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Effects of different foliar nitrogen fertilizers on cellular nitrogen metabolism and biomass of two shrub willow cultivars

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Abstract

We examined the effects of foliar supplementation of different nitrogen sources (urea, Nitamin®, NH₄NO₃ and arginine) to study their efficacy as fertilizers for growth of two clonally propagated shrub willow cultivars; namely, ‘Fish Creek’ (Salix purpurea) and ‘Preble’ (Salix viminalis x (S. sachalinensis x S. miyabeana)). Our objectives were to determine if: (1) foliar nitrogen application is an effective method of fertilization for the two shrub willows; and (2) different nitrogen sources are metabolized similarly by the plants. The analyses involved soluble leaf polyamines, amino acids, total protein, total nitrogen and carbon, and plant biomass in response to short-term treatments with four sources of nitrogen. The effects of foliar nitrogen application on leaf chemistry, biomass, and foliar nitrogen content varied according to the form of nitrogen used. The data indicate that: (1) urea is the most suitable nitrogen source for foliar spray (29% higher N accumulation vs. NH₄NO₃), whereas arginine is the least suitable; and (2) different nitrogen sources are metabolized differently by the plant. While the foliar nitrogen application method could become a practical and sustainable way to fertilize shrub willows and other short-rotation biofuel crops, it may also help reduce nitrogen loss to the environment.

Key words: Amino acids; Foliar application; Nitrogen assimilation; Polyamines; Shrub willows; Urea

Abbreviations: AA, amino acid; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; DW, dry weight; FW, fresh weight; PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine
Introduction

Global consumption of synthetic nitrogen (N) fertilizer increased almost 10-fold in 2015 vs. the early 1960’s (FAO data, http://www.fao.org/faostat/en/#data/GY). Although the norm, soil application of N fertilizer can be inefficient and a wasteful practice, especially if the fertilizer is not amended (slow/extended release, or enhanced with surfactants, humectants, etc.). On average, less than 50% of the applied N fertilizer is utilized by the intended crop (Raun and Johnson 1999; Kant et al. 2011; Witte 2011); the remainder is lost to the environment in groundwater, surface water by leaching, and to the atmosphere by microbial activity. Today, the estimated global loss of N through cropland leaching and runoff alone is about one-third of the total N used in agriculture as fertilizer and manure (Mekonnen and Hoekstra 2015).

Nitrate (NO$_3^-$) is one of the most common forms of N fertilizer but is also the form that is most easily lost to groundwater (Rekha et al. 2011; Syswerda et al. 2012; Eltarabily et al. 2017). Nitrate in drinking water has been steadily increasing in many regions of the world and agricultural fields have been cited as the major point source of this contamination (Tandia et al. 2000; Angelopoulos et al. 2009; Re et al. 2011). In aquatic ecosystems, NO$_3^-$ from surface runoff has been associated with eutrophication, hypoxia and depletion of animal life (Galloway et al. 2003; Diaz and Rosenberg 2008). Furthermore, N from unused fertilizer can also be released into the atmosphere via microbial interaction contributing to N$_2$O emissions and global warming (Galloway et al. 1995; Liu and Greaver 2009 and references therein). Due to the increase in N fertilizer usage to meet agricultural demands, N$_2$O emissions are predicted to rise exponentially (Shcherbak et al. 2014).

The majority of N fertilizer is applied to soil, but it has been suggested that foliar application could partially alleviate the risk of nutrient leaching as well as increase the efficiency
of N assimilation (Fernández and Brown 2013). Currently, foliar-application of fertilizer is commonly used to treat nutrient deficiencies (Hannam et al. 1984; Saxena et al. 1990; Shorrocks 1997; Kaya and Higgs 2002; Del Amor et al. 2009), to fertilize turf grass (Stiegler et al. 2010; Henning et al. 2013) or in conjunction with soil application to reduce annual use while not compromising yield (Akhtar et al. 2014). There are numerous reports on foliar nutrient applications to field-grown plants, whereas controlled greenhouse studies on foliar use of N fertilizer are rather limited (Fernández and Brown 2013; Voogt et al. 2013). Foliar application is efficient, and it can reduce the lag time between application and uptake by the plant. It was reported for turf grass that ~50% of N absorption occurred in as little as 1 h following application (Stiegler et al. 2011), although absorption time can vary greatly depending on species and the chemical properties of the fertilizer (Bowman and Paul 1992; Stiegler et al. 2010; Stiegler et al. 2011). To date, there have been a few studies showing the ability of non-agricultural shrubs and trees to absorb and retain N applied to the foliage (Tomaszewski et al. 2003; Gaige et al. 2007; Tomaszewski and Sievering 2007; Adriaenssens et al. 2012; Chiwa et al. 2012; Voogt et al. 2013).

Among the various forms of N used as fertilizer, urea (NH₂CONH₂), and its various formulations, are among the most common source of N used for foliar applications. Urea is an uncharged molecule with high solubility and a lower point of deliquescence (ability to dissolve in the moisture absorbed from the atmosphere) than some other N fertilizers [e.g. (NH₄)₂SO₄, KNO₃ or NH₄NO₃ - (Adams and Merz. 1929)]. For this reason, urea is absorbed by the foliage more rapidly than either NO₃⁻ or NH₄⁺ (Wittwer et al. 1963). Its cellular uptake involves passive transport channels as well as high-affinity active urea transporters in the cells (Witte 2011). In recent years, specially formulated forms of urea-containing fertilizers have become available,

Amino acids (AAs) have also been recognized as a suitable form of N fertilizer for crops (Xiaochuang et al. 2013; Hongjun et al. 2018). The energy used in assimilation of N from AAs is much lower as compared to the energy required for other forms of N. This is because AAs can be directly utilized without requiring additional steps such as those required for NO₃⁻ or NH₄⁺ assimilation into glutamic acid (Glu) (Wilson et al. 2013), which is the precursor for other AAs and polyamines (PAs) (Majumdar et al. 2016). In radish plants, Liu et al. (2008) demonstrated that foliar application of a ~5% solution of AAs at 15 and 22 days after sowing increased N content and biomass of the plant by day 35. Shafeek et al. (2012) further showed that foliar-applied arginine (Arg) and glutamine (Gln) fertilizer could be used to increase the total yield and bulb weight of onion. Earlier, Ohlund and Näsholm (2002) had observed that Scots pine seedlings fed with Arg had higher needle N content and higher N recoveries than those fed with NH₄NO₃. More recently, combinations of AAs or PAs have also been used to supplement root N fertilization for improvement of seed yield in Timothy grass (Radkowski and Radkowska 2018; Mahgoub et al. 2011). Development of efficient Arg production technology by fermentation has made Arg a cost effective and commercially feasible fertilizer (Pandey et al. 2012).

The major pathways for N assimilation from root have been well characterized, but much less is known about the metabolic pathways in plants following the direct foliar application of N (Fernández and Brown 2013). Overall, most of the N used as fertilizer follows a similar
assimilation pathway; mostly through Glu/Gln or aspartate/asparagine (Asp/Asn) as initial metabolites. The presence of metabolic enzymes for early assimilation of N into Glu and Gln has been known in several plants, including woody species (Cánovas et al. 2018). In addition to free AAs, PAs (putrescine -Put, spermidine – Spd, and spermine – Spm) also store and metabolize significant amounts of N due to their high N: C ratios and relatively high cellular concentrations. Polyamines play a multitude of physiological roles within plants, but their ability to rapidly utilize excess N, particularly under high N conditions, makes them reliable indicators of N uptake and assimilation (Minocha et al. 2000; Velikova et al. 2000; Hussain et al. 2011; Fariduddin et al. 2013; Minocha et al. 2014; Minocha et al. 2015; Handa et al. 2018).

The goal of the present study was to determine if foliar application of N in various formulations could potentially serve as a major source of N for two clonally propagated cultivars of shrub willows – ‘Fish Creek’ (Salix purpurea) and ‘Preble’ [Salix viminalis x (S. sachalinensis x S. miyabeana)]. These two willow cultivars are important short-rotation crops for biofuel production and for use in phytoremediation of contaminated lands (Cameron et al. 2007; Volk et al. 2011; Puckett et al. 2012; Serapiglia et al. 2012; Abrahamson et al. 2013). Examining N assimilation in shrub willows fertilized through foliar application has valuable applications for short rotation coppice systems and in maintenance of nursery stocks. Willow varieties grown for biomass production require N fertilization after each coppice, typically applied through the soil as urea or NH₄NO₃. In the absence of N fertilizer, willow plantations produce 35 to 40% less oven-dried biomass over the course of a rotation (Gonzalez-Garcia et al. 2012; Finnan et al. 2014). Yet, when plants are fertilized through the soil, there is typically >50% loss of N even in the densely planted field crops and it may be still higher for tree species. This loss is expensive for the farmer and will create the potential for increased N pollution with the expansion of
willow plantations. We tested four different N formulations including urea, Nitisin®, NH₄NO₃, and Arg for their efficacy to support growth and to study their effects on several cellular N-rich metabolites (i.e. AAs and common PAs). We also analyzed total N and C contents and soluble protein concentrations in the leaves following short term treatments with various N sources.

**Materials and Methods**

Dormant stem cuttings of two shrub willow cultivars, ‘Fish Creek’ (*Salix purpurea*) and ‘Preble’ (*Salix viminalis x (S. sachalinensis x S. miyabeana)*), were received from Double A Willow Nursery (Fredonia, NY - [www.doubleawillow.com](http://www.doubleawillow.com)). These cultivars are known to yield high biomass, to have resistance to diseases and pests, and to possess agronomic traits suitable for mechanical planting, harvesting, and post-harvest processing ([Abrahamson et al. 2013](#); [Cameron et al. 2007](#); [Volk et al. 2011](#)). The cuttings were 20 cm in length and 7 to 12 mm in diameter. For Experiment 1, two cuttings per pot were planted in 100 pots per cultivar in Fafard 52 mix (Conrad Fafard, Inc., Agawam, MA - [http://fafard.com](http://fafard.com)) in 16.5 cm diameter nursery pots to equal depth leaving 3 to 4 visible buds above the soil. For Experiment 2, cuttings were similarly planted but for ‘Fish Creek’ only. Each pot was considered a replicate. The plants were grown at the University of New Hampshire MacFarlane Greenhouse facility under ambient light and temperature conditions from June to August 2012 for a pilot study (called Experiment 1 here) with both cultivars and April to Aug 2013 for Experiment 2 (‘Fish Creek’ only). The cuttings were hand-watered for the first two weeks after potting, and then switched to drip lines and watered twice daily. The plants were fertilized weekly via driplines with fertilizer (Jack’s Professional 15-4-15 plus micronutrients – J.R. Peters Inc., Allentown, PA - [https://www.jrpeters.com/products_/16491/17592-jack-s-professional/16314-15-4-15-poinsettia-](http://https://www.jrpeters.com/products_/16491/17592-jack-s-professional/16314-15-4-15-poinsettia-))
feed-camg.html) at 150 mg L\(^{-1}\) N. No fertilizer was given for one week prior to the experimental treatments.

**Application of Foliar N as Fertilizer**

Experiment 1 was conducted in two Parts using the same plants; Part 1 in July and Part 2 in August of 2012. In Part 1, we tested two foliar N sources, urea (46-0-0) and Nitamin® (30-0-0). Nitamin® is a product specially formulated for use as a foliar spray (Koch Agronomic Services, Wichita, KS); it contains a total of 30% N (12% as urea + 18% in the form of slow-release N as Triazone and methylene urea). The objective of this experiment was to establish the tolerance level of the two cultivars to foliar-applied N in these two organic forms. Approximately 4 weeks after planting, randomly selected plants were treated with water (“unfertilized”) or foliar N in the form of either urea or Nitamin® (1% or 2% N equivalents). The foliar spray solutions included 0.05% (v/v) Silwet L-77 surfactant (Helena Agri-Enterprises, Collierville, TN), and were applied by hand spray using 500 mL plastic mist-spray bottles in the early evening. The treatments were applied until the spray ran off from the leaves. The pots were covered to prevent the fertilizer solution from dripping onto soil.

For Part 1 of the experiment, the plants were treated with N once, and leaf samples were collected at different times over the next two weeks for analysis of PAs, and total N and C contents. At the end of Part 1 of the experiment (all plants at this time were >1 m in height), the plants were cut back to a height of about 30 cm (removing most of the foliage) and treated with Jack’s Professional at 150 mg L\(^{-1}\) N through driplines. Then, for the next 3 weeks, the plants were treated with deionized water to induce N starvation before Part 2 of the experimental treatments were started. Due to the rapid growth of these plants after trimming, it was assumed that by the start of Part 2 of the experiment, most of the applied N had been metabolized. In
order to validate this assumption, on the day of the treatment for Part 2, a subset of these plants was sampled for metabolic analyses (time zero). The plants were then randomized before repeating the foliar treatments. The tissue samples were collected only from leaves that had emerged after the plants were cut back.

For Experiment 2 (2013), a new set of ‘Fish Creek’ cuttings were used. Replicate numbers of plants and the pots per treatment remained the same as for Experiment 1. In this Experiment we used urea, NH$_4$NO$_3$, and Arg as the N sources, with minor modifications of the spray solutions. These modifications included (i) adjusting the treatment solution pH to 5.7 (to mimic the pH of a typical tissue culture medium), and (ii) reducing the concentration of N to 0.5% vs. 1% and 2% (N equivalents) used in Experiment 1. Another difference was that in Experiment 2, the same plants were treated a second time with the respective treatment (without randomization) before harvesting the entire plant for biomass. Since there was severe leaf scorching in the ‘Preble’ cultivar, perhaps due to its thinner leaves (Fig. S1), this cultivar was not used in Experiment 2. Following the first foliar application, tissue samples were collected over a 2-week period for the analysis of PAs, AAs, soluble proteins, and total N and C. At three weeks after the second N application, when the plants were 9 weeks old, total plant material was collected for biomass measurements in different parts of the plants; i.e. leaves, stems and roots were collected and analyzed separately.

Tissue Preparation and Analysis of Soluble Foliar Polyamines and Amino Acids

In both experiments, 4 replicate tissue samples per treatment were collected at each time over the two-week period of treatment for PA analysis. Leaves from the two plants in the same pot were mixed for each replicate sample. Leaves were stored on ice during collection. Avoiding the mid vein and leaf edges, 6 to 12 discs (6 mm diameter) were taken per leaf using a paper-hole
punch from 2-3 healthy fully-elongated mature leaves per plant that were removed from the same position on the plant, about one-third below the branch apex. The leaves were thoroughly washed with cold water and surface-dried with paper towels before sampling. Fresh tissue discs (100 to 200 mg) were extracted in 5% PCA (1: 9 ratio for FW mg: μl volume) by three freeze-thaw cycles according to the procedure described in (Minocha et al. 1994), and dansylated as per Minocha et al. (1990) with the exception of using 50 mg ml\(^{-1}\) asparagine (Asn) to remove excess dansyl-chloride. Reverse phase gradient HPLC (Perkin Elmer, Waltham MA) system, fitted with a 3 μm particle size, 33 x 4.6 mm Pecosphere Cartridge Column (Perkin Elmer), was used to separate the three major PAs; i.e. Put, Spd and Spm (Minocha and Long 2004). From Experiment 2 samples, a subsample of the supernatant from the PCA extract was used for quantification of AAs by dansylation and HPLC as above.

**Total Nitrogen and Carbon**

On days 3 and 13 after treatment in Part 2 of Experiment 1, leaves were taken from 3 locations on a given branch per plant; 1 young leaf, 1 fully expanded mature leaf and 1 older leaf. The tissue was dried at 70°C for 72 h, and then ground by vigorous shaking in a stainless-steel vial with a ball pestle for 1 min. Samples were placed back in the oven (70°C) for an additional 16 h, and 2 to 5 mg samples were sealed in tin capsules. Total C and N were determined by elemental analysis using a Thermo-Scientific Flash EA Series 1112 CN analyzer (Thermo-Fisher Scientific Inc., Waltham, MA) as per EPA method 440.0 (Zimmerman et al. 1997).

For Experiment 2, 5 to 8 healthy, fully elongated mature leaves were removed from the middle of branches positioned one-third distance from the shoot tip on days 3 and 13 after treatment. Fresh tissue was dried in the oven for 72 h as above. All collected biomass was
ground using a mini Wiley Mill (Thomas Scientific, Swedesboro, NJ) and passed through a 0.85 mm sieve (#20 mesh). Samples were put back in a 70°C oven for additional 16 h before aliquots of 2 to 5 mg dry weight (DW) were sealed in tin capsules for analysis of C and N. Three replicate samples per organ per treatment were used for C and N analysis.

At harvest (9 weeks of growth), the total new biomass was separated into leaves, stems and roots. Leaves and stems of 4 pots per treatment were manually separated and weighed for total fresh weight (FW). The root ball was washed using a pressurized nozzle to remove soil. All parts were dried in a 70°C oven for 72 h before DW measurements.

**Soluble Proteins**

Leaf tissue from Experiment 2 was collected at days 5 and 13 after treatment, and at the time of final harvest. Soluble proteins were extracted in 1.0 mL of 0.1M phosphate buffer (pH 7.2) by freeze-thawing using 100 mg DW of tissue. The supernatant (centrifugation 13,000 x g for 10 min) was analyzed for total soluble protein concentration as per Bradford (1976), using Bio-Rad (Hercules, CA) protein assay dye reagent.

**Distribution of N in Different Metabolites**

With the data available to us and the information in the literature, we calculated the distribution of total foliar N into various metabolites that were analyzed in this study. For this, we calculated the proportional distribution of N in each of 16 AAs and 3 PAs at days 5 and 12 (and soluble proteins on days 5, 12, and at the time of harvest) in Experiment 2. The number of molecules of N per metabolite (e.g. 1 N in Gly vs. 4 N in Arg, and 2 N in Put vs. 4 N in Spm) were used to calculate the proportionate distribution of N in each entity. To calculate the amount of N in soluble proteins, the following assumptions were factored in: (i) the amount of soluble
protein (g⁻¹ FW or mg⁻¹ DW) equals the amount of AAs (g⁻¹ FW or mg⁻¹ DW); (ii) soluble proteins have a similar distribution of all 20 AAs; (iii) the average molecular weight (MW) of an amino acid in a polypeptide is 118.9 (Mean MW of all 20 AAs 136.9) minus 18 per peptide bond); and (iv) there is an average of 1.5 moles of N per mole of AA in protein (Table S2).

Calculated N in each entity is expressed as % of total N in the tissue at a given time of analysis and as % of N in the control treatment (Supplementary Tables S2-S5).

**Statistical analyses**

The effects of N treatment on PA and AA concentrations, C and N contents, soluble protein, and total biomass were tested using a one-way ANOVA followed by Tukey’s HSD post hoc test (XLSTAT: Data Analysis and Statistical Solution for Microsoft Excel) to determine the significant differences ($P \leq 0.05$) among treatments on a given day (C, N, PA, and biomass) and across days (AAs and soluble protein). The number of replicates for each data set are given in Figure/Table legends.

**Results**

In both parts of Experiment 1, there was evidence of some degree of leaf scorching in ‘Preble’ on the foliar-sprayed plants from 1% and 2% N (Fig. S1). The degree of scorching in the second experiment was minimal with 0.5% N. The major visual difference between the various N-treated plants and the controls were that the N-treated plants appeared taller, greener and fuller than the unfertilized plants.
Experiment 1

Soluble Foliar Polyamines

Overall, the response of foliar PA concentrations to N treatments was similar in the ‘Fish Creek’ and ‘Preble’ willow cultivars. In both cultivars, Spd was the predominant PA; its concentration being several-fold higher than Put and Spm on most days for ‘Fish Creek’ (Fig. 1) and somewhat lower for ‘Preble’ (Fig. S2). In the control (no fertilizer spray) plants of both cultivars, all three PAs either decreased or changed minimally over the two-week sampling period.

Foliar application of urea resulted in significantly higher (2 to 4-fold) leaf concentrations of Put at days 1 and 2 after treatment in both cultivars and in both parts of the Experiment 1 (Fig. 1 A, B; Fig. S2). However, the increases in Put were short-lived and by day 5, its concentration decreased to levels similar to the untreated plants. No significant changes in Spd or Spm were seen in response to foliar spray of urea in either part of Experiment 1 (Fig. 1 C-F).

The plants sprayed with Nitamin® showed similar responses in Put concentration to those of urea. Nitamin® produced a rapid, short lived increase in Put concentration lasting up to 2 days in ‘Fish Creek’ but a lesser effect in ‘Preble’ (Fig. 1 A, B; Fig. S2). By days 5 to 7 after treatment Put in the Nitamin®-treated plants had decreased to levels similar to the untreated control plants in both parts of Experiment 1 with ‘Fish Creek’. Except for urea spray, which caused an increase in Spd and Spm, their concentrations in the leaves remained quite stable over the remaining period of collections (Fig. 1 C-F; Fig. S2).
**Total Nitrogen and Carbon**

Foliar N treatments resulted in 10 to 60% increase in leaf N content (g⁻¹ DW basis) 3 days after N fertilization (Table 1). Changes in total C amongst the treatments were typically <5%; the range of C being 460-490 mg g⁻¹ DW for both cultivars (Table 1; Table S1). None of the treatments had a significant effect on %C content of the leaves. In ‘Fish Creek’, with the exception of 1% Nitamin®, all N treatments resulted in a significant increase in leaf N g⁻¹ DW compared to the untreated plants for day 3 as well as day 13 (Table S1). In all cases, including the control, the N content was lower on day 13 vs. day 3.

Leaf N and C contents in the N-treated ‘Preble’ plants at 3 days after treatment could not be compared to the unfertilized plants due to a loss of the untreated tissue during sample preparation. Overall, the N and C contents of the N-treated ‘Preble’ plants were comparable to those of N-treated ‘Fish Creek’ plants (see below); in the former, the N content declined from days 3 to 13 after N treatment. Additionally, no differences were seen in N or C content at 13 d among the different treatment groups.

**Experiment 2**

**Soluble Foliar Polyamines and Amino Acids**

Experiment 2 was conducted using a new set of cuttings of the ‘Fish Creek’ cultivar that were treated with foliar N in the form of Arg, NH₄NO₃, and urea. In this experiment, in addition to foliar PAs, the soluble AAs and soluble proteins were also analyzed to gather information about the distribution of N into major cellular N metabolites.

In all treatment groups, Put and Spd concentrations (g⁻¹ FW basis) decreased with time whereas Spm stayed mostly unchanged, a pattern similar to that seen in Experiment 1. The
dominance of Spd over Spm and Put was again seen in this experiment. Foliar application of urea had little effect on any of the PAs in the leaves whereas Arg and NH$_4$NO$_3$ caused a small decrease in Put during the first two days of treatment (Fig. 2).

Amino acids were quantified on days 1, 5, and 12 following the first application of Arg and NH$_4$NO$_3$. Of the 20 proteinogenic AAs, 16 were quantified by HPLC. Additionally, $\gamma$-amino butyric acid (GABA) and ornithine (Orn), two of the common non-protein AAs in plants were also quantified. In the untreated plants, typically, most AAs decreased (on g$^{-1}$ FW basis) from day 1 to 12. The exceptions were Lys, His and Met which increased, and Phe, Leu and Pro which remained unchanged (Fig. 3, 4). Changes from day 1 to day 12 were typically less than 2-fold, except for Gln, Ser, and Arg+Thr which declined more than two-fold. The most abundant AAs (>500 nmol g$^{-1}$ FW) were Glu, Ala, and Arg+Thr, His and GABA; all but His are related to the PA metabolism pathway (Fig. 3, 4). The least abundant (<100 nmol g$^{-1}$ FW) were Pro, Leu, Lys, Gly, Met and Orn; three of which (Pro, Met, and Orn) are also directly related to PA metabolism (Fig. 3, 4).

Foliar NH$_4$NO$_3$ application resulted in small significant decreases in Arg + Thr and an increase in Gln on day 1; Arg on the other hand had little effect on any AA (Fig. 3).

**Leaf Soluble Protein and Total Nitrogen and Carbon**

In comparison with control plants, the foliar treatments of Arg, NH$_4$NO$_3$, and urea made no difference to the leaf soluble protein content on any sampling day (Table 2). Soluble protein concentrations nearly doubled in all plants from day 5 to day 13 and remained at those levels until harvest (Table 2).
On days 5 and 13 after the urea treatment, no significant difference in total leaf N g\(^{-1}\) DW was seen in the ‘Fish Creek’ cultivar (Table 1). Foliar C content also remained unchanged on day 5 in all treatment groups, but at 13 days after application with Arg and NH\(_4\)NO\(_3\) there was a small increase in C compared to the untreated plants. With only minor differences in C content among treatments, no significant differences in C: N ratio were found at either day 3 or day 13. Leaf N g\(^{-1}\) DW in all plants decreased by as much as 50% between days 5 and 13 (Table 1). On day 13, the C: N ratio was almost double that of day 5, but still no differences among treatment groups were observed.

At harvest, total N (g\(^{-1}\) DW) varied greatly among treatment groups and among plant organs. Carbon content in the leaves and stems was greater in the N-treated plants compared to the untreated plants (Table 3). The major differences in N content and minor differences in C content lead to significant differences in the C: N ratios in some cases. In all plants, the leaves had 2 to 4 times more N than the stems and roots. The N stored in each organ varied significantly by type of foliar N treatment. For example, the plants treated with urea had more N in leaves, the plants treated with Arg had more N in the stems, and the plants treated with NH\(_4\)NO\(_3\) had more N in the roots than the other treatments (Table 3). Overall, at the whole-plant level, the urea treated plants had a higher N content compared to all other treatments.

**Distribution of N in Various Metabolites**

Urea and Arg had little effect on total N in the leaves on day 5. The percent of N in Put was higher in response to NH\(_4\)NO\(_3\) than urea, Arg, and the control plants (Supplementary Table S3). The proportion of N present in AAs was 1.41% of the total leaf N on day 5, which increased to 1.81% by day 12 (Supplementary Table S3). Among the AAs, Glu, Arg+Thr, Ala, and His represented the highest percent of N on both day 5 and 12 whereas Met and Orn represented the
least. When calculated as N% in a given AA in the control vs. each treatment, Arg application resulted in higher Lys and Arg at day 5, but by day 12, Orn showed the highest N. Most of N from NH₄NO₃ on day 5 showed an increase in GABA and Lys whereas on day 12 this N caused higher N accumulation in Orn, Gly, Leu, Ile, Val, Met and His relative to control. On day 5, the amount of N in 6 major amino acids related to N storage and PA biosynthetic pathway (Glu, Arg+Thr, Ala, Pro, GABA, and Orn) constituted about 45-60% of total AAs and their amounts decreased significantly by day 12 (Supplementary Table S4). In all treatments the N % in soluble proteins increased with time from day 5 to harvest by 4 to 5 times (Supplementary Table S5). As with the other N entities, there was decrease in the N % in soluble proteins in response to Arg at day 13. Urea treated plants showed the highest % in proteins on day 13.

**Biomass and Stem: Root Ratios**

At the end of Experiment 2, total dry biomass for each plant ranged from approximately 14 to 17 g, with the majority of it coming from the aboveground parts (stems + leaves, Fig. 5). No significant differences were seen in total plant biomass among treatments, but the urea treatment produced 30% more aboveground biomass than all other treatment groups, including the unfertilized controls (Fig. 5). The application of NH₄NO₃ showed a small decrease in root biomass production leading to an increase in the shoot: root ratio (Fig. 5). The Arg treatment did not affect either the aboveground or the belowground biomass production.

**Discussion**

The use of shrub willows in phytoremediation and in the production of biofuels has gained interest in recent decades due to the potential to produce high biomass in a short period, ease of vegetative propagation, broad genetic base, and coppicing ability following multiple harvests (Keoleian and Volk 2005; Zamora et al. 2014; Johnson et al. 2018). Recent findings suggest that
shrub willow crops have a greenhouse gas remediating potential of -42.9 metric tons CO$_2$ eq.ha$^{-1}$ at the end of seven 3-year harvest cycles (Pacaldo et al. 2012). In contrast to other agronomical crops, willows can be cultivated on marginal agricultural lands, and help improve site conditions, soil quality, and landscape diversity (Keoleian and Volk 2005). Willow biomass is also a versatile renewable feedstock that can be converted into a variety of bioenergy products; e.g. wood chips, ethanol and syngas (Volk et al. 2011). To remain productive, these plantations require a continual input of N fertilizer most of which is applied to the soil, which as mentioned earlier, is a rather inefficient method and a major cause of N pollution. In the present study, we examined the efficacy of foliar-applied supplemental N fertilizer to support the growth of two selected clonally propagated willow cultivars. We further studied the effects of several chemical formulations of N on selective (N-rich) foliar metabolites (i.e. PAs, AAs, proteins), and total N and C content in various tissues.

Preliminary data gathered in Experiment 1 indicated that foliar application of N was more effective on the ‘Fish Creek’ cultivar than ‘Preble’. The data showed that more N from foliar spray was assimilated into leaf N and foliar PAs. In Experiment 2, while the trends for the ‘Fish Creek’ cultivar were similar to those of Experiment 1, foliar N applications of a lower concentration of N (0.5% N) as urea, Nitamin®, NH$_4$NO$_3$, and Arg were not as effective as higher concentrations (1-2% N) used in Experiment 1. However, the effects of foliar-applied N on PAs, AAs and soluble proteins, and the distribution of stored N in different organs were affected by the form of N that was used.

The data further show that urea was the most effective form of N applied to shrub willow foliage as indicated by increases in total leaf N, cellular PA concentration, and aboveground biomass. The observations that PA concentration increased within the first day after N treatment
but was short-lived, and that the N treatments resulted in higher N accumulation than controls in
the different organs (leaves, roots and stem) indicate that: (a) the absorption and assimilation of
N from the leaves is rapid; (b) a significant amount of the absorbed N is converted into
aboveground biomass; and (c) the absorbed N is able to support the growth of plants and can be
translocated out of the leaf into stem and roots. Presumably, the first steps in N assimilation are
similar in the leaf and the root (i.e. N going through the Glu/Gln pathway) and require the
availability of carboxylic acids to assimilate NH$_3$. The latter steps of N conversion into other
AAs require photosynthetically produced reducing power (NADPH and ATP) as well. Further
work optimizing the form of N, its concentration, and environmental conditions for foliar N
application on shrub willow is necessary. In addition, it is important to compare N uptake and
assimilation between foliar and root applications under field conditions, to study the leaching of
foliar N into water systems, and include their effects on the soil microbiome, particularly the
microbial component that is involved in N interconversions in the soil. These studies are
currently underway in our lab.

**Foliar N Absorption and Metabolism**

A major advantage of foliar application of N fertilizer is the reduction in plant response time
to applied N. Foliar-applied urea has a relatively rapid response time, e.g. from a few hours in
wheat (Genter et al. 1998) up to 48 h in Arabidopsis (Majumdar et al. 2016). We found that in
the ‘Fish Creek’ cultivar, N urea apparently accumulated more rapidly and effectively (Table 3)
than Arg, and NH$_4$NO$_3$. Our results are consistent with the findings of Uscola et al. (2014), who
also reported that two forest trees (*Quercus ilex* and *Pinus halepensis*) were able to absorb urea
at a greater efficiency and at a faster rate than NH$_4^+$, NO$_3^-$, or Gly. The greater uptake of urea is
most likely because it is an uncharged molecule with a higher solubility than either NO$_3^-$ or
NH₄⁺, allowing it to more easily penetrate the cuticle and enter through the stomata. Urea is a smaller molecule than the other N sources tested in our study. This perhaps could be due to the leaf surface characteristics of willow [e.g. glandular and non-glandular trichomes, crystals, cuticle composition and thickness, stomatal density and aperture width, etc. - Binns and Blunden [1980]]; the larger molecules could not as easily breach these barriers and enter the leaf as did urea. Additionally, urea can enter plant cells both via specific transporters and passive transport channels in the membranes.

In Experiment 2, we found that foliar spray of 0.5% N as urea had no effect on PAs while in Experiment 1, rapid increases in Put with 1% and 2% urea N occurred within 1 to 2 days (depending on the cultivar). This may be the direct effect of additional N or perhaps a stress-induced PA accumulation of high N used in Experiment 1. The optimum concentration of urea N is therefore likely to be between 0.5-1% N. Although there was no visible increase in PAs from the urea treatment, at the time of harvest, the total DW in the aboveground and belowground biomass of ‘Fish Creek’ was significantly higher in these plants, even at 0.5% N (Fig. 5). This was accompanied by a significantly lower C: N ratios in the entire plant due to higher total N accumulation in the leaves and stems (Table 3). A higher total N accumulation in soluble proteins on day 13 in the urea-sprayed ‘Preble’ plants (Table S1) is also consistent with its effects on total biomass.

The decrease in Put on days 1 and 2 with foliar application of Arg and NH₄NO₃ in Experiment 2 was somewhat surprising (since Arg is a substrate for Put biosynthesis both directly by the ADC pathway as well as via Orn production and using the ODC pathway - Majumdar et al. 2016). One explanation for this decrease may be due to rapid leaf expansion (mostly through water uptake) shortly after Arg application diluting the concentration of Put.
calculated on g\(^{-1}\) FW basis. But by 3 days after treatment, the concentration of Put in the Arg-treated plants returned to levels similar to those in the controls, suggesting that by this time photosynthesis as well as PA biosynthesis had caught up with rapid leaf expansion effects.

In the NH\(_4\)NO\(_3\) sprayed plants, the observed increase in Glu and Gln on day 1 indicates its rapid assimilation into these two amino acids, especially since the appropriate enzymes and sufficient amounts of carboxylic acids and reducing power are available in the leaf. These AAs are the first products of NH\(_3\) assimilation. It can further be argued that these amino acids are rapidly converted to other amino acids and PAs and/or transported to other organs in the plant. While it is possible that the decrease in Put (observed 1 day after treatment) could be due to leaf expansion, an increase in Put (and GABA) on day 5 perhaps reveals processing of the added N into various PAs and AAs with time. This explanation is supported by the observed higher N % in Put and Spd on day 5 with NH\(_4\)NO\(_3\) (Table S3). This may partially explain the difference in response of these plants to urea vs. NH\(_4\)NO\(_3\). If the willow leaves treated with Arg were able to uptake the relatively large Arg molecule, it is possible that its lack of influence on PA concentration could also be due to the lack of ADC activity, combined with its faster metabolism into other AAs, proteins, nitric oxide, and Gln/Glu (Gao et al. 2009), and/or transport especially to roots. Free Arg can directly enter the PA biosynthetic pathway and has been used to promote nitric oxide and PA production in *Hyoscyamus niger* (Nasibi et al. 2013).

On a DW basis, the shoot: root ratios at the time of harvest were in the range of 5 to 8 across treatments. Li et al. (2011) showed that foliar-applied NH\(_4^+\) significantly impaired the number of lateral roots in *A. thaliana* seedlings. Their findings are consistent with the work of Peuke et al. (1998) indicating that NH\(_4\)NO\(_3\) may not be the most suitable form of N for use as a foliar spray on a long-term basis. Another possibility for the relatively lesser root biomass is that shortly after
foliar N application, leaf cells become N saturated, especially in the dark when C availability is
low. Leaves under these conditions can no longer safely metabolize N entering the leaf cells or
may even cause internal NH$_4^+$ toxicity (Sánchez et al. 2000; Roosta and Schjoerring 2007). It is
important for crops like willows, where aboveground parts are harvested on short rotations, for
there to be sufficient partitioning of N into the roots to sustain shoot biomass over time. It may
take a combination of root as well as foliar application of N to achieve this.

Our data further confirm earlier reports that the foliar uptake of N is rapid but short-lived
(Wittwer et al. 1963; Genter et al. 1998; Majumdar et al. 2016). An explanation for our results of
lower than expected biomass increase and shoot: root ratios in response to foliar N application is
not complete without further studies to show that other nutrients such as Mo, Fe, Mg or Ni
(structural components of several enzymes involved in N and C metabolism) were not limiting in
the leaves of these plants due to poor development of the root system. This may be related to the
experimental design in that our experiments were all conducted with freshly-rooted cuttings,
where the root biomass has a limited capacity to transport ions as well to store the assimilated C
and N. In an established plantation, where the shoots have been harvested once or twice, the pre-
existing root system may be a greater sink, providing a longer-term benefit to the plants. The
results of Hosseini and Khoshgoftarmanesh (2013) and Geetha et al. (2017) showed that the
addition of Ni to urea spray significantly increased N metabolism (urease/GS activity) in lettuce,
and increased vegetative growth in mulberry. However, this hypothesis requires further
investigation.

From the data on the distribution of N into different biochemical components of the cell, it
can be concluded that in spite of the limited growth effects of various low N treatments on ‘Fish
creek’ cultivar, the relative effects of the same amount of N distribution in different cellular
metabolites is different within the first day after treatment. Some of these effects are persistent over time up to the harvest day. Likewise, the effects of different N fertilizers on the distribution of total N into different metabolites is also affected by the form of N used (Supplementary Tables S3-S6), both in short-term as well as longer-term analyses. The results lead us to suggest that longer-term supplementation of foliar N would have a compounding positive effect in improving the total biomass of willows with minimal potential damage to the environment through N leaching. It could also be less costly than soil application in terms of the fertilizer cost and the amount to be used.

Conclusions and Future Direction

From the data presented here, we conclude that: (1) urea is the most suitable N source tested for foliar spray while NH$_4$NO$_3$ and Arg are the least suitable; (2) different forms of N fertilizer are assimilated into AAs, PAs and soluble proteins differently, and result in different growth responses; and (3) a combination of root and foliar application of N may be ideal in order to retain sufficient N in the roots for sustained increases in shoot biomass, especially in young plantations. For maximum benefit, foliar N may have to be applied: (1) more frequently than soil applications; (2) at modest concentrations to minimize scorching effects on the foliage; and (3) in combination with root applications to optimize root growth. Consistent with the previous studies, we have shown that the foliar N treatments result in rapid uptake and metabolism of N, perhaps due to the easy availability of photosynthetically fixed C in the leaves. Additional advantages of foliar N fertilization would be the potential for the reduction in total quantity of N used per season and a reduction in N lost into the environment and pollution, making it an environmentally friendlier practice. Further investigations of foliar fertilization in conjunction with micronutrient supplementation to optimize the benefits of this approach are warranted.
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References


Figure Legends

Figure 1. Concentration of polyamines [Put (A, B), Spd (C, D), and Spm (E, F)] in the leaves of ‘Fish Creek’ cultivar in Experiment 1 - Part 1 (A, C, E) and Part 2 (B, D, F) on different days of analysis. The plants received foliar applications of Nitamin® or urea at either 1% N or 2% N. The bars are mean (n = 4) ± SE. Different letters indicate statistically significant differences on a given day among treatment groups (P ≤ 0.05).

Figure 2. Concentration of polyamines [Put (A), Spd (B), and Spm (C)] in the leaves of ‘Fish Creek’ cultivar in Experiment 2 on different days of analysis. The plants received foliar application of 0.5% N as arginine, NH₄NO₃, or urea. The bars are mean (n = 4) ± SE. Different letters indicate significant differences on a given day among treatment groups (P ≤ 0.05).

Figure 3. Concentration of amino acids [Glu (A), Gln (B), Ala (C), Arg+Thr (D), Pro (E), Orn (F), GABA (G), and Met (H)] in the leaves of ‘Fish Creek’ cultivar in Experiment 2 on different days of analysis. The plants received foliar application of 0.5% N as either arginine or NH₄NO₃. The bars are mean (n= 3) ± SE. Different letters indicate significant differences among treatment groups across sampling days (P ≤ 0.05).

Figure 4. Concentration of amino acids [Gly (A), Lys (B), Val (C), Ile (D), Leu (E), His (F), and Trp+Phe (G)] in the leaves of ‘Fish Creek’ cultivar in Experiment 2 on different days of analysis. The plants received foliar application of 0.5% N as either arginine or NH₄NO₃. The bars are mean (n= 3) ± SE. Different letters indicate significant differences among treatment groups across sampling days (P ≤ 0.05).

Figure 5. Dry biomass of the whole plant, aboveground and belowground parts, and shoot: root ratio in the ‘Fish Creek’ cultivar collected at harvest in Experiment 2. The plants received foliar application of 0.5% N as arginine, NH₄NO₃ or urea. Values given for biomass exclude the original cutting. Data are mean (n = 4) ± SE. Different letters indicate significant differences (P ≤ 0.05) among treatment groups.
Table 1. Total nitrogen (N) and carbon (C) contents in the leaves of ‘Fish Creek’ cultivar on different days of analysis after treatments with different forms of N as indicated. Samples were collected on days 3, 5 and 13 for Experiment 1 and days 5 and 13 for Experiment 2. The values are mean (n = 3) ± SE. Different letters (if present) indicate significant differences (P ≤ 0.05) among treatment groups on a given day.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
<th>N (mg g⁻¹ DW) Mean ± SE</th>
<th>C (mg g⁻¹ DW) Mean ± SE</th>
<th>C:N Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>3</td>
<td>38.9 ± 0.6 a</td>
<td>464.4 ± 6.8 a</td>
<td>11.9 ± 0.1 a</td>
</tr>
<tr>
<td>Nitamin (1% N)</td>
<td>3</td>
<td>42.9 ± 3.6 a</td>
<td>489.0 ± 9.5 b</td>
<td>11.5 ± 0.9 a</td>
</tr>
<tr>
<td>Nitamin (2% N)</td>
<td>3</td>
<td>58.7 ± 0.6 b</td>
<td>479.1 ± 5.1 b</td>
<td>8.2 ± 0.1 b</td>
</tr>
<tr>
<td>Urea (1% N)</td>
<td>3</td>
<td>62.2 ± 3.8 b</td>
<td>470.6 ± 4.5 ab</td>
<td>7.6 ± 0.5 b</td>
</tr>
<tr>
<td>Urea (2% N)</td>
<td>3</td>
<td>56.4 ± 2.2 b</td>
<td>461.7 ± 3.8 a</td>
<td>8.2 ± 0.3 b</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>13</td>
<td>25.0 ± 1.2 a</td>
<td>468.2 ± 3.2</td>
<td>18.8 ± 0.8 a</td>
</tr>
<tr>
<td>Nitamin (1% N)</td>
<td>13</td>
<td>30.6 ± 2.8 ab</td>
<td>474.1 ± 2.1</td>
<td>18.2 ± 1.3 a</td>
</tr>
<tr>
<td>Nitamin (2% N)</td>
<td>13</td>
<td>36.1 ± 0.6 b</td>
<td>478.5 ± 9.6</td>
<td>13.3 ± 0.1 b</td>
</tr>
<tr>
<td>Urea (1% N)</td>
<td>13</td>
<td>37.3 ± 2.3 b</td>
<td>482.1 ± 4.9</td>
<td>13.5 ± 0.4 b</td>
</tr>
<tr>
<td>Urea (2% N)</td>
<td>13</td>
<td>35.3 ± 0.4 b</td>
<td>484.2 ± 1.2</td>
<td>13.0 ± 0.7 b</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>5</td>
<td>52.4 ± 1.0</td>
<td>480.2 ± 1.9</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>5</td>
<td>53.1 ± 1.9</td>
<td>488.4 ± 1.8</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td>NH₄NO₃ (0.5% N)</td>
<td>5</td>
<td>54.6 ± 1.5</td>
<td>491.9 ± 14.2</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>5</td>
<td>59.0 ± 2.8</td>
<td>478.6 ± 3.5</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>13</td>
<td>28.9 ± 0.9</td>
<td>475.8 ± 2.1 a</td>
<td>16.5 ± 0.6</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>13</td>
<td>31.6 ± 1.3</td>
<td>486.0 ± 1.3 b</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>NH₄NO₃ (0.5% N)</td>
<td>13</td>
<td>30.1 ± 1.1</td>
<td>485.5 ± 2.2 b</td>
<td>16.2 ± 0.6</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>13</td>
<td>28.4 ± 0.8</td>
<td>480.3 ± 0.7 ab</td>
<td>17.0 ± 0.5</td>
</tr>
</tbody>
</table>
Table 2. Soluble protein in the leaves of ‘Fish Creek’ cultivar in Experiment 2 on different days of analysis after treatment with different forms of N as indicated. Values are mean (n = 3 on days 5 and 13; n = 4 at harvest) ± SE. Different letters indicate significant differences ($P \leq 0.05$) among treatment groups and sampling days.

<table>
<thead>
<tr>
<th>Soluble Protein (mg g$^{-1}$ FW)</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td><strong>Day 5</strong></td>
</tr>
<tr>
<td>Unfertilized</td>
<td>6.4 ± 0.8 a</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>7.3 ± 0.6 a</td>
</tr>
<tr>
<td>$NH_4NO_3$ (0.5% N)</td>
<td>5.9 ± 0.6 a</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>6.8 ± 0.7 a</td>
</tr>
</tbody>
</table>
Table 3. Total nitrogen (N) and carbon (C) in different parts of the plant in ‘Fish Creek’ cultivar at harvest in Experiment 2 after treatment with different forms of N as indicated. The values are mean (n = 3) ± SE. Different letters indicate differences ($P \leq 0.05$) among treatment groups for a given organ.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Organ</th>
<th>N (mg g(^{-1}) DW) Mean ± SE</th>
<th>C (mg g(^{-1}) DW) Mean ± SE</th>
<th>C: N Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized</td>
<td>Leaf</td>
<td>24.2 ± 0.9 a</td>
<td>476.6 a ± 1.2 a</td>
<td>19.7 ± 0.8 a</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>Leaf</td>
<td>24.0 ± 0.5 a</td>
<td>481.5 b ± 0.2 b</td>
<td>20.1 ± 0.4 a</td>
</tr>
<tr>
<td>NH(_4)NO(_3) (0.5% N)</td>
<td>Leaf</td>
<td>24.4 ± 0.5 a</td>
<td>481.3 b ± 0.2 b</td>
<td>19.7 ± 0.4 a</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>Leaf</td>
<td>31.2 ± 0.3 b</td>
<td>486.2 c ± 0.9 c</td>
<td>15.6 ± 0.1 b</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>Stem</td>
<td>6.2 ± 0.4 a</td>
<td>481.3 a ± 1.4 a</td>
<td>77.5 ± 5.2 a</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>Stem</td>
<td>10.6 ± 0.6 c</td>
<td>473.5 b ± 1.0 b</td>
<td>44.9 ± 2.5 b</td>
</tr>
<tr>
<td>NH(_4)NO(_3) (0.5% N)</td>
<td>Stem</td>
<td>7.0 ± 0.0 ab</td>
<td>475.5 b ± 2.4 b</td>
<td>68.5 ± 0.5 ab</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>Stem</td>
<td>9.5 ± 0.1 bc</td>
<td>472.2 b ± 1.7 b</td>
<td>49.8 ± 0.7 b</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>Root</td>
<td>8.8 ± 0.3 a</td>
<td>482.6 ± 2.3</td>
<td>54.9 ± 1.9 a</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>Root</td>
<td>7.7 ± 0.3 a</td>
<td>483.9 ± 1.5</td>
<td>63.2 ± 2.4 c</td>
</tr>
<tr>
<td>NH(_4)NO(_3) (0.5% N)</td>
<td>Root</td>
<td>10.8 ± 0.0 b</td>
<td>479.4 ± 1.8</td>
<td>44.4 ± 0.1 b</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>Root</td>
<td>9.9 ± 0.2 ab</td>
<td>481.5 ± 1.2</td>
<td>48.6 ± 1.1 ab</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>Whole plant</td>
<td>13.1 ± 0.5 a</td>
<td>480.2 ± 1.4</td>
<td>50.7 ± 2.6 a</td>
</tr>
<tr>
<td>Arginine (0.5% N)</td>
<td>Whole plant</td>
<td>14.1 ± 0.3 ab</td>
<td>479.7 ± 0.6</td>
<td>42.7 ± 0.8 b</td>
</tr>
<tr>
<td>NH(_4)NO(_3) (0.5% N)</td>
<td>Whole plant</td>
<td>14.9 ± 0.8 b</td>
<td>498.9 ± 20.1</td>
<td>42.3 ± 1.9 b</td>
</tr>
<tr>
<td>Urea (0.5% N)</td>
<td>Whole plant</td>
<td>16.9 ± 0.2 c</td>
<td>480.0 ± 0.7</td>
<td>38.0 ± 0.6 b</td>
</tr>
</tbody>
</table>
Figure 1. Concentration of polyamines [Put (A, B), Spd (C, D), and Spm (E, F)] in the leaves of ‘Fish Creek’ cultivar in Experiment 1 - Part 1 (A, C, E) and Part 2 (B, D, F) on different days of analysis. The plants received foliar applications of Nitamin® or urea at either 1% N or 2% N. The bars are mean (n = 4) ± SE. Different letters indicate statistically significant differences on a given day among treatment groups (P ≤ 0.05).

162x138mm (300 x 300 DPI)
Figure 2. Concentration of polyamines [Put (A), Spd (B), and Spm (C)] in the leaves of 'Fish Creek' cultivar in Experiment 2 on different days of analysis. The plants received foliar application of 0.5% N as arginine, NH₄NO₃, or urea. The bars are mean (n = 4) ± SE. Different letters indicate significant differences on a given day among treatment groups (P ≤ 0.05).

114x228mm (300 x 300 DPI)
Figure 3. Concentration of amino acids [Glu (A), Gln (B), Ala (C), Arg+Thr (D), Pro (E), Orn (F), GABA (G), and Met (H)] in the leaves of 'Fish Creek' cultivar in Experiment 2 on different days of analysis. The plants received foliar application of 0.5% N as either arginine or NH$_4$NO$_3$. The bars are mean (n= 3) ± SE. Different letters indicate significant differences among treatment groups across sampling days (P≤ 0.05).
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