Progress Report

Title: Determining uptake of nitrate *vs* ammonium nitrogen in blueberry

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Introduction:

Blueberry (*Vaccinium* species) is a major fruit crop in the southeastern United States. In Georgia, it is cultivated in over 30,000 acres and has a farm gate value of over $225 million (2017 Georgia Farm Gate Value Report). Multiple aspects of blueberry plant nutrition are characteristic of these and others in the Ericaceae family. They perform better in soils with lower pH and prefer soils with relatively lower calcium available concentrations. They are better adapted to acidic soils where specific forms of nitrogen (N) such as ammonium (NH_4^+) are more available. It has often been reported that blueberries display a preference for the NH_4^+ form of N than the alternative, NO_3^- form (Claussen and Lenz, 1999; Poonnachit and Darnell, 2004; Alt et al., 2017).

Nitrogen is a macronutrient that makes up ~ 2% of the leaf dry weight in blueberry (Korcak, 1988). The two major inorganic forms in which N is available are: NO_3^- and NH_4^+. These forms are acquired by the root system and are then assimilated, translocated or stored within the plant. Most plants display a preference for the NO_3^- form of N, while a few plants such as blueberry and rice display potential preference for the NH_4^+ form of N. The form of N acquired has consequences for plant metabolism, growth and overall performance. Following its acquisition, NO_3^- needs to be reduced to NH_4^+ prior to assimilation. This is a 2-step process involving nitrate reductase (NR) and nitrite reductase (NiR) and involves intensive energy and reducing equivalent consumption thereby needing considerable carbon supply. The NH_4^+ form of N can be assimilated by glutamine synthetase with glutamate as a substrate to generate the organic N molecule, glutamine. In spite of additional energy requirements for NO_3^- acquisition and assimilation, it is often the preferred N source in plants owing to its ability to be effectively translocated and stored until needed. On the other hand, NH_4^+ is often toxic at high concentrations owing in part to its ability to dissipate pH gradients across cell membranes and is

therefore assimilated relatively rapidly after acquisition (Britto and Kronzucker, 2013). Hence, it does not accumulate and is not extensively translocated in the xylem (Britto et al., 2001). However, some plants such as those in the Ericaceae family are thought to be adapted to soils with an acidic pH and where NH_4^+ is the predominant form of available inorganic N. It has therefore been reported that blueberry plants within the Ericaceae family have better NH_4^+ acquisition characteristics. Further, it has been reported that blueberry plants display reduced or inhibited growth in the presence of NO_3^- (Townsend, 1969; Claussen and Lenz, 1999).

Preference for a specific form of N-source may be due to differences in the capacities for the acquisition, translocation, or assimilation of the N form. Previous research in the PI's lab suggested that acquisiton of N may be an important factor determining N-source preference. Interestingly, NH_4^+ and NO_3^- uptake rates using 2.5 mM ammonium sulfate and 5 mM KNO_3, respectively, were similar in rabbiteye and southern highbush blueberry plants (Alt et al., 2017). Often, high concentrations of N (~1-5 mM) have typically been used in several previous studies that have attempted to determine N-source preference using uptake studies (Poonnachit and Darnell, 2004). Plants utilize different systems for NO_3^- and NH_4^+ uptake based on their individual concentration ranges. At lower available NO_3^- concentrations ranging from 0 to around 0.1 mM, plants typically use the high affinity transport system (HATS), which is saturable (Aslam et al., 1992; Glass and Siddiqui, 1995). At concentrations higher than 0.1 mM and reaching up to almost 40 mM, plants use the low affinity transport system (LATS) which is constitutive and generally linear (Min et al., 2001). Similarly, NH_4^+ uptake at low concentration up to 0.1 mM is facilitated by the HATS and is saturable while at higher concentrations, the linear LATS is functional (Min et al., 2001). It is hypothesized that similar patterns of N uptake are present in blueberry and that differences in uptake kinetics between NO_3^- and NH_4^+ influence N-source preference in blueberry. Hence, in this study we sought to determine the uptake kinetics of blueberry at a range of low and high N concentrations.

Material and Methods:

Plant Material and Experimental Set-up

The southern highbush cultivar, Suziblue, was used in this study. Young (two-year old) plant material was utilized here. Plants were maintained in 1.6 L pots with 100 ppm N-fertilization using 20-20-20 (Peter's Professional Water-Soluble Fertilizer). The media consisted to 1:1 Fafard's 3B potting mix and pat moss. Plants were extracted from existing media and roots were thoroughly washed. The extracted plants were placed into hydroponics containers with the nutrient solution. A hydroponics system previously developed in the PI's lab through funding from the SRSFC was used to determine the N-uptake characteristics. The system was made of 4" tubing sealed on both ends with end-caps. Slits were made into the top end-cap to allow insertion of the plant. The cylinder held up to 1 L of nutrient solution during the experiments. Nutrients were provided to the plants through a modified Hoagland's solution as described previously (Poonnachit and Darnell, 2004; Alt et al., 2017). The pH of the nutrient solution was regularly monitored and maintained close to 5.0 to allow for the acidic pH preferred by blueberry.

Regulated air flow was provided to each container to aerate the nutrient solution. Prior to beginning the experiment, plants were provided with a low amount of N (0.25 mM NH_4NO_3) for around 3 d to allow for acclimatization to the hydroponics system. Prior to the initiation of the treatments, all plants were placed in the same nutrient solution without N for at least 2 d to deplete potential N reserves. Two ranges of N concentrations were tested by altering the concentration of KNO_3 for NO_3^- and $(NH_4)_2SO_4$ for NH_4^+ in the hydroponics solution. The two ranges, low range and high range, were used to determine the uptake characteristics for HATS and LATS, respectively. The experiments were conducted separately for NO_3^- and NH_4^+. For the NO_3^- experiment, the following eight concentrations of KNO_3 were evaluated to determine N uptake characteristics under the HATS: 0, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 mM. For the high range treatments to determine LATS kinetics, the KNO_3 concentration range in the hydroponics solution were: 0.5, 1, 5, 10, 25, 50 and 100 mM. Similarly, for the NH_4^+ experiment, the low range involved $(NH_4)_2SO_4$ concentrations of: 0, 0.01, 0.025, 0.05, 0.1, 0.25 mM, and 0.5 mM for HATS and the high range concentrations were: 0.5, 1, 5, 10, 25, 50 and 100 mM. Apart from changes associated with the N-source, there were no other alterations to the nutrient solution. A randomized complete block design was used as the experimental set up. Four replicates were used for each N concentration.

Sample collection

Samples from the nutrient solution were collected at 0 h, 6 h, 24 h and 48 h after the initiation of the N-source treatment. This timing was based on previous experiments which indicated complete N removal by about 3 d after treatment initiation. Sample collection involved the removal of 15 mL of the nutrient solution into storage tubes and storage at -20 C until further analysis. At each sampling, the amount of nutrient solution left in the hydroponics cylinder was measured. This information was used to determine the content of N left in the solution. At the end of the experiment, the plants were removed from the hydroponics systems and the roots were washed gently and blotted dry. Root fresh weight and dry weight measurements were recorded subsequently. Root dry weight was obtained after oven drying.

Measurements of nitrate and ammonium

Nitrogen uptake was measured by determining the amount of N left in the nutrient solution at each sampling time and comparing it to that at the initiation (known concentration and volume). Nitrate measurements were performed using a spectrophotometry-based assay as per Doane and Horwath (2003), which uses Vanadium (III) to reduce NO_3^- to NO_2^- which subsequently reacts with Griess's reagents. In the current method, the reagents were combined prior to assay to generate a working reagent. Two ratios of sample to working reagent were employed to accurately determine NO_3^- across a wide range of concentrations. Samples with NO_3^- concentration up to 50 µM were quantified using a ratio of 400:500 (sample to reagent). Concentrations from 50 to 500 µM were quantified using a ratio of 45:1000 (sample to reagent). Higher concentration samples were diluted with water prior to analysis. Samples were incubated

with the reagent for 20 h at rom temperature. Subsequently, samples were moved to a microplate and the absorbance was measured at 540 nm using a BioTek plate reader. Quantification was performed using standard curves generated for NO_3^- at each ratio. Standard curves were generated parallel to each assay.

Ammonium measurement was performed spectrophotometrically using a modification of the protocol described in Holmes et al., (1999) which is based on a fluorometric assay using OPA (orthopthalaldehyde). The working reagent consisted of borate buffer (21 mM), sodium sulfite (0.063 mM) and OPA (50 mL L^{-1} in ethanol). Samples (1 mL) were thawed for 2 d, added to the working reagent (4 mL), and incubated at room temperature for 2 h. Sample aliquots were transferred to a microplate and the absorbance at 415 nm was read on a BioTek plate reader. Quantification was performed using standard curves generated in parallel to the assays.

Results and Discussion

Analyses of NO_3^- uptake kinetics within the low range of N supply indicated HATS mediated transport (Fig. 1). Data from 0 to 250 µM of NO_3^- supply was used for these analyses as inclusion of the 500 µM data appeared to result in substantial deviation from Michealis-Menten kinetics suggesting initiation of LATS within this range. These data indicate that under low NO_3^- supply, transporters associated with nitrate uptake display high affinity and saturable transport characteristics.

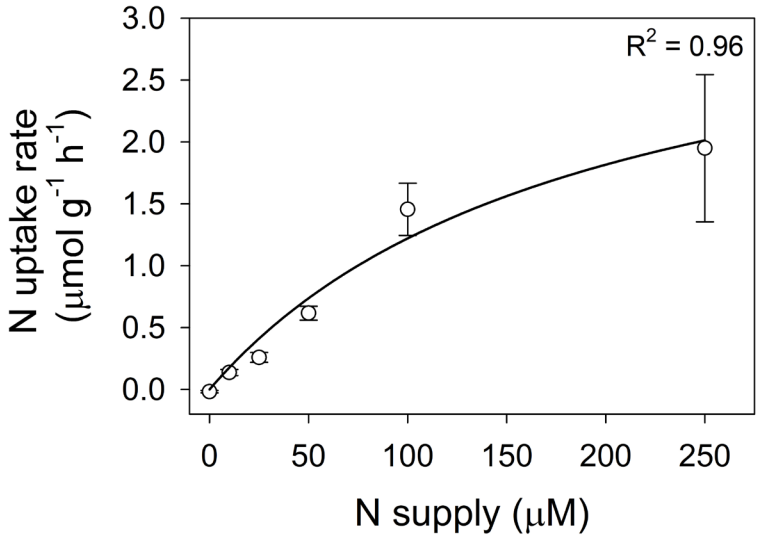

Fig. 1. Nitrate uptake kinetics in 'Suziblue' southern highbush blueberry. Michealis-Menten curve was fit to the data. Data were obtained from samples collected at 24 h after nitrogen supply in the form of KNO_3 at the indicated concentration (n = 4).

Ammonium uptake kinetics did not display saturable high affinity transport kinetics noted with nitrate uptake. The data suggested a strong linear relationship indicating that the extent of N uptake increased linearly with increasing NH_4^+ availability ($R^2 = 0.97$; Fig. 2). These data suggest that the mechanism of uptake associated with acquisition of NH_4^+ under low concentrations is not saturable, at least up to 500 μM.

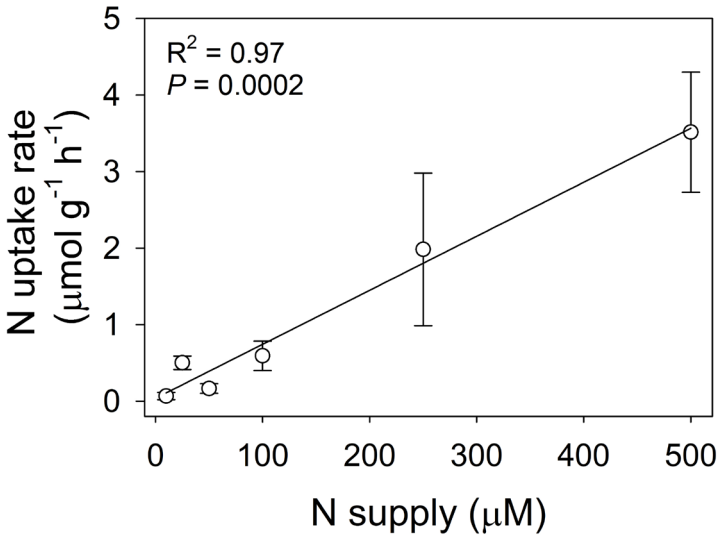

Fig. 2. Ammonium uptake kinetics under low external supply (up to 500 μM). Data were collected from samples obtained at 24 h after initiation of N supply at the indicated concentration (n = 4). For the 10 μM concentration, data from one replicate was excluded from the analysis as it indicated negative N uptake values. Although, 0 μM treatment was performed, NH_4^+ measurements were not performed with these samples.

Nitrate uptake kinetics were also determined at a higher range on N supply. In this experiment, the data for N uptake kinetics were found to be highly variable and indicated several errors, particularly at 0.5 mM and 1 mM concentrations. At these two concentrations, 1 (0.5 mM) to 3 (1 mM) replicates indicated negative N uptake values indicating either errors in experimental set-up or measurement. Those data were excluded from the analyses presented below (Fig. 3). A quadratic fit was performed with data from the high NO_3^- supply study. These data indicate that N uptake increased with increasing NO_3^- supply and that N uptake began to decline at higher NO_3^- concentrations. These data suggest that the transport system operational at higher NO_3^- supply is also saturable, particularly at extremely high N availability.

Ammonium uptake kinetics were determined at high N supply in the form of NH_4^+. In this experiment, one replicate under 0.5 mM and all four replicates under 1 mM N supply indicated erroneous negative uptake values. Hence, these samples were excluded from data analyses. No significant relationships were obtained between N supply and N acquisition in the form of NH_4^+ at high levels of N supply (Fig. 4).

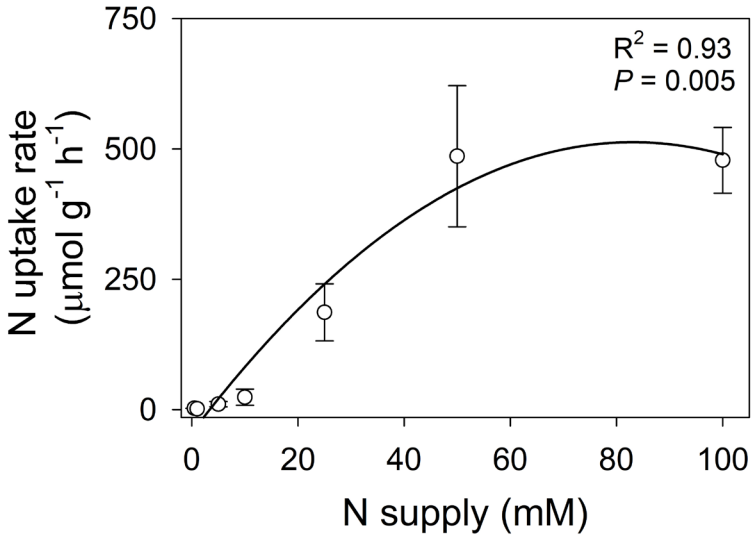

Fig. 3. Nitrate uptake in response to high N supply. Data were collected from samples obtained at 24 h after N supply at indicated concentrations (n = 4). One replicate at 0.5 mM and three replicates at 1 mM nitrate supply were excluded from the above analyses as the data indicated negative N uptake values. A quadratic relationship was observed between N supply and the N uptake rate.

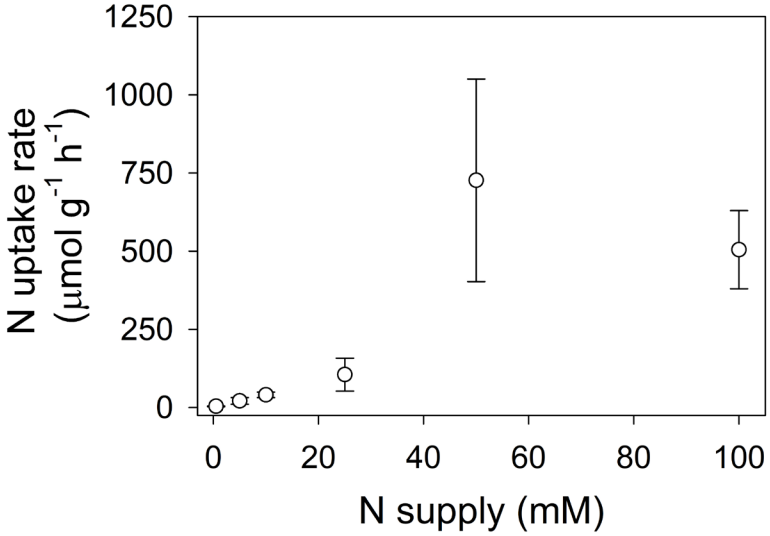

Fig. 4. Ammonium uptake in response to high N supply. Data are from samples obtained at 24 h after N supply at indicated concentrations (n = 4). One replicate at 0.5 mM and all four replicates replicates at 1 mM nitrate supply were excluded from the above analyses as the data indicated negative N uptake values. No significant relationship was observed between N supply and the N uptake rate in the form of NH_4^+.

Preliminary conclusions

Data from these studies indicate a saturable high affinity transport system associated with NO_3^- uptake in southern highbush blueberry. However, NH_4^+ uptake under similar N levels suggested a linear uptake mechanism. At higher N supply, NO_3^- uptake appeared to display saturable kinetics while no significant relationship could be established with that of NH_4^+. It should be noted that the experiment and the measurements were prone to substantial error in the high N supply studies. The sources of such error are currently unclear. Alternative approaches such as the use of labeled N sources and shorter duration studies may reduce the extent of such errors. Future efforts will involve the use of labeled N approaches to determine N uptake kinetics in blueberry.

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