PHYSIOLOGICAL AND MOLECULAR BASIS FOR LOW NITRATE (NO$_3^-$) ASSIMILATION IN BLUEBERRY

by

DOUGLAS S. ALT

(Under the Direction of Anish Malladi)

ABSTRACT

Blueberry (*Vaccinium* sp.) has recently emerged as a crop of significant economic value. The low ability of blueberry plants to assimilate the NO$_3^-$ form of nitrogen appears to limit its production in non-native soils. To investigate the physiological and molecular limitations of NO$_3^-$ assimilation, three different experiments were conducted using two types of blueberry, *V. corymbosum* ‘Sweetcrisp’ and *V. ashei* ‘Alapaha’: 1. Supplying NO$_3^-$ to the roots via a hydroponic solution; 2. Supplying NO$_3^-$ to the leaves via a foliar application; 3. Supplying NO$_3^-$ to the stem by placing a cut stem in a NO$_3^-$ solution. An ‘in situ’ nitrate reductase (NR) assay method was developed and used to quantify NO$_3^-$ assimilation in previously frozen root and leaf samples. NO$_3^-$ supplied to the roots induced a 3.1- and 1.8-fold (*V. ashei* and *V. corymbosum*, respectively) increase in root NR activity, but no additional NO$_3^-$ was transported in the xylem, which resulted in no significant changes in leaf NR activity. Foliar applied and stem supplied NO$_3^-$, induced NR activity in the leaves by 2- to 4-fold over the controls. Expression of NO$_3^-$ metabolism-related genes increased (up to 10-fold for *Nitrate REDUCTASE* and 40-fold for *NITRITE REDUCTASE*) when NO$_3^-$ was supplied to the cut end of the stem. These data indicate
that NR activity is limited by transcript accumulation as well as by post-translational mechanisms. Overall, the data indicate that blueberries are capable of assimilating NO$_3^-$ in the shoots albeit at relatively low levels. Root NO$_3^-$ uptake and its partitioning to the shoots may be significant limitations to nitrate assimilation in blueberries.

INDEX WORDS: Vaccinium sp., non-native soils, Nitrate assimilation, Nitrate reductase
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ASSIMILATION IN BLUEBERRY

by

DOUGLAS S. ALT

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by

DOUGLAS S. ALT

Major Professor: Anish Malladi
Committee: Marc W. van Iersel
            Miguel Cabrera

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this work to my wife, Valentina. You have stood beside me through the ups and downs as we build our family and pave our future, always offering your love and support.

I also dedicate this work to my sons,

With hard work and determination you can overcome any obstacle.

To my parents, who have supported me through life’s challenges,

   teaching me never to give up.

To my bothers, no matter how spread apart we are, the friendship and support is always strong.
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Blueberries (*Vaccinium* sp.) are flowering woody perennials that produce a small blue fruit containing high concentrations of anthocyanins. Recently identified health benefits along with a nutrition conscious society have been driving a strong market demand with annual per capita estimates 1.0 kg for 2013 up from 0.79 kg in 2009 (www.blueberry.org, September 2015). To meet increasing demand, blueberry production areas have increased from 28,327 hectares in 2005 to 49,776 hectares in 2012 with the majority of growth coming from the southeast and western growing regions (Brezelton, 2013). With limited native acid soils, production has moved into upland, agricultural soils (pH 6.0-6.5, low organic matter, and NO$_3^-$ as the primary nitrogen source), requiring costly soil amendments.

Belonging to the Ericaceae family (lime-hating), blueberries evolved near wetlands or forested areas with low pH soils (pH 4.0 – 5.5), high organic matter, and ammonium (NH$_4^+$) as the primary form of available nitrogen. Blueberries have small, shallow root systems that require specific soil conditions for productive growth and fruiting (Korcak, 1986; Korcak, 1988; Townsend, 1969). Low soil pH and high organic matter provide a steady supply of NH$_4^+$ nitrogen, leading to a suggested preference for the NH$_4^+$ form of nitrogen (Erb et al, 1993; Korcak, 1986; Ponnachit and Darnell, 2004; Townsend, 1969; Williamson and Lyrene, 1998). Some *Vaccinium* species have adapted to agricultural soils, but in production years, the addition of pine bark beddings with an initial establishment cost of $3740/hectare and new bark every three years ($2200) along with multiple applications of nitrogen fertilizers ($150/hectare) are
essential for productive crop yields (Korcak, 1986; Korcak, 1988; Eleveld et al., 2005; Townsend, 1969).

The Ericaceae family consists of over 4,000 species of flowering plants often found in acidic and non-fertile soils. There are nearly 400 Ericaceae species commercially cultivated for uses ranging from fruit production, ornamentals, herbs, and medicinal purposes (Galletta and Ballington, 1996). Of the cultivated species, Vaccinium species are intensely produced for their small fruit. Blueberries, northern highbush (V. corymbosum L.), southern highbush blueberry (V. corymbosum L. interspecific hybrids), and rabbiteye (V. ashei) make up the majority of the cultivated acreage along with cranberry (V. oxyccocus L.), large cranberry (V. macrocarpon), lowbush blueberry (V. angustifolium), half-high blueberry (hybrid of V. angustifolium × V. corymbosum), and lingonberry (V. vitis-idaea).

Many Ericaceae species are thought to have evolved near wooded wetlands where they developed specific soil condition preferences such as low pH, high soil organic matter, and the \( \text{NH}_4^+ \) form of nitrogen. Soil preference has also been seen in many gymnosperms species such as jack pine (Pinus banksiana) or white spruce (Picea glauca) found in the boreal forest range in northwest Canada (Ste-Marie and Pare, 1999). Jack Pine in particular has been shown to exude root extracts to lower soil pH and to reduce \( \text{NH}_4^+ \) nitrification (Ste-Marie and Pare, 1999). Griedanu et al (1972) and Korcak (1988) showed the growth of large cranberry and northern high bush blueberry seemed to be restricted when supplied with \( \text{NO}_3^- \) nitrogen, supporting Townsend’s work (1967) that suggested high bush blueberry grew better, producing longer shoots with an \( \text{NH}_4^+ \) source of nitrogen. Ingestad (1973) found V. vitis-idaea and V. myrtillus grew well with both nitrogen sources. However, Austin et al (1986) and Korcak (1986) found no difference in blueberry yield in relation to the source of nitrogen.
Some late successional forest species evolved mechanisms such as increased $\text{NH}_4^+$ uptake (20 times higher than $\text{NO}_3^-$) and can tolerate higher ammonium concentrations in the cytosol (10 times greater than $\text{NO}_3^-$) (Kronzucker et al, 1997). Poonnachit and Darnell (2004) reported a comparison of two *Vaccinium* species, southern highbush and sparkleberry (*V. arboretum* Marsh), that could uptake $\text{NH}_4^+$ two to nine times higher than $\text{NO}_3^-$. *V. arboretum* Marsh is a wild species that grows well in higher pH soils and $\text{NO}_3^-$ nitrogen due to its increased efficiency to uptake and assimilate both $\text{NO}_3^-$ and iron. The ability of blueberry to uptake high concentrations of $\text{NH}_4^+$ has been ignored as researchers find the lack of major nitrate assimilation to be of more importance (Havill et al, 1974).

Rabbiteye is native to the southeast United States while southern highbush was bred by crossing a high chilling requirement northern highbush and low chilling species *V. darrowi*, native to Florida (Galletta and Ballington, 1996; Lyrene, 1997). In Georgia, rabbiteye blueberries are grown in all regions, often serving as a processing variety due to perceived lower fruit quality and can serve as a later season variety in southern regions due to a longer growing season requirement. Southern highbush blueberries are also planted widely across the state but very low chill requirements make them susceptible to spring frosts in more northern growing regions.

**Plant Available Nitrogen**

Nitrogen is a macro plant nutrient and in blueberry constitutes 1.7-2.1% of the total plant dry weight serving as a building block for many plant compounds, such as proteins, chlorophyll, and nucleotides (Haynes, 1986, Retamales and Hancock, 2012). Nitrogen is often the most limiting nutrient to plant growth even with four potential sources of nitrogen: atmospheric nitrogen, $\text{NH}_4^+$ (inorganic), $\text{NO}_3^-$ (inorganic), and organic nitrogen (Crawford and Glass, 1998).
Atmospheric nitrogen is a diatomic molecule that makes up 79% of the atmosphere but is unavailable for direct plant uptake. Atmospheric nitrogen can be reduced to plant available nitrogen ($\text{NH}_4^+$ and $\text{NO}_3^-$) by nitrogen fixing bacteria, the Haber-Bosch method (fertilizer production), or by lightning. Nitrogen fixation is a process where the diatomic nitrogen is converted to reactive nitrogen forms, $\text{NH}_4^+$ or nitric oxide (Lawn and Brun, 1974). Legume plants are known for their symbiotic relationship with bacteria, rhizobia, living in root nodules where they fix $\text{N}_2$ into ammonia and provide it to the host plant in exchange for carbon supply (Lawn and Brun, 1974).

Nitrogen fertilizers are produced using the Haber-Bosch method where $\text{N}_2$ is converted to $\text{NH}_3$ under high pressure and temperature. The $\text{NH}_4^+$ can then be synthesized into many different concentrations or physical forms (anhydrous ammonium, urea, UAN, ammonium sulfate); it may also be oxidized to $\text{NO}_3^-$ to make additional nitrogen fertilizers. The fertilizers can be applied to the crops in a variety of methods: bulk application, in-furrow, fertigation, and foliar, all used to meet crop demand without being immobilized in the nitrogen cycle (Haynes, 1986).

To minimize N loss, split fertilizer applications and slow release fertilizer additives (polymers, nitrification and urease inhibitors) are used to try to prevent $\text{NO}_3^-$ leaching and $\text{NH}_4^+$ loss to volitization (Gioacchini et al., 2002). Nitripyrin is a nitrification inhibitor that keeps nitrogen in the ammonium form with a positive charge that can bind to negatively charged soil particles. This reduces the leaching loss of negatively charged $\text{NO}_3^-$ and losses from denitrification (Freney et al, 1993). Foliar applied nitrogen is when a nitrogen source, organic or inorganic is applied directly to the leaf foliage as a mist. Although application rates need to remain low to prevent leaf damage, foliar applied nitrogen is often considered to be much more efficient since it does not get immobilized in the soil.
Some forms of organic nitrogen and metabolites released in the soil from root exudates, decaying plant material, or microbes can be taken up directly through plant roots or provided to plants through interactions with other organisms. Some plants can uptake amino acids directly from the soil completely bypass nitrogen assimilation (Nasholm et al., 1998). Many symbiotic relationships such as the ericoid mycorrhizal fungi are known to form mutualistic relationships with Ericaceae species where the fungi mine the soil for organic nitrogen sources and exchanges amino acids for carbon sources in a coil region of root epidermal cells (Read, 1983). The complete impact of ericoid mycorrhizae on supplying plants with nitrogen is not fully understood, but it appears that colonization is very host specific and can improve nitrogen content when plants are fertilized with organic fertilizers, but no effect when mineral fertilizers are used (Haynes and Swift, 1985).

As leaves senesce in the fall, nitrogen rich proteins are broken down into amino acids and the N is remobilized and transported to storage organs such as roots and stems. Plant nitrogen reservoirs are very important in perennial crops that bloom in early spring because much of the soil nitrogen is still immobile and transpiration is low, so uptake and transport in the plant is limited. In early spring, the nitrogen reserves account for up to 90% of the nitrogen needed during anthesis and about 50% of the nitrogen supplied up till fruit maturity (Birkhold and Darnell, 1993). Proper senescence and fall nitrogen uptake can be critical to restore nitrogen reserves in the roots and shoots.

**Ammonium Uptake and Transport**

In the soil, NH$_4^+$ diffuses towards plant roots cells where it is moved across the plasma membrane into root cells by diffusion or across membranes by a uniporter (Logue and von
Diffusion can occur directly from the soil solution into the cell by way of potassium channels (K channels) (Logue and von Wiren, 2004; ten Hoopen et al., 2010). The majority of NH$_4^+$ uptake occurs by uniporters where proton pumps move hydrogen ions out of the cell, across the plasma membrane creating an electron gradient that allows for the movement of NH$_4^+$ ions into the cell (von Wiren et al., 2001). The excess hydrogen atoms pumped into the soil increase soil acidity as NH$_4^+$ is taken up (von Wiren et al., 2001).

Two transport systems, a high affinity transport system (HATS) and a low affinity transport system (LATS) regulate NH$_4^+$ uptake (Glass et al., 2002). At external NH$_4^+$ concentrations below 1 mM, the HATS is operational and at higher NH$_4^+$ concentrations, the LATS is functional (Forde and Clarkson, 1999; Glass et al., 2002).

Ammonium Assimilation

Once inside the root cell, it is rapidly assimilated through the glutamine synthetase and glutamine 2-oxoglutarate aminotransferase pathway (GS-GOGAT) (Glass et al., 2002; Mokhele et al, 2012). In this system, NH$_4^+$ reacts with glutamate in an initial step to form glutamine, a reaction catalyzed by glutamine synthetase (GS). Subsequently, glutamine and 2-oxoglutarate are reduced into two glutamates, a reaction catalyzed by glutamine oxoglutarate aminotransferase (GOGAT) (Lam et al, 1996). Glutamate, glutamine, asparagine and aspartate can be transported throughout the plant and provide nitrogen to synthesize other N-containing compounds.

Although shoots are capable of assimilating NH$_4^+$ to accommodate NH$_4^+$ produced in nitrate assimilation and photorespiration, plants do not appear to transport large quantities of NH$_4^+$ to the shoots due to the lack of ion sink (H$^+$ and OH$^-$) to buffer the cytosol and the risk of
NH₄⁺ toxicity (Andrews, 1986; Raven and Smith, 1976; Schjoerr et al., 2002). An H⁺ ion is released during ammonium assimilation. Root cells are in close contact with the large buffering capacity of the soil, while shoot cells need to synthesize additional organic compounds to buffer the cytosolic pH (Raven and Smith, 1976).

The NH₄⁺ uptake rates into root cells are highly regulated to avoid ammonia toxicity (Britto and Kronzucker, 2002). The pH of the cytosol is maintained near 7.5, so NH₄⁺ ions are deprotonated, and the uncharged ammonia can move freely across cell membranes. The ammonia may enter a vacuole with a low pH and become re-protonated (pick up a hydrogen ion), raising the pH in the vacuole. The changes in pH can alter normal cell function leading to a symptom referred to as ammonium toxicity. To prevent excess NH₄⁺, uptake rates are regulated to match assimilation through the GS-GOGAT pathway (Lam et al, 1996). Many species that evolved in soils with high NH₄⁺ concentrations seem to tolerate higher concentrations of cytoplasmic NH₄⁺. Some plants, such as rice (Oryza sativa ‘IR72’), are capable of cycling NH₄⁺ across the plasma membrane with an efflux rate up to 80% of the influx rate, keeping the GS-GOGAT system saturated while preventing ammonium build up (Britto et al, 2001).

Nitrate Uptake and Transport

NO₃⁻ is moved towards root cells by mass flow and across the plasma membrane by a symporter, following the proton gradient generated by active proton pumps (Glass et. al, 2002). Inside the cell, NO₃⁻ has three primary fates: immediate assimilation, storage in a root vacuole, or transport to the shoots (Crawford and Glass, 1998; Pate, 1980). The first step (rate limiting) of nitrate assimilation is the reduction of NO₃⁻ to NO₂ (Nitrite) by nitrate reductase (NR). Nitrite is further reduced to NH₄⁺ by nitrite reductase and enters the GS-GOGAT pathway (Haynes, 1986;
Lam et. al., 1996). Since nitrate reduction is the rate-limiting step, NR activity is often used to quantify nitrate assimilation in specific plant organs

$\text{NO}_3^-$ uptake requires active transport where a symporter moves $\text{NO}_3^-$ and two hydrogen protons across the plasma membrane of root cells (Glass et al., 2002). In contrast to $\text{NH}_4^+$ uptake, the removal of two $\text{H}^+$ ions causes a pH increase in the root rhizosphere during $\text{NO}_3^-$ uptake (von Wiren et al., 2001). There are three transport systems involved in $\text{NO}_3^-$ uptake: a constitutive, high-affinity transport system (cHATS), an inducible high-affinity transport system (iHATS), and a low-affinity transport system (LATS) (Glass et al., 2002). The cHATS system takes up $\text{NO}_3^-$ at very low soil concentrations (Glass et al., 2002). After the root cells first senses $\text{NO}_3^-$ uptake remains low for few hours until another high-affinity transport system (iHATS) is induced (Glass et al., 2002). When soil $\text{NO}_3^-$ concentrations exceeds 200 µM, the HATS becomes saturated and the LATS system takes over (Glass et al., 2002). These transport systems can be down regulated if $\text{NO}_3^-$ concentrations in the cell become too high.

Once inside the cell, $\text{NO}_3^-$ can be assimilated, stored, or transported (Crawford and Glass, 1998; Pate, 1980) depending on carbon availability and external $\text{NO}_3^-$ concentrations (Andrews, 1986). At low $\text{NO}_3^-$ concentrations, $\text{NO}_3^-$ will be assimilated in the roots if a carbon source is available, whereas at higher concentrations, some plant species transport $\text{NO}_3^-$ to the shoots to be assimilated (Andrews, 1986). In many woody perennials, higher nitrate assimilation occurs in the shoots, as it may be more efficient to transport $\text{NO}_3^-$ to the shoots where carbohydrates are actively synthesized and available to provide the energy required for nitrate assimilation (Andrews, 1986; Smirnoff and Stewart, 1985; Smirnoff et al, 1984).
**Nitrate Assimilation**

Nitrate assimilation is a two-step process, starting with the reduction of NO$_3^-$ to nitrite by nitrate reductase with two electrons from either NADH or NADPH (Kaiser and Huber, 1994). Nitrate reductase is a 100 kDa polypeptide complex consisting of an FAD, heme-iron, and molybdenum-molybdopterin (MO-MPT) cofactors connected by two hinge structures with a serine protein kinase located on the second hinge. The three cofactors serve as an electron transport chain with the electron donor starting a chain of redox reaction across the cofactors until the NO$_3^-$ ion is reduced (Bachmann et al., 1996). NO$_2$ is further reduced to ammonium by nitrite reductase (NiR). The ammonium enters the GS-GOGAT pathway to be assimilated into glutamate (Haynes, 1986; Lam et. at., 1996). Nitrate reduction is the rate-limiting step in NO$_3^-$ assimilation and can be used to quantify plant nitrate assimilation rates and plant N status (Srivastava, 1980).

As a quick response to NO$_3^-$ uptake, the cells maintain pools of NR that can be post-translationally regulated by phosphorylation and subsequent binding by a protein of the 14-3-3 binding family. The hinge 2 between the Fe and Mo cofactors, contains a serine 543 binding location that can be phosphorylated by a protein kinase. Once the site is phosphorylated, a 14-3-3 binding location can bind to the protein kinase, interrupting the electron transport and deactivating nitrate reductase (Bachmann et al., 1996). The plant cell maintains a population of deactivated protein that can quickly be dephosphorylated to an active state by phosphatase. The regulation is tightly controlled by both internal sugar and NO$_3^-$ availability and external factors, light and oxygen (Kaiser and Huber, 2010). If conditions are met for higher rates of nitrate assimilation, the cell will signal for NR transcription.
Nitrate Reductase Genes

*NR* gene expression is often induced when sufficient NO$_3^-$ concentrations are detected in the cell and the cell carbon status is sufficient (Wang et al, 2003). Studies in *Arabidopsis* have revealed two major genes encoding for nitrate reductase (Chang et al, 1988; Wilkinson and Crawford, 1991). The NIA1 locus is expressed primarily in the roots and accounts for 10% of the total NR activity. The NIA2 locus is expressed in both shoots and roots, accounting for 90% of the total NR activity (Wilkinson and Crawford, 1993). In blueberry two potential contigs, CUF8338 and CUF61184, have been identified from the blueberry draft genome using the Integrated Genome Browser (www.affymetrix.com, September 2015). These genes have not yet been functionally characterized.

Nitrate Assimilation in Blueberry

Previous studies have suggested that some Ericaceae species, such as lowbush blueberries and large cranberry, may not contain a nitrate reductase system (Greidanus et al, 1972; Townsend and Blatt, 1966). Subsequent work reported low amounts of NR activity in the roots with little to no nitrate assimilation in the shoots (Darnell and Hiss, 2006; Dirr et. al., 1972; Townsend, 1970). Poonnachit and Darnell (2004) reported that the difference between two *Vaccinium* species nitrate assimilatory rates was related to the plants’ ability to uptake NO$_3^-$. The low reported NR activity in the shoots may be related to low expression of the NR genes, due to the lack of NO$_3^-$ being transported to the shoots to induce NR activity, or due to other post-translational effects regulating NR activity.

A limitation in measuring NR activity in blueberry has been the use of the “*in vivo*” method where fresh tissue is assayed compared to an “*in vitro*” method where samples can be
frozen and the NR protein extracted and assayed. The “in vivo” method limits experiment sizes to the number of samples that can be processed immediately. Dirr et al (1973) demonstrated that adding the extract from certain Ericaceae species inhibited NR activity in *Zea mays* and suggested that tannins released during extraction compromised NR activity measurements. Dirr et al (1972) designed an “in vitro” method for *V. corymbosum* and *Rhododendron catawbiense* with a modification to the colorimetry step by filtering extracts directly into the coloring reagents, but this made it difficult to quantify NR activity on a weight basis.

**Statement of Research Hypothesis and Objectives**

**Research Hypothesis**

Blueberry roots are capable of assimilating all of the limited NO₃⁻ taken up by the plant and thus none is transported to the shoots to induce leaf NR activity.

**Objectives**

1. Determine if any root supplied NO₃⁻ is transported to the shoots for assimilation
   
   a) Design a method to measure low NR activity in frozen blueberry tissue.
   
   b) Measure NO₃⁻ concentrations transported in blueberry shoots.

2. Determine if NR activity and NR gene expression can be induced in blueberry shoots by supplying exogenous NO₃⁻.

**Research Significance:**

If blueberry could uptake and assimilate higher amounts of NO₃⁻, less soil modifications may be needed when growing blueberries in sub-optimal soil conditions. If the shoots are capable of assimilating NO₃⁻ but none is being transported there, foliar NO₃⁻ applications can be
used to enhance N fertilization in blueberry. Alternatively, breeding approaches can be focused on developing plants with higher uptake rates on NO$_3^-$.

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Introduction

Blueberries (*Vaccinium* sp.) are a popular fruit that are consumed fresh, frozen and processed, and known for their health benefits (Howell, 2008). Blueberry production in North America has grown from 28,327 hectares in 2005 to 49,776 hectares in 2012 (Brazelton, 2013). Blueberries evolved in forested wetlands with specific soil preferences such as high organic matter, low pH (<5.5) and ammonium (NH$_4^+$) nitrogen. The expansion of blueberry acreage is leading to the production of blueberries in agricultural soils (low soil organic matter, pH 6.0-6.5), nitrate (NO$_3^-$) nitrogen), requiring expensive soil amendments (organic matter and NH$_4^+$ based fertilizers) to maintain high production. Without these soil amendments, many *Vaccinium* species experience stunted shoot growth and decreased yields often associated with a decreased ability to uptake and assimilate nitrogen (Korcak, 1986; Korcak, 1988; Ponnachit and Darnell, 2004; Townsend, 1969).

Nitrogen is a macronutrient that can be taken up in the form of the inorganic ions ammonium (NH$_4^+$) and NO3- (NO$_3^-$). NH$_4^+$ is moved across the plasma membrane by a uniporter in passive transport while NO$_3^-$ is carried across by a symporter through a secondary active transport system (Glass et. al, 2002; Mokhele et al, 2012). *Vaccinium* sp. display a relatively greater ability to uptake NH$_4^+$, which in some species is 1.7 to 8.5 times higher than that of NO$_3^-$ (Poonnachit and Darnell, 2004). The lower ability to uptake NO$_3^-$ may be related to low nitrate
assimilatory rates in blueberry (Glass et al., 2002; Poonnachit and Darnell, 2004).

In most woody perennials, nitrate assimilation takes place in the roots at low external NO$_3^-$ concentrations but is partitioned to the shoots as concentrations increase (Andrews, 1986). Nitrate assimilation is a two-step process catalyzed by the reduction of NO$_3^-$ to NO$_2$ (nitrite) by nitrate reductase (NR), and NO$_2$ being further reduced to ammonia by nitrite reductase (NiR) (Glass et al, 2002; Kaiser and Huber, 1994). Subsequently, NH$_4^+$ is assimilated into amino acids through the glutamine synthetase and glutamine oxoglutarate aminotransferase pathway (GS-GOGAT) (Glass et al, 2002; Mokhele et al, 2012). Due to the high energy required for nitrate assimilation many plants transport NO$_3^-$ to the shoots for assimilation (Andrews, 1986). Blueberries reportedly display low levels of NR activity in the roots and little to no NR activity in the leaves (Claussen and Lenz, 1999; Dirr et. al., 1972; Poonnachit and Darnell, 2004; Townsend, 1970).

Many internal factors, such as sugar and NO$_3^-$ availability, and external factors, such as light intensity and oxygen concentrations, can regulate NR either prior to or after translation (Kaiser and Huber, 2001). NR gene expression is often induced by the presence of NO$_3^-$ in the cytosol. If NO$_3^-$ concentrations are too low, or under conditions of low NO$_3^-$ uptake, NR gene expression is typically down-regulated (Glass et al., 2002; Vincentz et al., 1993). Plants also maintain a pool of deactivated NR protein that can quickly be dephosphorylated to rapidly initiate nitrate assimilation when conditions are optimal (Bachmann et al., 1996). The low rates of NR activity generally reported in blueberry roots may be the result of such a pool of inactive NR. Such NR activity may be sufficient to assimilate the NO$_3^-$ taken up by the roots under NO$_3^-$ nutrition conditions. The inability to partition large amounts of NO$_3^-$ assimilation to the shoots may limit the plant’s ability to efficiently assimilate NO$_3^-$. Hence, it was hypothesized that low
Nitrate assimilation in blueberry shoots was due to limited NO$_3^-$ transport from the shoots.

*V. corymbosum* (southern highbush blueberry) and *V. ashei* (rabbiteye blueberry) are two commercially cultivated blueberry species that are thought to grow best in acidic soils and with the NH$_4^+$ form of nitrogen, although rabbiteye blueberry types are known to better adapt to agricultural soils with NO$_3^-$ nitrogen. It was hypothesized that better adaptability of *V. ashei* to NO$_3^-$ nutrition is associated with greater NO$_3^-$ uptake and transport to the shoots for assimilation.

Another objective of this work was to design an alternative method to measure NR activity from previously frozen tissues of blueberry roots and leaves. Current “in vivo” NR activity methods limit the size of experiments to the number of samples that can be processed as live tissue. If tissue could be frozen, it could all be collected at a single time point and analyzed at a later date.

**Materials and Methods**

**Plant Culture**

On January 27, 2014, rooted stem cuttings of *V. corymbosum* ‘Sweetcrisp’ and *V. ashei* were planted in a peat:pine bark nursery mix (Faford) in 1-L pots, fertilized (4 g pot$^{-1}$) with a slow release fertilizer (Osmocote: 18-6-12, N-10% NH$_4^+$ + 8% NO$_3^-$), and placed in a glass house. On June 20, 2014, plants were transferred to 4-L pots and again fertilized (9 g pot$^{-1}$) with Osmocote. Shoots and roots were pruned in September 2014 to maintain a compact plant to fit into the hydroponics system and maximize growing points. Blueberries were maintained in the glass house as “evergreen” plants over the winter to minimize nitrogen remobilization and storage and to increase potential dependence on root supplied nitrogen. On December 14, 2014, plants were fertilized with another 9 g pot$^{-1}$ with Osmocote.
In the spring of 2015, as plants flowered, floral structures were removed to maintain largely vegetative growth on the plant. The plants were grouped according to size and 12 plants from each species were randomly selected. In March 2015, plants were further grouped by size into four blocks with two plants from each cultivar, removed from the pots, and allowed to soak in water for 1 h. Roots were thoroughly washed to remove attached substrate, and randomly placed into 2.5 L plastic buckets covered with heavy duty aluminum foil. To keep plants centered, plants were inserted through a hole in the lid also covered in aluminum foil. A single air pump (AAPA 110L, Active Aqua, Phoenix, USA) connected with plastic tubing and valve regulated distributors supplied 1 L min\(^{-1}\) of air to an air-stone placed in the bottom of each bucket. Plants were supported with a string attached to an overhead tube (PVC) that held the plant stems above the nutrient solution. Temperature (°C) in the glass house ranged from 18.9±0.1 to 24.4±0.4 and a daily light integral of 8.7±0.8 (mol m\(^{-2}\) d\(^{-1}\)) during acclimation and 19.0 ± 0.1 to 24.8 ± 0.8 and a daily light integral of 9.9± 0.9 during the treatment period.

Plants were acclimated for two weeks, supplied a Hoagland based nutrient solution (2 L bucket\(^{-1}\)) and nitrogen in the form of NH\(_4\)\(^{+}\) (1 mM) in an attempt to deplete NO\(_3\)\(^{-}\) reserves. The nutrient solution contained (mM): 0.5 mM K\(_2\)HPO\(_4\), 1.0 mM MgSO\(_4\), 0.5 mM CaCl\(_2\), 0.08 mM Fe-Ethylenediaminetetraacetic acid (Fe-EDTA), 0.045 mM H\(_3\)BO\(_3\), 0.01 mM MnSO\(_4\), 0.01 mM ZnSO\(_4\), and 0.2 µM NaMoO\(_4\) (according to Darnell and Hiss, 2006; Poonachit and Darnell, 2004). Nitrogen was supplied as either 5.0 mM KNO\(_3\) or 2.5 mM (NH\(_4\))\(_2\)SO\(_4\). To prevent nitrification, 4.0 µM of nitrapyrin (2-Chlor-6-(trichloromethyl)pyridin) was added to the solution (Rosen et al., 1990) and KCl was used to adjust for the K\(^{+}\) differences created from different nitrogen sources. Solution pH was monitored and adjusted every other day to a pH of 5.0 using a
portable pH meter (760 BK Precision, Linda, CA). Solutions were changed every five days to maintain a continual supply of nitrogen.

Nitrogen Treatments

Two NO$_3^-$ treatments were applied through the hydroponic solutions: 5 mM NO$_3^-$ (KNO$_3$) and 0 mM NO$_3^-$ control (2.5 mM (NH$_4$)$_2$SO$_4$ + 5 mM KCl). The NH$_4^+$ treatment served as a 0 mM NO$_3^-$ control. Two buckets, each with a different N treatment, were used as controls without plants for determining water evaporation and nitrogen loss. During solution changes, water losses (evaporation) were measured and subtracted from the plant water uptake levels. Nutrient solutions were also collect from the control buckets to measure nitrogen losses from denitrification and NH$_4^+$ volitization. Treatments were initiated in April 2015 as the plants were well acclimated to the nutrient solution and displayed adequate new growth by this time. At the beginning of each change of hydroponics solution, root tips (about 300 mg) and upper fully expanded leaves were collected, frozen in liquid nitrogen, and stored at -80 °C. Plant solution uptake was recorded and 1 mL of nutrient solution (for nitrogen uptake analysis) was also collected at the time of solution change at 0, 6, 12, and 16 d after treatment.

At 17 d after treatment, the blueberry plants (still in solution) were moved into the laboratory. Stems were removed 1 cm above the crown and a 10 cm piece of stem was cut off, under de-ionized (DI) water. Bark and cambium was removed 1 cm back from the shoot end of the stems and washed with DI water to remove any contamination from the phloem. The stem was then placed in a pressure chamber with the shoot end protruding out and a constant pressure was applied using a nitrogen gas cylinder at a pressure lower than 0.2 MPa to prevent cellular contamination (Alexou and Peuke, 2013; Grassi et al, 2002). Exuding sap was collected with a pipette tip, placed in a 1.5 mL tube, immediately frozen in liquid nitrogen, and stored at -80 °C.
Subsequently, the roots were cut 1 cm below the crown and oven dried at 70°C for 72 h to obtain
the root dry weight.

NO₃⁻ and NH₄⁺ Uptake

NO₃⁻ and NH₄⁺ concentrations remaining in the buckets at the time of change of
hydroponics solution were measured spectrophotometrically using an auto-analyzer (Cabrera et
al., 2005). To calculate the nutrient uptake, the final nitrogen content minus nitrogen loss was
subtracted from the initial NO₃⁻ content to get a potential maximum nitrogen uptake (Frith and
Nicholas, 1974; Malagoli et al., 2000; Poonnachit and Darnell, 2004).

Quantifying NR Activity

Leaf and root samples were removed from the -80°C freezer, thawed (first freeze-thaw
cycle), and cut into small pieces (3-4 mm). Sliced tissue (300 mg) was placed in a 4 mL glass
tube and immediately frozen (30 s) in liquid nitrogen then thawed again in a 30°C water bath (2
min). This process was repeated for an additional four freeze-thaw cycles (Maurino et al., 1985;
Rhodes and Stewart, 1974). An extraction buffer containing 100 mM KH₂PO₄ (pH 7.5), 2 % 1-
propanol, 1 mM EDTA (ethylenediaminetetraacetic acid), 50 mM KNO₃, and 5 mM NADH was
added (4 ml g⁻¹) and mixed vigorously. An initial sample (0.4 mL) was removed and placed in a
new 1.5 mL tube, and was used to determine the initial nitrite concentration. The remaining
buffer with the tissue was vacuum-infiltrated for 5 min in the dark, and then placed into a
shaking incubator for 1 h at 30 °C in the dark. Samples were then vacuum-filtered through a 5
mL column to remove the plant material, and to stop the reaction. The filtered extraction (0.4
mL) was placed in a 1.5 mL tube as a final sample.

Prior to measuring nitrite concentration with a colorimetry method, samples were treated
to improve color development (Scholl et al., 1974). Initially, 40 µL of 0.5 M Zn-acetate was
added and allowed to react for 5 min then centrifuged for 1 min at 20,000 g to remove precipitates. Subsequently, 20 µL of 1 mM PMS was added to oxidize any remaining NADH. Samples were allowed to react in the dark for 10 min and then centrifuged for 10 min at 20,000 g. NO₂ concentrations were measured with an Azo colorimetry method at 540 nm using a spectrophotometer (Genesys 2, Spectronic, Pittford, USA) based on methods from Poonnachit and Darnell (2004). NR activity was determined from the difference of initial and final NO₂ concentrations.

Measuring NO₃⁻ in Xylem Sap

Frozen samples were thawed on ice and centrifuged for 5 min at 5000 g. The supernatant was collected and diluted 5-fold with distilled water (Grassi et al, 2002). NO₃⁻ levels were quantified using a single reagent method (Doane and Horwath, 2003) where NO₃⁻ was reduced with vanadium (III) chloride and allowed to react for 24 h, then measured in a spectrophotometer (modified from Smirnoff and Stewart, 1985).

NO₃⁻ Uptake vs. Assimilation

Plant total nitrogen uptake was calculated for the 6 day period from 6 to 12 d after treatment by multiplying the measure daily nitrate uptake for day 12 (Figure 2.1) by six days. Total nitrate assimilated was calculated using the rates of NR activity from 12 d after treatment (Figure 2.2) and multiplying it by an estimated total root fresh weight. NR activity was assumed to be constant across the entire root system. NR activity was considered to be constant over the entire time period, so NR activity per root system was multiplied by total hours in the time period (144 h). Root fresh weight was estimated using known dry weight from day 17 of the experiment and using a formula modeled from previous fresh weight vs. dry weight plant data:

Estimated root fresh weight \[ Y = \left( X + 6.49994 \right) / 0.2567 \]
\[ Y = \text{fresh weight}; \ X = \text{dry weight} \]

Leaf NR activity typically follows a diurnal pattern, highest at mid-day, but root NR activity operated at a more constant rate for the entire 24 h period in cucumber (de la Haba et al., 2001). Using our root NR values collected at mid-day, we assumed the activity to be a basal rate that was constant over the 24 h period for 6 d. Dividing total NR activity by total \( \text{NO}_3^- \) uptake and multiplying by 100 calculated the percent of nitrate assimilated within the roots.

**Experimental Design**

The experiment was a 2 x 2 factorial design using a complete randomized setup with each plant as an experimental unit. There were four replicates of each treatment factor: species (two levels) and nitrogen treatment (two levels). Analysis of variance was used with log transformed data (n =4).

**Results**

**Nitrogen Uptake**

Nitrate and ammonium uptake rates generally remained constant for the duration of the experiment, except in the \( V. \ ashei \) \( \text{NH}_4^+ \) treatment that steadily increased (not significant) from 0.25 to 0.45 mmoles N plant\(^{-1}\) day\(^{-1}\) (Figure 2.1). Nitrogen uptake in the form of \( \text{NH}_4^+ \) was higher than that in the form of \( \text{NO}_3^- \) at 12 and 16 d after treatment. At 12 d after treatment there was a significant treatment effect, ammonium uptake was 2- and 2-fold higher in \( V. \ ashei \) and \( V. \ corymbosum \), respectively (\( P = 0.0199 \)). Analysis of variance further showed that at 6 d after treatment, there was a significant cultivar effect illustrating that \( V. \ corymbosum \) displayed greater N uptake than \( V. \ ashei \) (\( P = 0.026 \)).
Root NR Activity

NR activity in the roots of *V. corymbosum* and *V. ashei* were similar and generally low at around 50 nmoles g\(^{-1}\) (FW) h\(^{-1}\) with ammonium as the only source of nitrogen. When NO\(_3^-\) was supplied to the roots through the hydroponic nutrient solution, NR activity in the roots transiently increased in both species at 12 d after treatment (Figure 2.2). At 12 d after treatment, NR activity under NO\(_3^-\) nutrition was 3.1- and 1.8-fold higher than under NH\(_4^+\) nutrition in *V. corymbosum* and *V. ashei*, respectively (*P* = 0.0045). At 16 d after treatment, NR activity in all treatments declined to basal levels. However, NR activity under NO\(_3^-\) nutrition was still significantly higher than that under NH\(_4^+\) nutrition (*P* = 0.0076). There was no interaction between species and nitrogen treatment factors.

Nitrate Concentrations in the Xylem Sap

Low concentrations of NO\(_3^-\) were measured in the xylem sap extracted from blueberry stems, ranging from 1.68 mM under ammonium nutrition to 1.92 mM under NO\(_3^-\) nutrition in *V. corymbosum* and 1.09 mM to 1.46 mM in *V. ashei* (Figure 2.3). There was no significant effect of nitrate nutrition on the concentration of nitrate transported within the xylem sap. Additionally, there was no significant difference between *V. corymbosum* and *V. ashei* in the NO\(_3^-\) concentration within the xylem sap.

Leaf NR Activity

NR activity in the leaves was generally low ranging from 9 to 33 nmoles g\(^{-1}\) (FW) h\(^{-1}\) (Figure 2.4). The source of nitrogen nutrition did not significantly affect leaf NR activity over the duration of the experiment.
Total Root NR Capacity

The total root NR activity followed a similar trend to the root NR activity (Figure 2.2). The initial values ranged from 2 to 4 nmol NO$_2$ root$^{-1}$ h$^{-1}$ (Figure 2.5). NR activity was significantly induced at 12 d after treatment with a 3-fold increase in V. ashei and a 2-fold increase in ‘Sweetcrisp’ ($P = .005$). There were no significant interaction or species effects.

Nitrate Uptake vs. Nitrate Assimilation

The root dry weight was not consistently affected by the treatments or species effect (Table 2.1). From day 6 to 12, V. ashei was estimated to take up 1.16 nmol NO$_3^-$ and estimated to assimilate 1.31 nmol, i.e. 118% assimilation compared to uptake. V. corymbosum was estimated to assimilate 92% of the NO$_3^-$ taken up.

Discussion

The two Vaccinium species were able to take up both nitrogen forms, but showed an increased ability to uptake NH$_4^+$, V. corymbosum took up 2.2 to 2.3 times more NH$_4^+$ than NO$_3^-$ while V. ashei 1.4 to 2.1 times more over the 16 d treatment period. Previous work reported similar patterns with increased NH$_4^+$ uptake 2.0 to 8.5 times higher than that of NO$_3^-$ (Merhuat and Darnell, 1995; Poonnachit and Darnell, 2004). The increased ability to take up high rates of NH$_4^+$ appears to be a common trend in species that evolved in acidic soils. Malagoli et al. (2000) observed that Larix decidua (European Larch) and Pinus sylvestri (Scots Pine) seedlings take up to 20 times higher NH$_4^+$ than NO$_3^-$ uptake rates whereas apple seedlings that prefer high pH soils showed no significant difference in the uptake rates between the two N forms (Frith and Nicholas, 1975). The uptake rates of both forms were much lower than that many other crops suggesting that blueberries have a decreased ability or requirement for the uptake of inorganic
nitrogen. Blueberries in commercial production are known to have low nitrogen requirements, but nitrogen acquired from other sources such as organic nitrogen or symbiotic relationships have not been sufficiently well described.

There was a significant difference between species seen 6 days after treatment \( (P = 0.026) \) but at day 12, the uptake rates where no longer significantly different. This was due to the \( V. \) *ashei* \( \text{NH}_4^+ \) uptake rate being low relative to \( V. \) *corymbosum*, then increasing the uptake rate 1.5 times by day 12. The plants had been acclimated in an \( \text{NH}_4^+ \) solution for two weeks prior to treatment, so \( \text{NH}_4^+ \) uptake may have been downregulated during the first time period due to adequate nitrogen previously assimilated then upregulated as nitrogen reserves were depleted.

The low \( \text{NO}_3^- \) uptake was adequate to significantly induce root NR activity in both species at times 12 and 16 d (Figure 2.2). On a per weight basis, \( V. \) *corymbosum* was able to assimilate higher amounts of \( \text{NO}_3^- \), 135 nmoles \( \text{NO}_2 \) g (FW) \(^{-1} \) h \(^{-1} \) compared to \( V. \) *ashei* at 92, but on a whole plant basis, \( V. \) *ashei* assimilated more due to a larger root mass (Table 2.1). Although the NR rates are low, the plants were capable of assimilating nearly all (98 and 118%) the \( \text{NO}_3^- \) taken up during time period 6 to 12 within the roots (Table 2.1). On day 16, root NR activity decreased in all treatments suggesting that NR activity was down regulated due to some abiotic factor such as carbon source or oxygen concentration (Glass et al., 2002). The drastic decrease seen in the \( \text{NO}_3^- \) treatments may have also been regulated by a decreased amount of available \( \text{NO}_3^- \) since the uptake to assimilation ratio in table 2.1 was at or over one.

The low NR activity in the \( \text{NH}_4^+ \) treatment is consistent with a constitutive NR mechanism previously suggested in blueberry and reported in strawberry and raspberry (Clausen and Lenz, 1999; Dir et al., 1972; Ponnachit and Darnel, 2004). The basal rates measured in the
roots were similar to the low NR rates measured in the leaves, which suggests a similar 
constitutive NR system expressed in both organs.

The consistency of the “in situ” method in measuring NR activity similar to the values 
reported in the literature of ranging from 50 to 400 (nmoles NO$_2$ g (F.W) $^{-1}$ hr $^{-1}$) in the roots 
illustrates that the method is functioning. The low NR activities measured in the control roots 
and leaves illustrate an improved sensitivity previous only available through an infusion method 
in fresh tissues (Dirr et al., 1972). The ability to accurately process frozen samples improves our 
ability to further study nitrate assimilation processes in these plants.

Supplying 5 mM NO$_3^-$ to the roots of blueberries did not significantly increase the 
amount of NO$_3^-$ being transported in the xylem and therefore did not induce NR activity in the 
leaves. The NO$_3^-$ concentrations in the blueberry xylem sap described here (1-2 mM) are 
generally low compared to other crops (4 to 12 mM) which are known to assimilate high 
amounts of NO$_3^-$ in the shoots (Andrews, 1986). The basal amounts of nitrate traveling in the 
xylem in the NH$_4^+$ treatment may have potentially originated from root stored NO$_3^-$ sourced from 
the application of controlled release fertilizer applications. The formulation of the controlled 
release fertilizer used in this study prior to the treatment applications contained up to 8% N in the 
form of NO$_3^-$.

As suggested in Andrews (1986), many temperate plants will assimilate all the NO$_3^-$ in 
the roots until a threshold value of external NO$_3^-$ is sensed. If blueberries are capable of 
partitioning nitrate assimilation to the shoots, then the 5 mM NO$_3^-$ treatment was not adequate to 
induce this system. Another issue may be that blueberries lack a nitrate transport system capable 
of taking up high amounts of NO$_3^-$. Alternatively, blueberries may not have a high requirement 
for nitrogen as has been suggested previously (Poonnachit and Darnell, 2004).
In conclusion, *V. corymbosuum* and *V. ashei* did uptake higher rates of NH$_4^+$ than NO$_3^-$ nitrogen, yet both species were able to uptake and assimilate NO$_3^-$ within the roots. The form of nitrogen supplied to the roots did not affect NO$_3^-$ concentrations transported in the xylem sap and did not induce NR activity in the shoots. This supports our hypothesis that the roots are capable of assimilating the vast majority of the NO$_3^-$ being taken up by the plant and therefore none is being transported to the shoots to induce NR activity. Alternatively, additional NO$_3^-$ may not be transported to the shoots because of an overall inability of blueberry plants to assimilate high amounts of nitrogen.
Table 2.1. Percent of NO$_3^-$ uptake vs. nitrate assimilated. Represents total nitrogen uptake and total NR activity from time 6 to 12 days after nitrogen treatment supplied to the roots. Root fresh weight were estimated using dry weight data obtained at 17 d after treatment. Reports estimated root potentials to immediately assimilate NO$_3^-$ taken up from the nutrient solution. Shows average values (n=4) with standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root D.W. (g)</th>
<th>Estimated Root F.W.(g)</th>
<th>Total NR Activity (mmoles NO$_2$ plant$^{-1}$)</th>
<th>Total N uptake (mmoles)</th>
<th>Percent of NO$_3^-$ Assimilated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. ashei</em> NH$_4^+$</td>
<td>8.83±1.85</td>
<td>59.72±0.13</td>
<td>0.44±13.34</td>
<td>2.39±0.53</td>
<td>na</td>
</tr>
<tr>
<td><em>V. ashei</em> NO$_3^-$</td>
<td>18.81±5.20</td>
<td>98.60±20.25</td>
<td>1.31±0.32</td>
<td>1.16±0.28</td>
<td>118±26%</td>
</tr>
<tr>
<td><em>V. corymbosum</em> NH$_4^+$</td>
<td>16.84±4.56</td>
<td>90.92±17.36</td>
<td>0.52±0.11</td>
<td>3.79±0.97</td>
<td>na</td>
</tr>
<tr>
<td><em>V. corymbosum</em> NO$_3^-$</td>
<td>9.52±1.63</td>
<td>62.40±6.35</td>
<td>1.19±0.19</td>
<td>1.56±0.54</td>
<td>92±27%</td>
</tr>
</tbody>
</table>

*Assumes NR activity is equal across the entire root system.
*Assumes NR activity is constant over entire treatment period.
Figure 2-1. Nitrogen uptake from the nutrient solution. The average amount of nitrogen depleted from the nutrient solution over time is displayed. Data are means ± SE (n = 4).
Figure 2.2. Root nitrate reductase (NR) activity. Nitrate reductase activity in the roots supplied with either 5 mM NO$_3^-$ or 5 mM NH$_4^+$. Data are means ± SE (n = 4). Asterisk indicates significant difference between the ammonium (NH$_4^+$) and the nitrate (NO$_3^-$) treatments.
Figure 2.3. Nitrate concentrations in the xylem sap of blueberry stems. NO₃⁻ concentrations in the xylem sap in two *Vaccinium* species subjected to ammonium (NH₄⁺) or nitrate (NO₃⁻) nutrition are shown. Stems were cut and the xylem sap was extracted using the pressure bomb apparatus. Data are means ± SE (n = 4).
Figure 2.4. Leaf nitrate reductase (NR) activity in two *blueberry* species. The effect of the form of N nutrition on leaf NR activity is presented. Two nitrogen treats were supplied to the roots: 5 mM NO$_3^-$ or 5 mM NH$_4^+$. Data are means ± SE (n = 4).
Figure 2.5. Total root nitrate reductase (NR) activity in two *Vaccinium* species. Total root NR activity was estimated using estimated root fresh weight and root NR activity (Figure 2.2) from 12 d after treatment, assuming the NR root activity to be a constant rate over a 24 h period. Data shows means ± SE (n = 4).
References


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Chapter 3

INDUCTION OF NO3- ASSIMILATION AND EXPRESSION OF ASSOCIATED GENES IN BLUEBERRY SHOOTS

Introduction

Blueberry (Vaccinium sp.) consumption per capita has been steadily increasing from 0.79 kg in 2009 to 1.0 kg in 2013, at least in part due to their reportedly high anthocyanin contents and associated health benefits (www.blueberry.org, September 2015). To meet the increasing demand, blueberry production has been moving to more upland soils, lacking in some key conditions normally present in their native soils. Blueberries are thought to have evolved near wooded wetlands, often being considered facultative anaerobes with strict soil preferences of low soil pH (4.0 - 5.5), high soil organic matter content, and nitrogen primarily in the ammonium (NH₄⁺) form. These conditions are contrary to the higher soil pH (6.0 – 6.5), low soil organic matter, and nitrogen primarily in the nitrate (NO₃⁻) form, normally found in the agricultural, upland soils. Blueberry has shown a decreased ability to adapt to upland soils and requires expensive soil amendments (pine bark bedding, NH₄⁺ fertilizers) to maintain productive yields and plant growth (Korcak, 1986; Korcak, 1988; Ponnachit and Darnell, 2004; Townsend, 1969).

Nitrogen is a plant macronutrient accounting for 1.7-2.1% of the total plant dry weight in blueberry and serving as a building block for many plant compounds, such as proteins, chlorophyll, and nucleotides (Haynes, 1986, Retamales and Hancock, 2012). Plants can uptake nitrogen directly as NH₄⁺ (inorganic), NO₃⁻ (inorganic), and amino acids, but also form mutualistic relationships with fungi and bacteria that can supply nitrogen in exchange for a
carbon source or shelter (Crawford and Glass, 2002). Inorganic nitrogen sources are widely used in agricultural production primarily due to their low cost and ease of application. Plants can take up inorganic nitrogen directly, but these forms need to be assimilated into amino acids to aid in biological functions.

NH$_4^+$ uptake and assimilation is highly regulated where influx and efflux are used to maintain high assimilatory rates yet avoid toxic overloading of NH$_4^+$ in the cell (Glass et al., 2002). NO$_3^-$ uptake is less regulated as it does not need to be immediately assimilated, but can be stored in vacuoles or transported directly to the shoots for storage or assimilation (Crawford and Glass, 1998; Glass et al., 2002).

Nitrate assimilation is a two-step process catalyzed by the reduction of NO$_3^-$ to NO$_2$ (nitrite) by nitrate reductase (NR) and further to ammonia by nitrite reductase (NiR) (Glass et al., 2002; Kaiser and Huber, 1994). Subsequently, NH$_4^+$ is assimilated into amino acids through the glutamine synthetase and glutamine oxoglutarate aminotransferase pathway (GS-GOGAT) (Glass et al., 2002; Mokhele et al., 2012). Many woody perennials appear to transport the majority of NO$_3^-$ to the shoots where carbohydrates are synthesized and available to provide the energy required for nitrate assimilation (Andrews, 1986; Smirnoff and Stewart, 1985; Stewart et al., 1989).

Blueberries were initially thought to not contain an active nitrate assimilation system in the shoots (Townsend and Blatt, 1966), but subsequently low levels of NR activity have been reported in the shoots (Darnell and Hiss, 2006; Dirr et al., 1972; Townsend, 1970). Previous work (Chapter 2) suggests that blueberries conduct the majority of nitrate assimilation in the roots and may not partition additional NO$_3^-$ to the shoots even when high concentrations of NO$_3^-$ are supplied to the roots. It may be hypothesized that the inability to induce NR activity or
expression of nitrate metabolism associated genes in the shoots may limit nitrate assimilation within these tissues.

NR activity is regulated by sugar and NO$_3^-$ availability and external factors such as light and oxygen (Kaiser and Huber, 2001). For low NO$_3^-$ concentrations, plant cells may maintain a population of deactivated protein that can quickly be dephosphorylated to an active state by a phosphatase (Bachmann et al., 1996). If conditions are met for higher rates of nitrate assimilation, cells can induce transcription of nitrate metabolism-related genes (Wang et al., 2003). It is possible that the inability to activate NR or induce expression of nitrate metabolism-related genes may limit the amount of NO$_3^-$ being partitioned for shoot assimilation.

We hypothesize that if nitrate assimilation in the shoots was low due to the inability to induce it, NR activity and expression of nitrate metabolism-related genes would not be inducible in blueberry leaves when supplied directly with high NO$_3^-$ concentrations. Hence, the objectives of this study were to determine changes in NR activity and associated gene expression could be induced by supplying NO3- directly to the shoot tissue either through the cut ends of the shoot or through foliar application.

Materials and Methods

Two experiments were conducted: a foliar application of NO$_3^-$ and supplying NO$_3^-$ to a cuts stem. Prior to treatment, plants were acclimated to only a NH$_4^+$ nitrogen source using a deep-water culture hydroponic system.

Plant Culture

Rooted stem cuttings of southern highbush blueberry (V. corymbosum) ‘Sweetcrisp’, and rabbiteye blueberry (V. ashei) ‘Alapaha’, were procured and transplanted in a glasshouse to 1-L
pots with a peat:pine bark nursery mix (Faford) and fertilized (4 g pot⁻¹) with a slow release fertilizer (Osmocote 18-6-12; 8% NO₃⁻ and 10% NH₄⁺) in January 2014. The plants were moved to larger pots (4-L pots) in June 2014 and fertilized with an additional 9 g pot⁻¹ of Osmocote. In September 2014, plants were pruned to increase growing points while condensing plant size to fit into the hydroponic system. Plants were kept as “evergreen” in a temperature controlled glass house (18.5°C to 24°C) to minimize nitrogen remobilization and storage and to increase potential dependence on root supplied nitrogen. An additional 9 g pot⁻¹ of Osmocote fertilizer was added to each pot in December 2014.

To keep plants in a vegetative growth stage, floral structures were removed as they emerged. After flowering had commenced, plants were sorted by size and 12 plants from each cultivar were randomly selected. In March 2015, 24 plants (12 from each cultivar) for the foliar NO₃⁻ application experiment were further sorted by size into six blocks. Plants were removed from the pots, loose substrate removed, and the roots were allowed to soak in water for 1 h. Remaining substrate was manually removed and plants were placed into the designated blocks in 2.5 L plastic buckets covered with heavy duty aluminum foil. Plants were inserted through a hole in the bucket lid to keep them centered and aluminum foil wrapped around the base of the plant. Air-stones placed in each bucket supplied 1 L min⁻¹ of air from a single air pump (AAPA 110L, Active Aqua, Petaluma, CA, USA) connected with plastic tubing and valve regulated distributors. A string attached to an overhead tube (PVC) held the plant stems above the nutrient solution.

Plants were acclimated to a Hoagland based nutrient solution (2 L bucket⁻¹) providing only the NH₄⁺ form of nitrogen (1.0 mM) trying to deplete any stored NO₃⁻ reserves. The nutrient solution consisted of (mM): 0.5 mM K₂HPO₄, 1.0 mM MgSO₄, 0.5 mM CaCl₂, 0.08 mM Fe-
Ethylenediaminetetraacetic acid (Fe-EDTA), 0.045 mM H$_3$BO$_3$, 0.01 mM MnSO$_4$, 0.01 mM ZnSO$_4$, and 0.2 µM NaMoO$_4$ (according to Darnell and Hiss, 2006; Poonachit and Darnell, 2004). Nitrogen was supplied as 1.0 mM (NH$_4$)$_2$SO$_4$ and nitrification was inhibited with 4.0 µM of nitrapyrin (2-Chloro-6-(trichloromethyl)pyridine) (Rosen et al., 1990). A portable pH meter (760 BK Precision, Linda, CA, USA) was used to measure solution pH every other day and adjusted to pH 5.0 using 0.1 mM KOH or 0.1 mM HCl.

Foliar Application of NO$_3^-$

In April 2015 two foliar applications were applied directly to the leaf surface of the hydroponically grown plants: 1) distilled water with 0.1% surfactant (Latron B1956, Sticker Spreader) (control); and 2) 5 mM KNO$_3$ with 0.1% surfactant. To prevent cross contamination, plants (still in buckets with ammonium based nutrient solution but with air-stones and support string removed) were spread across the lab bench. The foliar application was applied until dripping was noticed. The plants were allowed to dry prior to being placed back into their designated location in the hydroponic system. Two leaf samples were collected for NR activity and gene expression analyses at 0, 24, 48, and 72 h after treatment. Samples were immediately frozen in liquid nitrogen and stored at -80 °C. Glass house temperature (°C) ranged from 18.9±0.1 to 24.4±0.4 and a daily light integral of 8.7±0.8 (mol m$^{-2}$·d$^{-1}$) during the acclimation period and 19.0 ± 0.05 to 24.6 ± 0.1 and a daily light integral of 11.8 ± 2.5 during the treatment period.
Stem Supply of NO$_3^-$

In April 2015, 24 plants (12 from each cultivar) were sorted and paired by size into six blocks with treatments randomly assigned. Plants were placed into the hydroponic system described above and acclimated to only the NH$_4^+$ form of nitrogen for two weeks as described previously. In May, stems were cut 1 cm above the roots and placed in a 500 mL Erlenmeyer flask covered with heavy-duty aluminum foil. The flasks were filled with 500 mL of one of the two treatments: 5 mM KNO$_3$ solution or distilled water (control). Parafilm was wrapped around the stem and top of the flask to reduce water loss to evaporation. Two leaf samples were collected at times: 0, 24, 48 and 72 h after treatment. Samples were immediately frozen in liquid nitrogen and stored at -80 °C. After 72 h, the remaining solution was measured in each flask, including a non-plant control, and solution samples were collected and stored at -20 °C. In the glass house, temperature (°C) ranged from 19.2 ± 0.2 to 24.8 ± 0.1 and a daily light integral of 8.0 ± 1.2 (mol m$^{-2} \cdot$ d$^{-1}$) during the acclimation period and 18.7 ± 0.3 to 24.9 ± 0.2 and a daily light integral of 11.1 ± 3.4 during the treatment period.

Water Uptake

Plant water uptake from the stem supplied NO$_3^-$ experiment was determined by measuring the remaining solution in the flask. The remaining solution was normalized for loss to evaporation using the control flask without a plant and subtracting it from the initial volume in the flask (500 mL).

NO$_3^-$ Uptake

NO$_3^-$ concentrations of the remaining solution in the stem supplied NO$_3^-$ experiment were measured spectrophotometrically using an auto-analyzer (Cabrera et al., 2005). The measured NO$_3^-$ concentration were multiplied by the remaining water to get moles of NO$_3^-$ then adjusted
for any nitrogen loss measured in the control flask. This was then subtracted from the initial NO$_3^-$ amount to get NO$_3^-$ uptake.

Quantifying NR Activity

NR activity was quantified using the “in situ” method where the leaf samples were subjected to five freeze-thaw cycles (Maurino et al., 1985; Rhodes and Stewart, 1974). The first cycle was started when the samples where frozen immediately after collection, they were then removed from the -80 °C freezer, and allowed to thaw. The leaf samples were cut into small pieces (3-4 mm) and 300 mg was placed in a 4 mL glass tube. Four more freeze-thaw cycles were applied by freezing the samples in liquid nitrogen (30 s) then thawing them in a 30 °C water bath (2 min). An assay buffer containing 100 mM KH$_2$PO$_4$ (pH 7.5), 2 % 1-propanol, 1 mM EDTA (Ethylenediaminetetraacetic acid), 50 mM KNO$_3$, and 5 mM NADH was added (4 ml g$^{-1}$) and mixed vigorously. An initial time point sample was collected (0.4 mL) from the assay and placed in a new 1.5 mL tube and served to determine the initial nitrite concentration. The remaining buffer with the tissue was vacuum-infiltrated for 5 min in the dark. The samples were allowed to react for 1 h in a shaking incubator at 30 °C in the dark. To extract only the assay and stop the reaction, samples were placed in a 5 mL column and vacuum filtered and 0.4 mL of the filtered extraction was placed in a new 1.5 mL tube as a final sample.

As a step to improve color development, two additional reactions were used to remove potential color inhibitory compounds (Scholl et al., 1974). First a 5 min reaction with 40 µL of 0.5 M Zn-acetate was used to form a precipitate that was settled out with centrifugation for 1 min at 20,000g. To oxidize the remaining NADH, the following step involved the addition of 20 µL of 1 mM PMS. Samples were placed in the dark for 10 min to react then centrifuged for 10 min at 20,000g. An Azo colorimetry method using a spectrophotometer (Genesys 2,
Spectronic, Pittford, NY, USA) was used to measure the nitrite concentrations to determine the amount of NO$_3^-$ reduced.

Gene Expression Analyses

Using duplicate samples collected from time points 0 and 48 h after treatments, RNA was extracted, purified, and quantified using methods described previously in Vashisth et al. (2011). The RNA was further treated with DNase to remove any DNA contamination and reverse transcribed to generate cDNA. To obtain the NO$_3^-$ REDUCTASE and NITRITE REDUCTASE genes from blueberry, the blueberry genome sequence was queried on the Integrated Genome Browser (www.affymetrix.com, September 2015) and the gene sequences were compared to the Arabidopsis nitrate metabolism related genes. Two putative NR (CUF61184 [NR1] and CUF8338 [NR2]) genes and one NiR gene were identified through this analysis. The sequence information for the two NR genes was found not to be complete with NR1 primarily containing the C terminus and NR2 containing primarily the N terminal portions of the predicted proteins. NR1 displayed 78% identity with the Arabidopsis NR1 while NR2 displayed 87% identity with the Arabidopsis NR2.

Expression of the nitrate metabolism-related genes was studied using quantitative RT-PCR as described previously in Vashisth et al. (2011). Briefly, a 12 µL reaction containing cDNA, primers and 6 µL of the master mix (Veriquest SYBR green Master Mix, Affymetrix, Santa Clara, CA) was used. The thermal cycling conditions were: 95 °C (10 min), and 40 cycles of 94 °C (30 s) followed by 60 °C (1 min). Melt-curve analysis was performed at the end of the thermal cycling. Control reactions included reactions without a template and without the reverse transcriptase. The primer efficiencies (E) were determined using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). Relative quantity (RQ) for each sample was calculated as: $1/E^{C_q}$,
where Cq is the quantification cycle (Rieu and Powers, 2009). The expression data were normalized to the geometric mean of the expression of two reference genes, *RNA HELICASE-LIKE 8 (RH8)* and *CLATHRIN ADAPTOR COMPLEXES MEDIUM SUBUNIT FAMILY PROTEIN A (CACSa)* which were previously demonstrated to be stable reference genes in blueberry (Vashisth et al., 2011). The normalized data were log2 transformed prior to statistical analysis.

Experimental Design

Two separate experiments were conducted at separate times: 1. Foliar Applied NO₃⁻ and 2. Stem Supplied NO₃⁻. Each plant was treated as an experimental unit and placed into a completely randomized 2 cultivar x 2 N treatment factorial design. Each experiment had six replicates. The species treatment included the levels *V. corymbosum* and *V. ashei* while the N treatment included the levels 0 mM (water) and 5 mM KNO₃. The experiment was conducted in a temperature controlled glass house with blocks being designated in a hydroponic setup on a workbench. The foliar application was conducted first, then the stem supplied NO₃⁻ experiment used the same setup after the foliar experiment had finished. Data analysis was done on NR activity using analysis of variance with Log transformed data and a general linear model of Log2 transformed data for relative gene expression. A t-test was used to determine treatment significance in cultivar × treatment interactions.

**Results**

**Foliar Applied NO₃⁻ Experiment**

Leaf NR activity in the foliar N application study was initially at a low basal rate of around 20 nmoles g FW⁻¹ h⁻¹ (Figure 3.1). At 24 h after application, NR activity in the water
controls increased slightly to around 50 nmoles g\(^{-1}\) (FW) h\(^{-1}\) while the NO\(_3^-\) treatment increased NR activity significantly higher than that in the controls to 74 and 135 nmoles g\(^{-1}\) (FW) h\(^{-1}\) in *V. ashei* and *V. corymbosum*, respectively (*P* = 0.005). This resulted in a 2.7-fold and 1.7–fold increase over the controls. NR activity declined in the NO\(_3^-\) treatments by 48 h and by 72 h all the treatments displayed similar NR activity of around 40 nmoles g\(^{-1}\) (FW) h\(^{-1}\). There was no species effect or an interaction between species and treatments.

**Water and NO\(_3^-\) Uptake (Stem Supplied NO\(_3^-\) Experiment)**

Water uptake through the cut stem was consistent for all species and treatments, averaging 0.165 L d\(^{-1}\) (Figure 3.2).

The *V. corymbosum* species took up 0.259 mmoles of NO\(_3^-\) from the solution while the *V. ashei* species took up 0.186 mmoles over the duration of the experiment (Figure 3.3). There were no significant differences.

**NR Activity in leaves within the Stem Supplied NO\(_3^-\) Experiment**

Leaf NR activity in *V. corymbosum* and *V. ashei* were similar in the control plants at the initial time point and measured only a low basal rate, averaging between 6.8 to 11.5 nmoles g FW\(^{-1}\) h\(^{-1}\) (Figure 3.4). The NR activity of leaves from the NO\(_3^-\) treatment initially displayed low basal rates but increased (not significant) to around 25 nmoles g FW\(^{-1}\) h\(^{-1}\) by 24 h after treatment. By 48 h after treatment, NR activity in the NO\(_3^-\) treatment had increased, *V. ashei* displaying a 4.4–fold increase while *V. corymbosum* displayed a 3.4-fold increase over the controls (*P* = 0.002). All NR values decreased by 72 h with *V. corymbosum* dropping to basal rates while *V. ashei* remained slightly higher resulting in a significant species effect (*P* = 0.034). There was no interaction between the species and the treatment.
Expression of Nitrate Metabolism Related Genes in the Stem Supplied NO₃⁻ Study

The transcript levels of NR1 were significantly higher at 48 h after 5 mM NO₃⁻ was supplied to the cut stem. The increase in expression of this gene was by 10-fold and 9.3-fold in *V. ashei* and *V. corymbosum* respectively, in comparison to that in the controls (Figure 3.5; *P* < 0.0001).

At 48 h after treatment, the 5 mM NO₃⁻ supply significantly increased NR2 transcript accumulation expression in both *V. ashei* and *V. corymbosum* (*P* < 0.0001). The induction in *V. ashei* (7-fold) trended slightly greater than that in *V. corymbosum* (5.7-fold), Figure 3.6).

Relative expression of the NiR1 gene at time point 48 h yielded a significant interaction between species and treatment (*P* < 0.0001; Figure 3.7). When both species were supplied with NO₃⁻, NiR transcript accumulation was greater in *V. ashei* compared to ‘Sweetcrisp’ by 42-fold and 19-fold (*P* = 0.0004 and 0.0022), respectively. When both species were supplied with NH₄⁺, there was no effect on NiR1 transcript accumulation.

**Discussion**

Foliar application of 5 mM NO₃⁻ was adequate to significantly induce NR activity in the leaves in both *Vaccinium* species by 24 h after application. The induction was measured by 24 h after treatment, then returned to basal rates by 48 h after application. Since it was only a single application of NO₃⁻ the leaves may have assimilated much of the NO₃⁻ that was taken in or may have stored the remaining NO₃⁻. In any case, these data indicate that NR activity in blueberry leaves may be inducible by direct supply of NO₃⁻ to the shoots. Although significant, the extent of the NR activity achieved was still low compared to that reported in other plants, 800 nmoles g
FW⁻¹ h⁻¹ in strawberry, 990 in raspberry, and 12,500 in maize (Clausen and Lenz, 1999; Huber et al., 1994).

Supplying a NO₃⁻ treatment to a cut stem provided a continuous supply of NO₃⁻ to the leaves. The consistency of the water uptake (Figure 3.2) and NO₃⁻ uptake (Figure 3.3) across both species and N treatments confirms that the stems were transpiring. Supplying 5 mM NO₃⁻ to cut stems appeared to increase NR activity by 24 h after treatment but resulted in a significant induction measured only at 48 h after treatment (Figure 3.4). The rates of NR activity reported here under NO₃⁻ supply were similar to those previously reported by Wang and Korcak (1996) in a similar study with a rabbityeye and Northern highbush species.

There appears to be a down regulation of NR activity in the stem supplied NO₃⁻ treatment at 72 h similar to the down regulation seen in root NR activity in Chapter 2. A similar trend was reported in the roots of *V. corymbosum* cv. ‘Misty’ while comparing it to *V. arboretum* which is known to assimilate higher rates of NO₃⁻ (Poonnachit and Darnell, 2004). NR activity in ‘Misty’ was rapidly induced 3 weeks after treatment then declined back to basal rates at 9 weeks after treatment even though there was a continual supply of NO₃⁻. However, NR activity in *V. arboretum* was induced and remained high during the same period. This trend over different species and cultivars at different environmental conditions suggests that NR activity is being downregulated through the feedback inhibition of excess glutamine and glutamate.

The stem supplied NO₃⁻ treatment induced the expression of both nitrate reductase genes identified in this study, *NR1* and *NR2* as well as the nitrite reductase gene, *NiR1*. These data clearly demonstrate that the expression of nitrate assimilation genes in the shoots is inducible by the direct supply of NO₃⁻ to these tissues. The magnitude of increased transcript accumulation of the *NR* genes, 9- 10-fold for *NR1* and 4- 7-fold for *NR2*, was relatively higher than the increase
noted in NR activity (3.4 – 4.4 fold increase). The extent of gene expression is commonly higher than protein level or activity. In tobacco leaves (*Nicotiana glauca*) supplied with NO$_3^-$, changes in NR expression averaged 10% higher than changes in NR activity (Vincentz et al., 1993). In maize, gene expression is higher than NR activity for the majority of the photoperiod, but as light intensity decreases, gene expression decreases prior to NR activity (Huber et al., 1994). The increase in NR gene expression and an associated increase in NR activity suggest that nitrate assimilation is at least in part under transcriptional regulation. However, the lower magnitudes of increase in NR activity in comparison the transcript accumulation suggest that post-translational mechanisms are also important in regulating nitrate assimilation in the leaves. In fact, NR is known to be regulated, extensively at the post-translational level (Kaiser and Huber, 2001).

The induction of *NiR1* expression under the NO$_3^-$ treatment illustrates that *NiR1* transcript accumulation in blueberry is regulated by the NO3- concentration in the cell. These data together with the regulation of *NR* expression suggest that NO$_3^-$ sensing and signal transduction mechanisms leading to changes in gene expression (Castaings et al., 2011) are operational within blueberry leaves. NO$_3^-$ is known to be a signal molecule in plants that can lead to changes in the expression of multiple genes associated with NO$_3^-$ transport and metabolism, as well as a multitude of genes associated with other metabolic processes (Castaings et al., 2011). In arabidopsis, NRT1.1 functions as a dual affinity NO$_3^-$ transporter as well as a NO$_3^-$ receptor involved in NO$_3^-$ sensing (transceptor; Ho et al., 2009). It may be speculated that such NO$_3^-$ transceptor mediated NO$_3^-$ sensing and downstream signaling leads to the increased expression of NO$_3^-$ metabolism-related genes in blueberry. The large increase in *NiR1* expression suggests that it is not as tightly regulated as *NR* genes by NO3-; consistent with the idea that NR regulation is the rate limiting aspect of NO$_3^-$ assimilation. The regulation of nitrite reductase gene expression...
in tobacco leaves and Scott Pine whorls has been shown to be predominantly controlled by light and was reported to be less sensitive to large changes in NO3- concentrations (Nolminger et al, 1994; Vincentz et al., 1993). The generally higher NR and NiR1 gene expression in *V. ashei* is consistent with a higher NR activity. It may be speculated that these trends in nitrate assimilation may potentially contribute to the ability of *V. ashei* and other rabbiteye blueberries to better adapt to upland soils.

In conclusion, it is demonstrated in this study that blueberry shoots are capable of assimilating NO3- when supplied directly. NO3- is capable of inducing nitrate metabolism-related gene expression as well as NR activity, while other regulatory mechanisms may still limit the capacity to assimilate large amounts of NO3-. Although we supplied NO3- to the shoots, we do not know how much was actually taken in because leaves still need to unload NO3- from the xylem. Blueberries may be capable of higher rates of nitrate assimilation if larger amounts of NO3- are present within the cell. It is suggested that nitrate transport mechanisms are a major limitation to nitrate assimilation in blueberry.
Figure 3.1. Effect of foliar application of NO$_3^-$ on leaf nitrate reductase (NR) activity in two blueberry species. Water or NO$_3^-$ (5 mM) was applied and the leaf NR activity was monitored at regular intervals. Data are means ± SE (n = 6). Asterisk indicates significant difference between the water and NO3- application treatments (α = 0.05).
Figure 3.2. Total water uptake through the cut end of the stem by two blueberry species. The mean water uptake ± SE (n = 6) by plants through the cut end of the stem after immersion into either water or a 5 mM solution of KNO₃ are shown.
Figure 3.3. Nitrate uptake through a cut stem end in two blueberry species. Stems were cut and the cut end was immersed in a 5 mM KNO₃ solution. The NO₃⁻ remaining in the solution was determined at the end of the experiment (72 h after treatment) and the amount of NO₃⁻ absorbed per day was determined. Data are means ± SE (n = 6).
Figure 3.4. Leaf nitrate reductase activity (NR) in two blueberry species in response to NO$_3^-$ supplied through the cut end of the stem. Stems were cut and immersed in either water or 5 mM KNO$_3$ for 72 h. Data are means ± SE (n = 6). Asterisk indicates significant difference between the water and NO$_3^-$ treatments (α = 0.05).
Figure 3.5. Expression of a putative blueberry nitrate reductase gene, *NR1*, in response to NO$_3^-$ supplied from the cut stem end in two blueberry species. Stems were cut and immersed in either water or 5 mM KNO$_3$. Expression of *NR1* was determined at 0 and 48 h after treatment. Data are means + SE (n ≥ 3). Asterisk indicates significant difference between the treatments (α = 0.05).
Figure 3.6. Expression of a putative blueberry nitrate reductase gene, NR2, in response to NO$_3^-$ supplied from the cut stem end in two blueberry species. Stems were cut and immersed in either water or 5 mM KNO$_3$. Expression of NR2 was determined at 0 and 48 h after treatment. Data are means + SE (n ≥ 3). Asterisk indicates significant difference between the treatments (α = 0.05).
Figure 3.7. Expression of a putative blueberry nitrite reductase gene, \textit{NiR}\textsubscript{1}, in response to NO\textsubscript{3}\textsuperscript{-} supplied from the cut stem end in two blueberry species. Stems were cut and immersed in either water or 5 mM KNO\textsubscript{3}. Expression of \textit{NiR}\textsubscript{1} was determined at 0 and 48 h after treatment. Data are means ± SE (n ≥ 3). Asterisk indicates significant difference between the treatments within each species (α = 0.05).
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Chapter 4

CONCLUSIONS AND FUTURE RESEARCH

The aim of this research was to investigate the assimilation and transport to the shoots of root supplied NO$_3^-$, measure nitrate reductase (NR) activity from previously frozen blueberry root and leaf samples, and induce NR activity and NR related gene expression in the shoots from exogenous NO$_3^-$. Three separate experiments were performed:

Supplying 5 mM NO$_3^-$ to the roots of two blueberry species *V. ashei* and *V. corymbosum* induced low NR activity, agreeing with previous literature and confirming the efficiency of the ‘in situ’ NR assay developed here. No additional NO$_3^-$ was transported in the xylem sap, and therefore supplying NO$_3^-$ to the roots did not induce leaf NR activity. Estimates of potential root NR activity suggest that with low NO$_3^-$ uptake rates, the roots are capable of assimilating the majority of the NO$_3^-$, therefore no additional NO$_3^-$ was partitioned to the shoots. Further research should examine gene expression levels of nitrate transporters genes and nitrate metabolism-related genes that may provide more information about low NO$_3^-$ uptake and NR activity. Also, performing additional studies with short-term nitrogen starvation prior to NO$_3^-$ re-supply may provide additional information of the general basal ability of blueberry plants to assimilate NO$_3^-$. It is hypothesized that supplying NO$_3^-$ to nitrogen-starved plants may induce higher uptake rates, and subsequently higher NR activity in the roots and shoots.

Foliar applied NO$_3^-$ induced NR activity in the leaves (3 to 4-fold), confirming that blueberry shoots are capable of assimilating NO$_3^-$ when supplied directly. Continued research will investigate the NR metabolism-related gene expression in the foliar application study and
measure changes in leaf nitrogen concentrations to determine how much NO$_3^-$ was actually taken in to induce NR activity. Efficient uptake and assimilation of foliar applied NO$_3^-$ may provide growers an additional method of amending plant N status.

Supplying NO$_3^-$ to a cut stem also induced NR activity in the shoots. Nitrate metabolism-related gene expression was also induced in this study. The correlation between low NR activity induction (3 to 4-fold) and high induction of gene expression (7 to 10-fold) suggest that the NR activity maybe be limited at least in part by post translational regulation. Future research may investigate the translation efficiency of induced NR mRNA to better identify additional limitations in NR activity. Inducing NR activity in a more controlled environment while supplying adequate carbon and NO$_3^-$ directly to the cell may results in higher rates of NR activity.