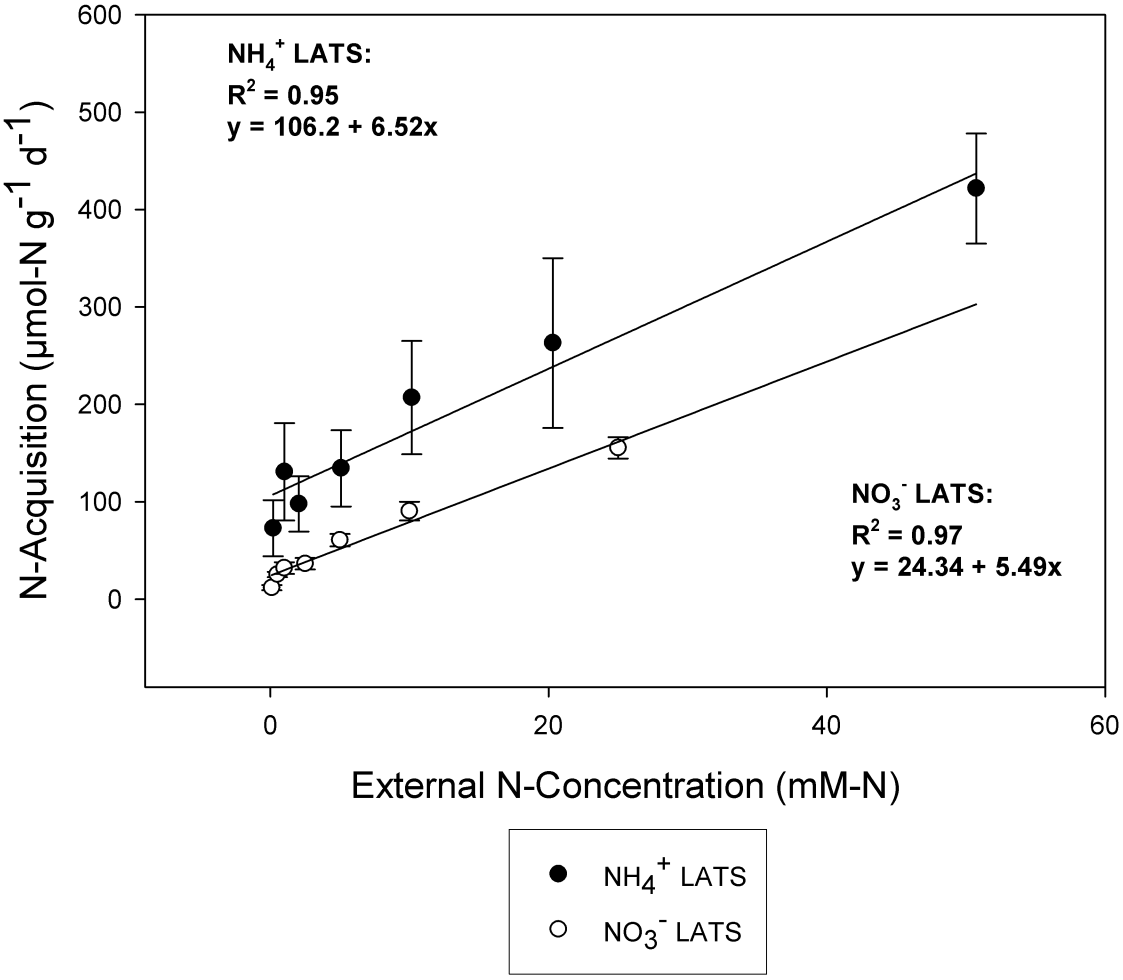

Figure 2.9 – Ammonium and Nitrate LATS Activity: Nearly 'parallel' LATS activity with respect to NH_4^+ and NO_3^- indicates that HATS systems set the pace for uptake of NH_4^+ in 'Suziblue' at the millimolar level. The y-intercepts for NH_4^+ LATS equation is closely associated with the V_{max} value observed for NH_4^+ HATS (V_{max} = 85.8 µmol N g^{-1} d^{-1}).

While analyzing HATS and LATS data, the y-intercept of NH_4^+ LATS (106.2 µmol N g^{-1} d^{-1}) was closely related with the V_{max} from our NH_4^+ HATS (85.8 µmol N g^{-1} d^{-1}). Plotting NH_4^+ HATS and LATS together indicated that HATS was still operating in the background of LATS activity. Further analysis of LATS uptake for both NH_4^+ and NO_3^- was conducted to determine the functions of the LATS systems without the background presence of HATS (Figures 10 and 11).

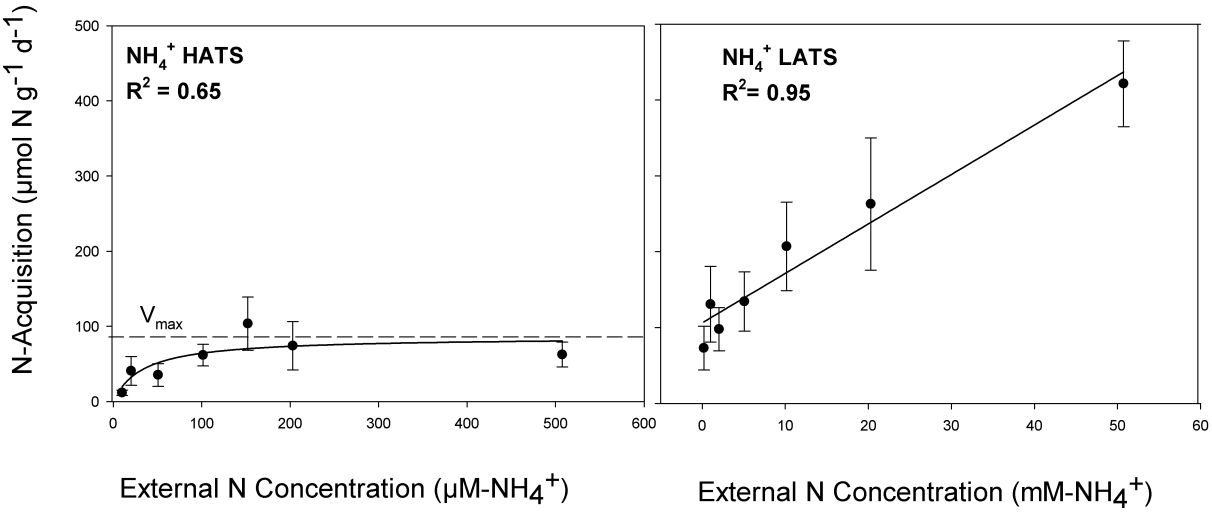

Figure 2.10 – Ammonium HATS and LATS Activity: Ammonium HATS V_{max} (85.8 µmol N g-1 d-1) was similar to the y-intercept (106.2 µmol N g-1 d-1) obtained from the linear model applied for LATS. This similarity may indicate that ammonium HATS is still functional in the background while LATS activity is present. Further evaluation of LATS activity in the

mM range was conducted to determine uptake for both inorganic forms of N at higher concentration range (mM).

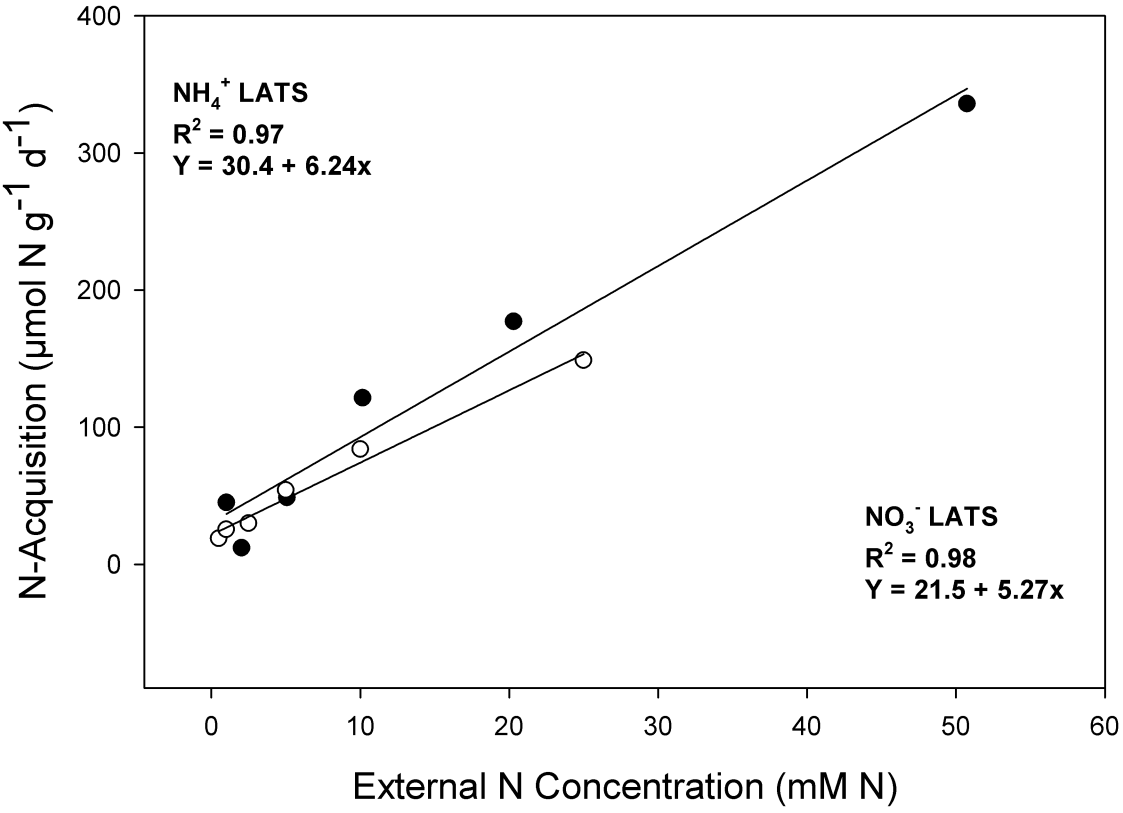

Figure 2.11 – Removed of HATS Vmax rates from LATS Uptake Activity under mM Concentrations: Presence of HATS activity during LATS uptake conditions based on similarity between V_{max} from HATS and y-intercept from LATS resulted in re-evaluation of LATS uptake rates. Removal of V_{max} values from observed LATS activity determined that ammonium LATS uptake was still greater than nitrate uptake, however, at mM concentrations, HATS activity may be more prevalent in the acquisition of N.

Discussion

Nitrate uptake in blueberry clearly displayed a distinct, operational, and saturable HATS in the µM range of available N. This is consistent with presence of such distinct mechanisms in other plant species [24, 25]. The K_m of the HATS was around 17 µM in blueberry and is within the range noted in other plant species [24, 25] including that reported previously for rabbiteye blueberry (23 µM) [13]. The V_{max} of nitrate uptake under low N availability as determined from the current analysis was lower than that described in other plants, including that in rabbiteye blueberry [8, 13, 24]. These data suggest a low capacity for NO_3^- uptake in 'Suziblue' southern highbush blueberry. In other plant species, HATS has been further divided into additional components: constitutive and inducible. In the current study, the uptake rate analyses were performed after the plants were subjected to a short starvation period of 2 d following acclimation at 250 µM for 5d. Inducibility of HATS peaked around 2-3 d after exposure to NO_3^- [24]. It is likely that the 2 d starvation period resulted in downregulation of NO_3^- uptake capacity. Hence, HATS characteristics described in the current study with blueberry likely represent a constitutive component (cHATS).

Ammonium uptake also displayed a distinctly operational HATS that was saturable, consistent with patterns noted previously in other plant species [9, 26]. The saturable NH_4^+ HATS was also consistent with previous reports of a saturable uptake component at low external NH_4^+ concentration in rabbiteye blueberry [17]. Further, the V_{max} noted here for NH_4^+ uptake in 'Suziblue' was similar to that described in the previous study with rabbiteye blueberry (~ 139 µmol g^{-1} d^{-1}), but the K_m was about 2 to 3-fold higher.

Comparison of HATS uptake kinetics between NO_3^- and NH_4^+ reveal several important features. The K_m of the NO_3^- uptake system was about 2-fold lower than that of the NH_4^+

suggesting that the operational NO_3^- HATS displayed higher affinity for this form of N. However, the V_{max} of the NO_3^- uptake system was 13-fold lower than that for the NH_4^+ uptake system within the low range of available external N. Lower V_{max} of the NO_3^- uptake system may reflect either lower activity of this system in transporting this form of N, lower amount of the transport system or a combination of the two. In any case, these data clearly indicate a lower overall capacity for NO_3^- uptake in 'Suziblue' blueberry in comparison to that of NH_4^+. Further, these data indicate that N-source preference in blueberry is facilitated by multiple-fold lower capacity for NO_3^- uptake at low external N concentration [27, 28]. Similarly, based on uptake kinetic parameters (V_{max}), rabbiteye blueberry was also suggested to display around 5-fold lower capacity for N uptake as NO_3^- compared to that as NH_4^+ [13]. Together, these data indicate that N-source preference at low external N availability in blueberry is manifest (at least in part) through a lower capacity for NO_3^- uptake.

At higher concentrations of N availability (mM) increase in N uptake rate was linear, for NO_3^- and NH_4^+, consistent with previous reports of a linear LATS for NO_3^- and NH_4^+ in plants [15, 16, 24, 25]. A LATS component for NO_3^- or NH_4^+uptake has not been described previously in blueberry. Comparative analyses of the LATS data indicate several important features. **1.** The uptake rate of NH_4^+ was greater by about 2 to 3-fold than in comparison to that of NO_3^- indicating that even at high external N concentration, 'Suziblue' plants displayed a higher capacity for uptake of NH_4^+ than that for NO_3^-. This indicates operational N-source preference even at higher external N concentrations. However, the magnitude of this preference was substantially lower than that observed with HATS (~13-fold difference in V_{max}). **2.** Although, the uptake rate for NO_3^- was lower than that for NH_4^+, there was still considerable N uptake occurring in the form of NO_3^- when it was available to the plant. Further, this indicates that when

external NO_3^- concentration is high, blueberry plants are capable of acquiring this form of N. Hence, under high N availability conditions, acquisition of NO_3^- may not be the main limiting factor affecting plant performance. Potentially, translocation to the shoot and/or assimilation may limit the utilization of the acquired NO_3^-, thereby affecting plant performance [29-31]. **3.** Previous studies indicated that HATS is potentially functional even under conditions where LATS serves as the major system allowing for N uptake [8, 16]. When, the V_{max} from HATS was removed from the LATS data for NO_3^- and NH_4^+, the slopes of the resulting responses were not altered, and were generally similar between NO_3^- and NH_4^+. However, the difference between the uptake rates of NO_3^- and NH_4^+ reduced to less than 2-fold. These data suggest that under high concentrations of external N, differences between the uptake rates of NO_3^- and NH_4^+ could be influenced substantially by underlying operational HATS activity, and that the absolute LATS activity may not be substantially different between NO_3^- and NH_4^+.

Differences in the extent of translocation of acquired N to the shoots based on the form of N-acquired was noted in this study. N translocation to the shoots was very limited under low N availability conditions, ranging from 5-15% for NH_4^+. This was substantially lower at less than 3% with NO_3^- as the N source. Under both forms of N, translocation to the shoots was not related to the external N availability. At higher N availability, translocation to the shoot appeared to be greater, ranging from 10-28% with NH_4^+ as the N-source and from 4-36% with NO_3^- as the N source. Further, extent of translocation to the shoots increased with increasing N acquired in the form of NO_3^-. At the highest level of NO_3^- uptake rate, about 35% of the ^{15}N was translocated to the shoots. Comparatively, at a similar uptake rate of NH_4^+, about half the label was recovered from the shoots. These data suggest that as NO_3^- uptake increases, its storage and assimilation capacity in the roots become limiting allowing for greater N translocation to the shoots, likely in

the form of NO_3^-. The proportion of N translocated to the shoots did not increase with increasing uptake in the form of NH_4^+, suggesting higher storage capacity in the roots, either as NH_4^+ or after it is assimilated into amino acids. As plants typically do not accumulate NH_4^+ to large concentrations within cells , it is likely that the acquired NH_4^+ is converted to amino acids in the roots [27]. Analysis of carbon backbone consumption (from photosynthates), glutamine synthetase activity, and accumulation of amino acids in the roots in relation to NH_4^+ acquisition can help determine the capacity for assimilation in the roots.

Conclusion

Evaluation of uptake kinetics of NO_3^- and NH_4^+ indicate the presence of saturable HATS and a non-saturable LATS for both N-forms in blueberry. N-source preference was strongly evident at the level of N acquisition under conditions of low N availability and reflected in a 13-fold higher capacity for NH_4^+ uptake in comparison to that of NO_3^-. At higher N availability, N-source preference for NH_4^+ acquisition was still apparent but to a lower extent. At higher external NO_3^- availability, blueberry plants acquired substantial N, at least within the period of this study. Further, an increasing proportion of N was translocated to the shoots with increasing uptake of NO_3^-, likely as NO_3^- itself.

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CHAPTER 3

Dual Approach to Investigating Acquisitional Nitrogen-Source Preference in Southern Highbush Blueberry[2]

[1] Doyle, John, Malladi, Anish, Cabrera, Miguel, and Coolong, Timothy. To be submitted to Horticulturae.

Abstract

Evaluation of Nitrogen-Source preference in southern highbush blueberry cultivar, ‘Suizblue’ was conducted either by nutrient depletion from hydroponic media or through evaluation of ^{15}N enrichment in plant tissues. ‘Suziblue’ was provided ammonium and nitrate, either solely or simultaneously to determine if acquisition was a source of nitrogen-source preference. In both approaches, plants supplied solely ammonium displayed a greater capacity to acquire ammonium compared to nitrate at both 50 and 500µM-N. Plants provided ammonium and nitrate simultaneously displayed a greater capacity to acquire ammonium compared to nitrate. Shoot translocation of ^{15}N was investigated in the isotope evaluation study and was minimal across all treatments except for ammonium treatments at 500µM-N. Plants that received ammonium at 500µM-N display greater translocation of ^{15}N to shoot tissues compared to plants that received nitrate. This pattern of translocation held true for plants provided ammonium solely or simultaneously. Plants under limiting [N] displayed greater capacity to acquire ammonium compared to nitrate, indicating a preference for ammonium under limiting conditions at the acquisition level.

Introduction

Understanding nutrient acquisition by plants will greatly increase our ability to manage different plant systems under conditions that are not always favorable for enhanced growth and development. Nitrogen use efficiency (NUE) is a concept of great importance to plant scientists for understanding how plants acquire and use nitrogen (N) received from the soil. With increasing interest and development in precision agriculture, NUE is becoming even more vital for increasing crop yields. Blueberry (*Vaccinium* sp) is thought to display a low demand for nutrients and prefers forested upland soils that are high in organic matter (OM), have a low pH (<5.5) and ammonium (NH_4^+) is the primary form of inorganic N [1, 2]. Blueberry is a member of the *Ericaceae* family and other members of this family are also thought to prefer NH_4^+ over nitrate (NO_3^-), a phenomenon referred to as N-source preference. However, the extent of this preference and the mechanisms underlying it are not well understood.

Since the early 2000s, blueberry production has increased rapidly both in total yield and production area and as of 2020 the United States is the leading producer of blueberry, contributing to approximately 679 million pounds or 1/3 of global production [3]. In the state of Georgia, blueberry is the leading commercial fruit crop with production accounting for more than 31,000 acres and valued at over $300 million [4]. Blueberry production in Georgia consists primarily of southern highbush and rabbiteye cultivars and has expanded drastically in the last 2 decades such that production now occurs where soil conditions are not optimal for growth and development [5]. As production expands, soil characteristics traditionally associated with blueberry are not able to be maintained. This requires better understanding of what forms of inorganic N blueberry prefers as well as understanding the mechanisms associated with acquisition under varying soil N concentrations.

Current management practices associated with commercial blueberry production suggest use of NH_4^+ based fertilizers [6]. While, increased rates of fertilizer application can result in enhanced growth and development, it can result also in damage to roots and increased losses from the soil due to ammonia volatilization [7]. Kozinski et. al. (2004) tested the effects of N fertilization rate and mulching materials on 'Bluecrop' blueberry. Fertilization rates applied consisted of 0, 60, 120, 180 kg N ha^{-1}. They identified that fertilization rates above 60 kg N ha^{-1} decreased yield. However, they identified that sawdust had a positive effect on yield compared to pine bark, indicating the influence of OM on blueberry growth and development [8]. These findings represent the importance of fertilizer rate and that increasing rates continually does not always positively influence yield or growth and development. These findings also indicate that OM plays a significant role in N availability, especially for blueberry production. Organic matter in the soil can greatly contribute to N availability to plants and blueberry are traditionally planted in pine bark mulch beds to allow for higher OM and to reduce soil pH to optimal levels for growth and development.

In this study, N-source preference in the southern highbush cultivar 'Suziblue' was investigated by simultaneously supplying NH_4^+ and NO_3^- to the split roots of a plant's root system. The objective of this study was to investigate which inorganic form of N was preferred by blueberry plants when provided both forms were provided at concentrations that represent nutrient limiting soil conditions.

Methods and Materials

Plant Material

Blueberry cuttings were purchased from Alma Nursery and Berry Farms in Alma, GA and transported to the Riverbend Greenhouse Complex in Athens, GA. The plants were transplanted into 3.78 L containers filled with a 2:1 mixture of pine bark mulch and peat moss. Plants were fertilized every 2-weeks using Peter's Professional Acid Special 21-7-7 fertilizer at an initial rate of 50 mg L^{-1}-N and then increased to 100 mg L^{-1}-N as plants grew and nutrient requirements increased. Cuttings were grown out for a month to allow for root development and to allow the architecture of the plants to develop. Thirty-four 'Suziblue' plants were used for the investigation of N-Source preference using ^{15}N in each split-root study.

Split-Root Hydroponic System

The hydroponic system was constructed using 68 1-L paint buckets purchased from a local hardware store. The volume of each cup was around 800 mL and allowed enough volume for the plant roots to be suspended in solution. One-half of the plant root system was placed into 1 cup (2 cups per plant) and suspended from a trellis system in the greenhouse. The root collar of the plant was suspended 1´´ above the solution in the cup. Air was supplied to each cup *via* an air pump and airline tubing with an air-stone on the end. The hydroponic solution was modified from Hoagland's Solution and consisted of 0.5 mM potassium phosphate, 1 mM magnesium sulfate, 0.5 mM calcium chloride, 0.08 mM Fe-EDTA, 0.045 mM boric acid, 0.01 mM manganese sulfate, 0.01 mM zinc sulfate, 0.02 µM sodium molybdate and ^{15}N-source of either ammonium sulfate or potassium nitrate at 5 atom percent enrichment (APE) [9, 10].

Plants were first subjected to an acclimation period of 5 d with the modified Hoagland's solution with 50 or 500 µM N as ammonium sulfate or potassium nitrate. After acclimation, plants received a 2-d starvation period consisting of Hoagland's solution without N. Following

starvation, four treatments were applied to the split root system. Only one part of the split-root system received ^{15}N-labeled N as NH_4^+ or NO_3^-, while the other side received non-labeled N at the same concentration. This resulted in four treatments as indicated: $^{15}NH_4^+|NH_4^+$, $^{15}NO_3^-|NO_3^-$, $^{15}NH_4^+|NO_3^-$, and $^{15}NO_3^-|NH_4^+$. Studies using the split-root approach with ^{15}N were performed at two levels of N, separately: the first using 50 µM N and the second using 500 µM N.

^{15}N Stock Solution Preparation and Calculations

Calculations for ^{15}N stock solutions were performed following Cabrera and Kissel (1989) using average molecular weight. Natural ^{15}N abundance of 0.366% was used in these calculations [11].

Sample Collection and Preparation

Root and Shoot samples were collected at 24h after initiation of treatments. Root samples were washed three times; the first and second wash consisted of the modified Hoagland's solution with non-labelled N-sources, and the final wash consisted of the modified Hoagland's solution without N. Root wash timeframes were kept consistent between washes and among root samples. Washes lasted < 1 minute and were conducted to remove any labeled N-sources adhering to the surface of the root tissue. Once roots were washed, they were blotted dry and placed into 50-mL tubes. Shoot samples were collected and entire sample placed in 50-mL tubes. All sample tubes were pre-frozen in liquid N_2 with care taken to prevent contamination of the tubes and then re-frozen once sample was in place. Samples were stored at -80 °C until freeze drying for 24 h. After freeze drying, samples were submitted to the Stable Isotope Ecology

Laboratory at the Center for Applied Isotope Studies at the University of Georgia, Athens, GA (SIEL-UGA) where they were prepared and processed for analysis.

Isotope Ratio Mass Spectrometry Analysis

Samples submitted to SIEL-UGA were first lyophilized/ freeze-dried for at least 24 h as indicated earlier. After drying, the sample material was finely ground and 2-3 mg was added to encapsulation tins after weighing on a microbalance accurate to 0.001 mg. These tins were then placed in a 96-well microtiter plate and submitted for analysis. Analysis consisted of combusting the encapsulated samples at 1100 °C and the gases produced were delivered *via* continuous-flow for analysis of $\delta^{15}N$ using isotope ratio mass spectrometry (IRMS) where enrichment of samples was compared to natural abundance of ^{15}N in the atmosphere (0.37 atom %). Results were presented as Atom % ^{15}N and this was used to calculate to Atom % Excess (APE) for each sample. The APE was then used to calculate ^{15}N incorporated (during acquisition) into the plant tissues. The analysis was conducted by using the samples with the least amount of enrichment first and moving to samples with the highest enrichment to reduce contamination within the lab setting. All materials used for the analysis were thoroughly cleaned with ethanol between each sample to further reduce chances of contamination [12].

Experimental Design

A randomized complete block design with eight replications was used in these studies. Even though a single plant's roots were split across 2 containers receiving separate treatments, the entire root system was considered an experimental unit. Plant roots not receiving ^{15}N were collected and weighed to ensure that the root system was evenly split between the 2 containers.

Statistical analyses were conducted using R software and JMP software and figures were created using SigmaPlot 14.0 [13].

Results

Acquisition of N – 50 µM N

In 'Suziblue', acquisition of N in the form of NH_4^+ was greater than that of NO_3^- by around 1.83-fold (21.6 and 11.8 µmol g^{-1} d^{-1}, respectively) when supplied at an external concentration of 50 µM (Fig. 1) as indicated by analyses of contrasts. The rate of acquisition of NH_4^+ by one part of the split-root system was not dependent on the form of N supplied to the other part over the duration of the experiment (24 h). Similarly, the rate of acquisition of NO_3^- by one half of the split-root system was not influenced by the form of N supplied to the other half. Further, the rate of acquisition of N as NH_4^+ was significantly greater than that of NO_3^- irrespective of the form of equivalent N supplied to the other part of the split-root system.

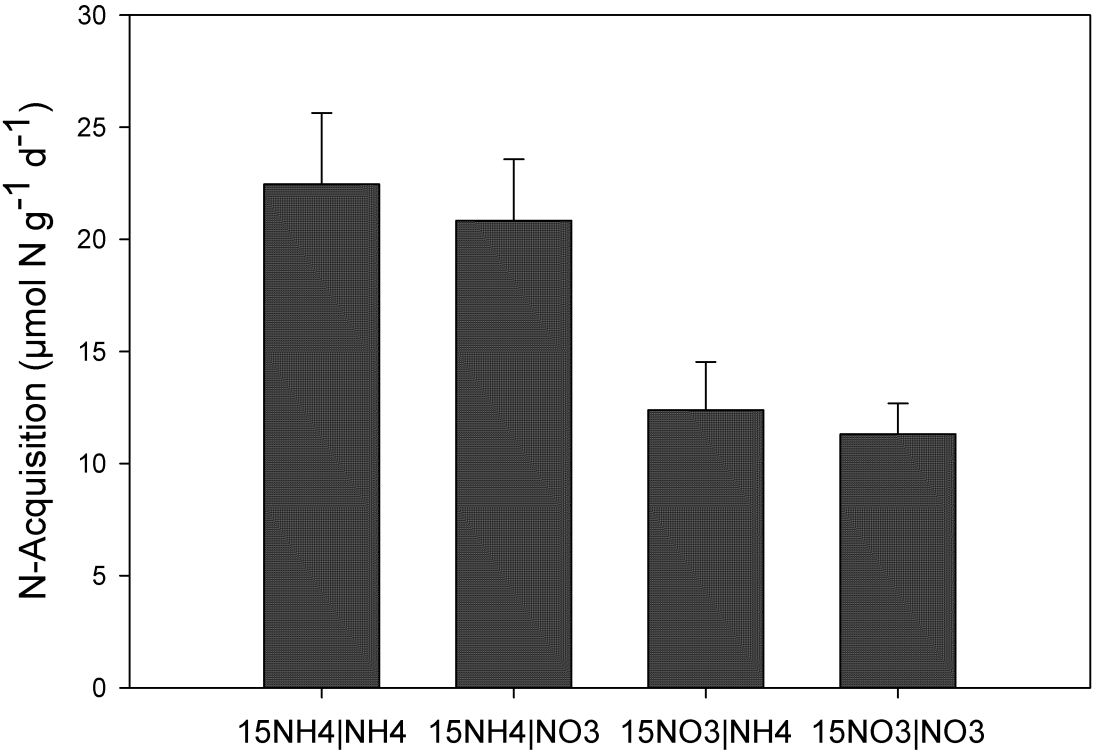

Figure 3.1: 'Suziblue' Split Root Acquisition at 50 µM-N. Whole plant single source treatments in 'Suziblue' displayed greater uptake capacity for ammonium compared to nitrate. Ammonium mean uptake under sole source conditions was 22.45 µmol-N g^{-1} d^{-1} and nitrate mean uptake was 11.31 µmol-N g^{-1} d^{-1}. Ammonium uptake was significantly greater than that of nitrate at 50 µM-N ($p = 0.0003$). 'Suziblue' plants were also provided ammonium and nitrate simultaneously at 50 µM-N and acquisition was evaluated. Ammonium uptake was approximately double that of nitrate uptake, even in the presence of nitrate. The mean ammonium uptake rate was 20.84 µmol-N g-1 d-1 compared to the mean nitrate uptake rate of 12.39 µmol-N g-1 d-1. Ammonium uptake was significantly greater than that of nitrate when provided both forms simultaneously in 'Suziblue' .

Acquisition of N– 500 µM - N

At higher external N concentration (500µM-N), 'Suziblue' continued to display a greater capacity to acquire NH_4^+ compared to NO_3^- by around 7.4-fold (Figure 2). N acquisition as NH_4^+ was always greater than that of NO_3^-, irrespective of form of N supplied to the other half of the root system. The mean uptake rate when NH_4^+ was supplied was 102.7 µmol N g^{-1} d^{-1} (mean of two treatments with $^{15}NH_4^+$) compared to 13.9 µmol N g^{-1} d^{-1} (mean of two treatments with $^{15}NO_3^-$) when NO_3^- was supplied ($P < 0.001$). N acquisition rate as NH_4^+ was not affected by the form of equivalent N supplied to the other half of the root system. Similarly, the N acquisition rate as NO_3^- by one side of the root system was not affected by the form of equivalent N provided to the other half.

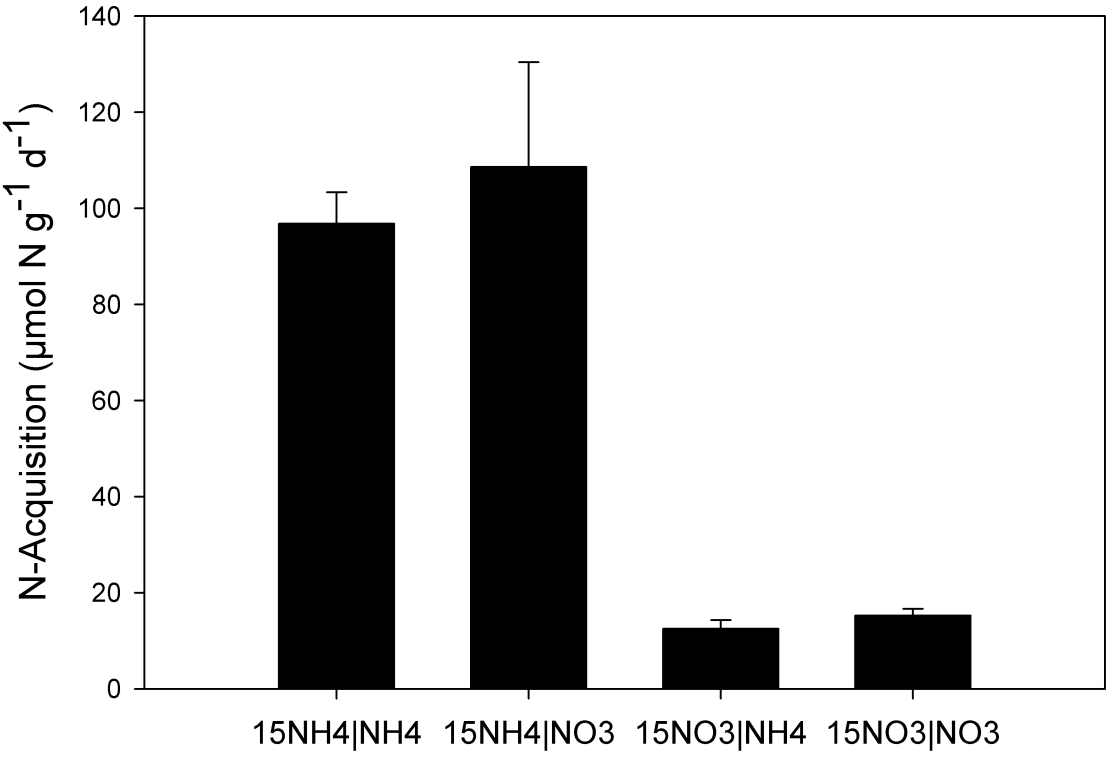

Figure 3.2: Acquisition of ammonium and nitrate at 500µM-N. 'Suziblue' displayed a greater capacity to acquire ammonium over nitrate when provided one form or the other. The mean uptake of ammonium at 500 µM-N was 96.76 µmol-n g^{-1} d^{-1}, while the mean uptake rate of nitrate at 500 µM-N was 15.26 µmol-N g^{-1} d^{-1}. Ammonium uptake was significantly greater than that of nitrate (6-fold greater) in 'Suziblue' plants provided 500µM-N ($p < 0.0001$). 'Suziblue' plants were hydroponically grown and provided both ammonium and nitrate simultaneously at a concentration of 500 µM-N. Ammonium uptake was significantly greater than nitrate and approximately 8-fold higher at 500 µM-N , even in the presence of nitrate. The mean ammonium uptake rate was 108.59 µmol-N g^{-1} d^{-1} compared to the mean uptake rate of nitrate which was 12.51 µmol-N g^{-1} d^{-1}.

Translocation of ^{15}N to Shoots

At a concentration of 50 µM external N, translocation to the shoots was minimal among all treatments within the 24 h duration of the experiment. It ranged from around 0.37 to 2.1 % across the treatments. Further, it was not significantly different across the treatments evaluated (Figure 3).

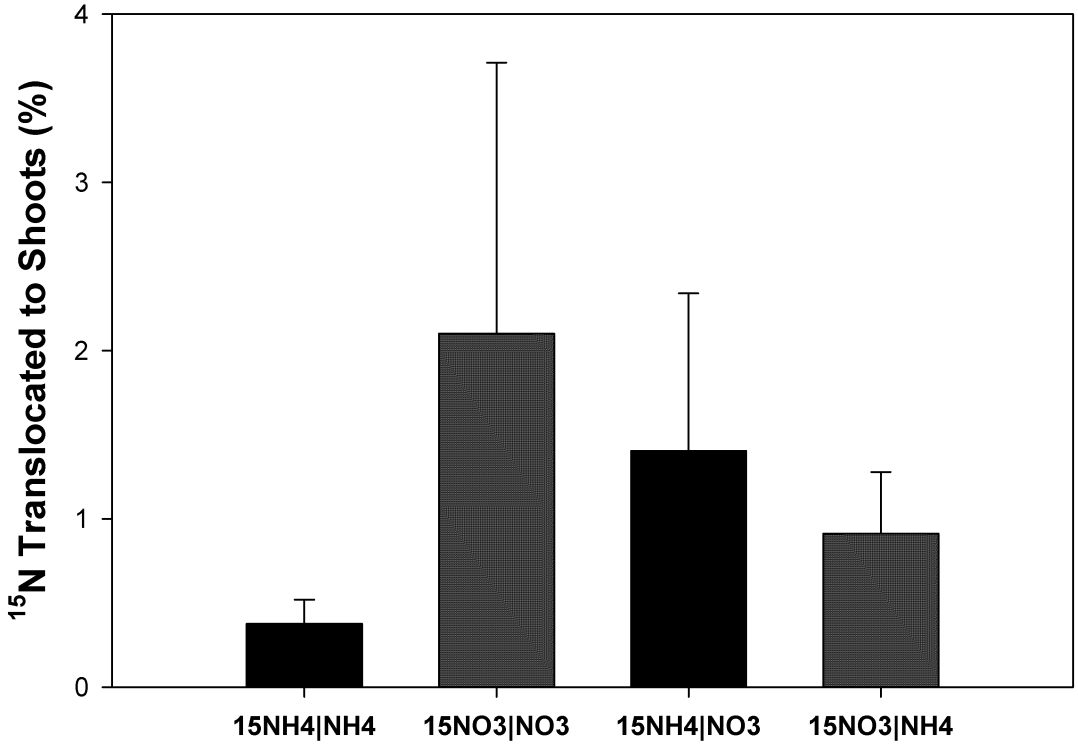

Figure 3.3: Translocation of ^{15}N to shoot tissues in 'Suziblue' at 50µM-N. Translocation of N to the shoot tissues in 'Suziblue' was minimal during the 24 h study period at an external concentration of 50 µM-N. Plants provided only nitrate displayed the most translocation to the shoots at approximately 2%, however this was not significantly different when compared to other treatments.

The translocation of ^{15}N at higher external N concentration (500 µM) displayed differences among treatments during the 24h study period. Plants provided with NH_4^+ displayed significantly higher translocation of ^{15}N to the shoots than in comparison to those supplied with NO_3^-, irrespective of the form of N supplied to the other half of the root system (19.6 and 3.5%, mean values of $^{15}NH_4^+$ and NO_3^- treatments respectively; $P < 0.0001$). The extent of ^{15}N

translocation to the shoots when supplied with NH_4^+ was not affected by the form of N supplied to the other half of the split-root system. Similarly, the extent of translocation ^{15}N when supplied with NO_3^- was not affected by the form of N supplied to the other half of the roots (Figure 4).

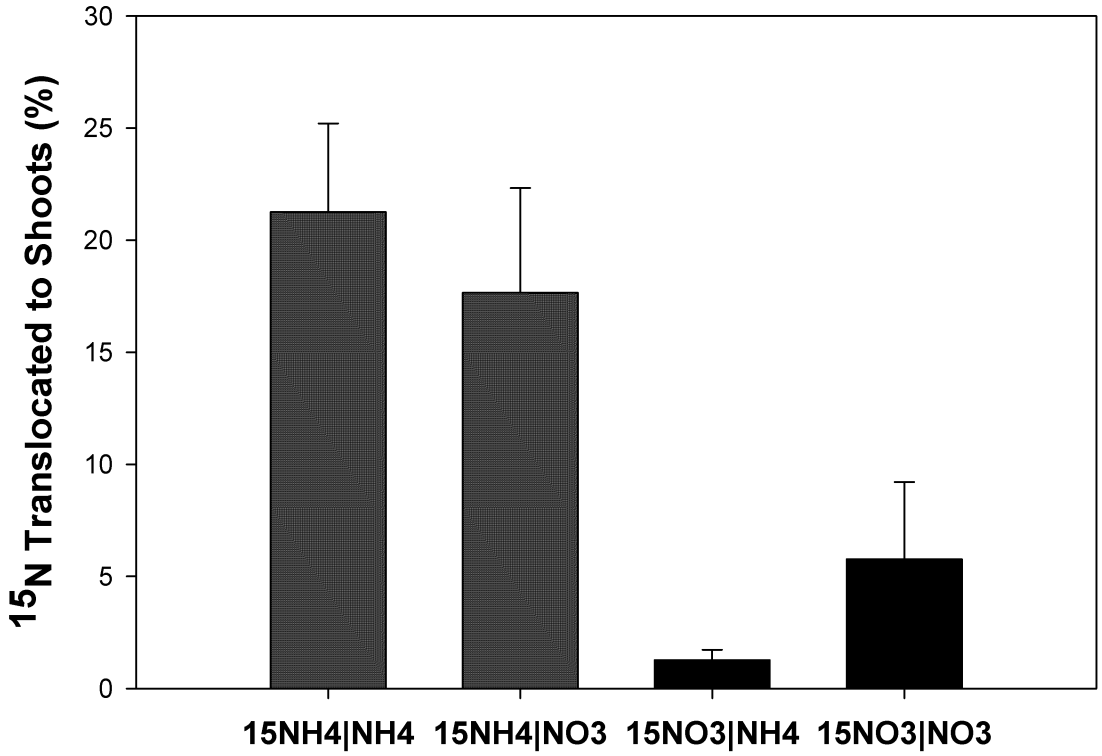

Figure 3.4: Shoot translocation of ^{15}N in 'Suziblue' provided 500 µM-N for 24 h.

Translocation of N to the shoot tissues at 500µM-N was greater in plants provided ammonium. The treatment of only ammonium was significantly different from the treatment of only nitrate ($p = 0.0194$). Additionally, the treatment of ammonium|nitrate was significantly different from the treatment of nitrate|ammonium ($p = 0.0125$) and the treatment of only ammonium was significantly different from nitrate|ammonium ($p = 0.0019$). Indicating that more ^{15}N was translocated to the shoots under ammonium treatments. The form of ^{15}N that was translocated to the shoots needs to be evaluated in future research, but the most likely form is amino acids.

Discussion

The rate of N acquisition in the form of NH_4^+ was around 1.8-fold greater than that in the form of NO_3^- when N was supplied at a low rate of 50 µM. This was further apparent when N uptake was evaluated using the split-root system at an external N of 500 µM, where the rate of NH_4^+ uptake was over 7-fold higher than that of NO_3^-. These data clearly indicate higher capacity for NH_4^+ acquisition than for NO_3^- acquisition in blueberry and are consistent with results from the previous study where N uptake kinetics were investigated (Chapter 2). While the 50 µM N concentration, was clearly within the range of HATS for both forms of N, the 500 µM likely represented a concentration where the HATS was saturated and where N uptake was beginning to be facilitated additionally by LATS. Between the low (50 µM) and moderate (500 µM) N levels, NH_4^+ uptake rate increased by around 4 to 5-fold, indicating that saturation of NH_4^+ uptake rate and likely initiation of LATS had occurred. However, across the same range, NO_3^- uptake rate was not substantially altered, suggesting that the HATS was saturated at a lower external N supply and that the LATS was not yet functional to contribute to N uptake. The absolute difference in the rates of NH_4^+ and NO_3^- uptake noted at the level of moderate N supply was around 90 µmol g^{-1} d^{-1} which was comparable to the absolute difference between the V_{max} of the HATS displayed by these two N forms in the previous study (Chapter 2). Together, these data clearly indicate the substantially higher capacity for N uptake in the form of NH_4^+ compared to NO_3^-, particularly under low and moderate levels of N supply. Hence, N acquisition is an important contributor to N-source preference in blueberry under low and moderate N supply conditions. Similarly, in white spruce (*Picea glauca*), a conifer with a similar N-source

preference for NH_4^+ as blueberry, N influx capacity for NH_4^+ was about 20-fold higher than that for NO_3^- within the lower N (≤ 1 mM) external N supply range [14].

Nitrogen translocation to the shoots was very limited (≤ 2%) under conditions of low N supply and no influence of a systemic signal in regulating it was observed. At moderate N supply, while an effect of a systemic signal was still not observed, an effect of the N-source on translocation was evident. Nitrogen supplied as NH_4^+ resulted in substantially greater translocation of N to the shoots. These data suggest that greater uptake of N (in the form of NH_4^+) likely resulted in higher assimilation and translocation to the shoots and that the low levels of N acquired in the form of NO_3^- were likely stored as such or after assimilation within the roots.

It was hypothesized that the form of N supplied on one side of the roots and thereby the N status of the plant may influence N uptake rates on the other half. In Arabidopsis, local and systemic signals are involved in modulating root growth and foraging responses to N supply and the N status of the plant [15, 16]. In the current study, N uptake rates remained unaffected by the form of equivalent N supplied to the other half of the root system. This suggests that a systemic signal was not likely involved in altering N uptake characteristics. Systemic signals are likely a reflection of the plant N status and mediated by assimilation products such as amino acids [15]. The lack of an influence of NO_3^- supply may be attributable in part to the limited uptake and translocation of this form of N. However, substantial uptake and translocation of NH_4^+ N was observed, particularly under moderate N supply conditions. Hence, it is likely that NH_4^+ uptake does not systemically down-regulate NO_3^- uptake in blueberry. It may also be speculated that the short duration of the experiment (24 h) did not allow for a potential systemic signal to manifest. Future experiment aims could explore longer duration of differential N-source supplied under

split-root conditions to investigate potential systemic signaling. Additionally, in future studies, supply of both forms of N simultaneously to the same roots may also be explored. However, the influence of the uptake of one form on the pH of the medium and the membrane potential may influence the interpretation of results from such a study [17].

Conclusions

Overall, data from the current study indicate that availability of one form of inorganic N does not systemically affect the uptake of the other under conditions of low and moderate N availability. Data from this study also clearly indicate higher N uptake capacity in blueberry when supplied in the form of NH_4^+. These data support those from studies on uptake kinetics that southern highbush blueberry plants display N-source preference for NH_4^+ in a significant part through its acquisition.

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CHAPTER 4

Conclusions and Future Work

Evaluation of uptake kinetics for NO_3^- and NH_4^+ indicated the presence of saturable HATS and non-saturable LATS for both forms in southern highbush blueberry. These data indicate acquisition as a level of N-source preference in blueberry. At low external concentrations (µM), blueberry displayed 13-fold greater capacity to acquire NH_4^+ compared to NO_3^-. At high external concentrations (mM), N-source preference for NH_4^+ was still observed, just at a lower extent. Substantial NO_3^- was still acquired at high external concentrations during this study and resulted in an increased proportion of ^{15}N translocated to the shoots, likely in the form of NO_3^-. Split source evaluation of acquisition at low and moderate external concentrations of N displayed a greater capacity to acquire NH_4^+ compared to NO_3^-. The presence of both inorganic forms to the (split) roots of one plant did not alter the acquisition of the other under low and moderate external concentrations in blueberry. Together, these data indicate that acquisition is a level of N-source preference in southern highbush blueberry.

Future evaluation is critical to improving our understanding of N acquisition and N-source preference in blueberry. Evaluation of the transport proteins involved in acquisition under low and high N concentrations, as well as the effects of pH on N acquisition under these conditions, would aid in our understanding of the basis of N-source preference at the level of acquisition, in blueberry.