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Arginine and the shade tolerance of white spruce saplings entering winter dormancy

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ABSTRACT: Shade-tolerant white spruce saplings grown at 100, 45, 25, and 13% natural light for four years, and entering winter dormancy, modified their growth habit and redistributed the total soluble N among needles, roots, and stems with buds mainly to arginine N. Most free amino acid N was found in roots in saplings at full light, and the least at 13% light. Glutamate, glutamine, and aspartate N contributed to the accumulation of soluble arginine N. Arginine-derived γ-guanidinobutyric acid, agmatine and an unidentified guanidino compound accumulated mainly in stems with buds at 25 and 13% light. The profiling N metabolism and arginine-derived guanidino compounds extend models for shade tolerance based mainly on photosynthesis, respiration and carbon gain.

Keywords: amino acids; arginine; guanidino compounds; nitrogen; *Picea glauca*; shade tolerance; winter dormancy

White spruce (*Picea glauca* [Moench.] Voss.) is a shade-tolerant conifer in forest understories. Prior studies focused on the shade-induced changes in leaf morphology and physiology, photosynthesis, respiration and dry matter (Kramer, Kozlowski 1979; Mitamura et al. 2008). Although arginine (2-amino-5-guanidinovaleric acid) was first isolated from the soluble nitrogen (N) and proteins of *Picea*, *Pinus*, and *Abies* seedlings (Schulze 1896), very little is known about how arginine metabolism relates to shade tolerance and to the survival of saplings under field conditions and in N-poor forest soils.

Isotopic studies with conifers demonstrated that arginine was synthesized *de novo* via the urea or ornithine cycle and enriched the soluble N pool by protein turnover (Durzan 1968, 1969). The fate of the carbon of arginine in white spruce trees entering winter dormancy was traced to several guanidino compounds (Durzan 1968, 1969). The transfer of the amidino moiety [-C(=NH)-NH_2] of arginine to γ-aminobutyric acid was responsible for the formation of γ-guanidinobutyric acid. Agmatine is formed by the decarboxylation of arginine. Guanidino compounds are known respiratory inhibitors (Wilson, Bonner 1970; Bidwell, Durzan 1975, 2009).

This study investigates arginine N and its derived guanidino compounds in white spruce saplings habituated after four years of shading under controlled field conditions. It asks how continuous shading redistributed amino acid N in the soluble amino acid N pool of needles, stems with new buds and roots in response to prior hours of sunshine and air temperature. It demonstrates how the sequential diversion of aspartate, glutamate and glutamine N to arginine N and guanidino compounds correlated with the recovery of organ biomass during the onset of winter dormancy in a shade tolerant conifer.

MATERIALS AND METHODS

Four-year-old white spruce saplings were grown from seed obtained from a tree breeding seed bank at the Petawawa Forest Experiment Station, Chalk

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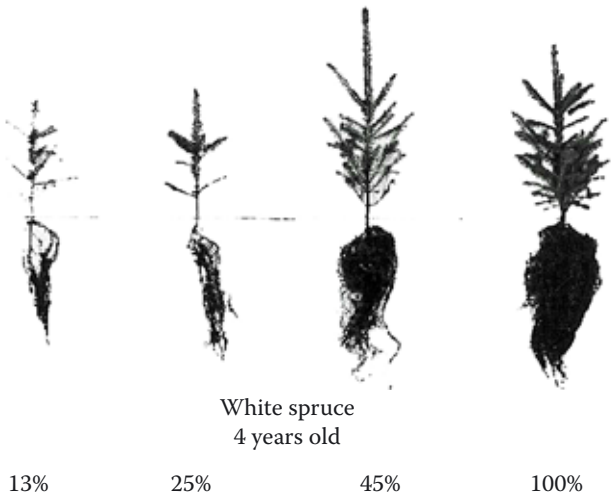

Fig. 1. The effects of shading on the morphology and redistribution of biomass in shade-tolerant white spruce saplings compared to full light

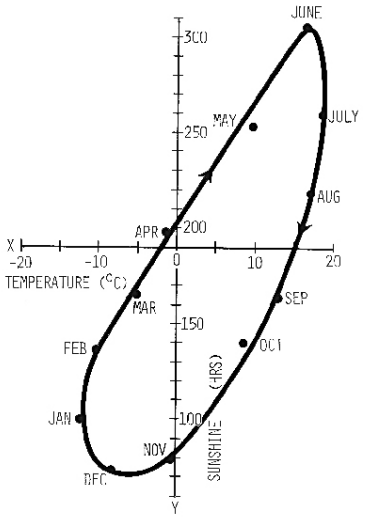

Fig. 2. Hysteresis is demonstrated in the annual relationship between the monthly average hours of sunshine and temperatures at the experimental field site. Shoot elongation ceased in early July. The following year's buds were visible at the end of July. Saplings were harvested on October 13

River, Ontario, Canada. Seeds originated from a local population at 45°08'N, 81°27'W. Seedlings of uniform size were initially selected in 1966 to minimize genetic variation and planted in sandy loam in an open forest area (Logan 1969). Soil, light intensity, amount of growing space and climate were controlled factors throughout sapling development over four years. Shade was maintained in three shelters of lath with fibreglass screening.

The quantity of the light from dawn to dusk, measured in the shelters on clear sunny days was 13, 24, and 45% full light. Results with a Bellani pyranometer, which integrates total solar radiation received on a spherical surface, showed that the percent radiation in shelters during May to July was similar to the percent illumination obtained from spherical illuminometers. Differences in sapling morphology caused by environmental factors, other than the effects of shade, were small so that the major variable affecting sapling growth was the quantity of light (Logan 1969). In full light, the lag between monthly hours of sunshine and air temperature (hysteresis) over the year formed a closed path in the *x*, *y* plane (Fig. 2). Both factors preconditioned seedlings for bud set and the onset of dormancy. By October 13, buds on shoots already developed for the following year (Fig. 1).

On this date, the total needles, shoots with buds, and roots were quickly separated and harvested between 2:30 to 3:30 pm to minimize diurnal and translocation variations in amino acid content. Duplicate harvests of organ biomass were weighed fresh and fixed immediately in the field and in 80% ethanol (v/v). Morphological measurements were based on the means of three saplings, one of which was not used for biochemical analyses. The third replicate was kept in the event that the extraction of one of the two selected saplings was accidentally lost. The main experimental limitation was the cost of amino acid and guanidino analyses.

Organs were homogenized in a Waring blender with 80% ethanol for the extraction of all Sakaguchi and ninhydrin-positive substances. Extracts were filtered and quickly dried at 20°C in a jet of N gas. Residues were dissolved in a known volume of 0.2N (Na) citrate buffer and refrigerated after adding a few ml of chloroform to maintain asepsis and to collect pigments which separated at the bottom of sample vials. Free amino acids in the buffer were determined in triplicate and quantitatively within ± 3% by the method of Benson and Patterson (1965) using a Model 120C Beckman Amino Acid Analyzer.

Guanidino compounds (Tables 1–3) in the same buffer were determined within ± 6% by a modification of the Amino Acid Analyzer where the Sakaguchi reaction replaced the ninhydrin (Durzan 1969). Agmatine and the Sakaguchi-reactive guanidino compound (J) are expressed as colour equivalents based on the reaction with arginine. The chromatographic locations of these products of ^{14}C-L-arginine are reported in earlier publications (Durzan 1968, 1969).

Fresh weights of whole seedlings, total needles, roots, stems with buds, and the significance of changes in total soluble N and arginine N contents were evaluated using *F* values based on orthogonal comparisons of equally spaced data using linear,

quadratic, and cubic partitions of light intensity (Steel, Torrie 1960; Durzan 1971).

RESULTS AND DISCUSSION

The survival of a spruce seedling is estimated to require at least 20% light transmittance (Grossnickle 2000). Survival is equivalent to about half the growth achieved in full light. For shade tolerance, an evergreen habit, reduced shoot biomass, and a large root biomass are considered beneficial (Billings 1974). For white spruce saplings, this benefit became evident after four years of shading (Fig. 1).

By October, full and 45% light produced the highest total biomass, the most robust saplings, the highest density of needles, and most side branches (Fig. 1, Tables 1–3). Leader-shoot height and needle length were greatest at 45% light. At 13% light, total sapling biomass was less than one fourth of that of full and 45% light. Roots now accounted for nearly half of the sapling biomass. Stems with buds had the highest biomass density ($g.cc^{-2}$).

Orthogonal comparisons over shade treatments for the response of total soluble ($N.g^{-1}$) f wt gave highly significant *F* values (Tables 1–3). Most soluble N was distributed to roots (full light, Table 2), followed by needles (45% light, Table 1), and stems with new buds (25% light, Table 3). Least soluble N was recovered from roots (25 and 13% light, Table 2), needles (13% light), and stems with new buds (full light). The greatest decline occurred in roots and needles. In roots, the soluble N fell from 870 (full light) to 86 μg N (13% light, Table 2). In leaves it fell from 770 (45% light) to 173 μg N (13% light).

Table 1. Needle parameters and the composition of free amino acid N and guanidino compounds in the soluble N pool of four-year-old white spruce saplings exposed to continuous natural light and shading under field conditions (% total soluble N)

Treatment	Natural	45%	25%	13%
Amino acid N				
Glutamate**	11.1	6.7	6.6	5.7
Glutamine**	12.1	20.8	21.0	9.7
Aspartate	1.1	0.7	0.5	0.8
Asparagine	6.9	3.9	7.7	4.8
Arginine*	4.0	7.2	7.9	15.1
Ornithine	0.5	0.3	0.4	0.8
Proline	3.1	5.3	3.3	2.9
Glycine	1.4	1.2	0.9	1.3
γ-Aminobutyrate	13.4	13.3	8.9	11.4
Subtotal % N	53.6	59.4	57.2	52.5
μg soluble $N.g^{-1}$/f wt*	**481.0**	**770.0**	**484.0**	**173.0**
Guanidino compounds		**arginine colour equivalents/g f wt**		
γ-Guanidinobutyrate*	1.3	3.3	9.4	5.6
Agmatine	0.8	0.3	0.9	5.4
Unidentified J	0.9	1.1	5.2	2.9
Total colour equivalents*	**3.0**	**4.7**	**15.5**	**13.9**
		needle biomass		
Needle length mm*	12.1	14.0	11.0	9.3
% g f wt*	24.8	26.1	50.0	34.5
Total g f wt/sapling**	**106.5**	**105.3**	**46.0**	**23.3**

Guanidino compounds are expressed as arginine equivalents based on the colour reaction with the Sakaguchi reagent. *F* values significant at 1** and 5*%; f wt – fresh weight

Table 2. The responses of free amino acid N and guanidino compounds in the soluble N pool of the roots of four-year-old white spruce saplings exposed to continuous natural light and shading under field conditions (% total soluble N). This is only place where traces (t) were observed

Treatment	Natural	45%	25%	13%
Amino acid N				
Glutamate*	24.5	36.6	21.9	18.5
Glutamine**	4.6	12.0	18.8	24.2
Aspartate	4.5	5.0	6.6	3.4
Asparagine	6.6	8.1	2.9	13.6
Arginine*	22.6	8.4	10.9	30.0
Ornithine	0.3	0.2	2.2	0.5
Proline	1.2	1.6	0.9	1.0
Glycine	0.5	0.7	1.3	1.3
γ-Aminobutyrate	2.6	1.4	1.1	3.0
Subtotal % N	67.4	74.0	66.6	95.5
μg soluble N.g^{-1}/f wt**	**870.0**	**655.0**	**92.0**	**86.0**
Guanidino compounds		**arginine colour equivalents/g f wt**		
γ-Guanidinobutyrate*	14.1	7.0	14.4	40.6
Agmatine	6.4	9.0	2.9	5.7
Unidentified J	t	t	1.0	t
Total colour equivalents	**20.5**	**16.0**	**18.3**	**46.3**
		root biomass		
% g f wt	61.5	58.8	38.0	45.2
Total g f wt/sapling**	**106.5**	**105.3**	**46.0**	**23.3**

Guanidino compounds are expressed as arginine equivalents based on the colour reaction with the Sakaguchi reagent. *F* values significant at 1** and 5*%; f wt – fresh weight

Arginine N originates mainly from glutamic acid, glutamine, and aspartic acid (Durzan, Steward 1983). Glutamic acid N is a precursor for glutamine N. The latter is a main translocated form of soluble N. Aspartic acid N is required for the synthesis of argininosuccinic acid, which is a transient and immediate precursor for the N in the guanidino moiety in arginine. It is also a precursor for asparagine.

In response to shading, the percent N changes for glutamic acid, glutamine and arginine N in all organs were highly significant (Tables 1–3). Glutamic acid N declined in all organs. Glutamine N declined in needles and stems with buds but increased in roots (25 and 13% light). Arginine N accumulated in all organs. Percent arginine N was greatest in roots and in stems with new buds. At 13% light, glutamic acid, glutamine, aspartic acid and arginine N contributed 76% to the total soluble N of roots. Changes in aspartic acid N were not significant.

Protein turnover or synthesis either added to or subtracted arginine N in the soluble N pool. The accumulation of arginine N indicated that reduced light may have limited the synthesis of N-rich storage proteins. Proteins are turned over in the following spring to provide amino acid substrates and energy for growth and development (Durzan 1969).

In white spruce shoots, respiration declines from a high in June to a low in late August (Clark 1961). When shoot elongation ended in mid-July at Petawawa, arginine N started to accumulate in terminal shoots (Durzan 1968). By early September and after the first frost, the synthesis of γ-guanidinobutyric acid and other guanidino compounds from [UL-^{14}C]-L-arginine was already in progress.

The transfer (transamidination) of the amidino moiety of arginine to γ-aminobutyric acid is required for the synthesis of γ-guanidinobutyric acid (Durzan 1969). The decarboxylation of arginine

Table 3. Size parameters and the responses of free amino acid N and guanidino compounds in the soluble N pool of stem and buds of four-year-old white spruce saplings exposed to natural light and shading under field conditions in mid-October

Treatment	Stem and Buds			
	Natural	45%	25%	13%
Amino acid N		% total soluble N		
Glutamate**	10.5	6.1	4.2	4.3
Glutamine**	29.7	42.4	22.2	20.4
Aspartate	1.3	0.7	0.8	1.0
Asparagine	4.0	3.9	3.7	3.1
Arginine*	17.1	28.2	52.1	47.7
Ornithine	0.3	0.1	0.1	0.5
Proline	4.5	2.9	1.9	2.5
Glycine	1.1	0.6	0.6	0.9
γ-Aminobutyrate	6.8	1.7	2.4	4.8
Subtotal % N	75.3	86.6	88.0	85.2
µg soluble N.g^{-1}/f wt**	**352.0**	**513.0**	**648.0**	**498.0**
Guanidino compounds		**arginine colour equivalents/g f wt**		
γ-Guanidinobutyrate*	40.9	104.4	299.0	175.8
Agmatine*	23.5	50.7	161.3	90.9
Unidentified J	3.3	0.9	3.4	16.8
Total colour equivalents*	**67.5**	**156.0**	**463.7**	**238.5**
		stem and bud biomass		
Shoot height cm*	7.4	9.8	6.5	6.4
Shoot density (g/cc^{-3})*	3.7	3.9	4.1	5.9
Mid shoot dia. mm*	2.6	2.3	1.7	1.3
% g f wt/sapling	13.6	15.2	12.0	20.2
Total g f wt/sapling**	**106.5**	**105.3**	**46.0**	**23.3**

Guanidino compounds are expressed as arginine equivalents based on the colour reaction with the Sakaguchi reagent. *F* values significant at 1** and 5*%; f wt – fresh weight

accounts for the formation of agmatine. Although the structure of compound J remains unknown, its chromatographic properties indicated that it is a more basic compound than arginine. Another guanidino compound in spruce, α-keto-δ-guanidinovaleric acid (Durzan, Richardson 1966), is formed by transamination and decarboxylation, as distinct from transamidination. Only traces were detected in full light.

With increasing shade, total guanidino compounds accumulated in stems with new buds followed by roots and needles (total colour equivalents, Table 3). γ-Guanidinobutyric acid (all organs) and agmatine (stems with buds) showed a significant response to shading. The inhibitory properties of guanidino compounds and their differential distribution in saplings indicate that not all organs may have become dormant at the same time. [1-^{14}C]-Guanidinoacetic acid, a candidate for one of the trace unidentified guanidino compounds, when added to excised shoot primordia of white spruce in October, inhibited respiration (Durzan 2009).

In spring, the prior accumulation of free arginine spares energy as adenosine triphosphate (ATP) for

the *de novo* synthesis of arginine when ATP is needed for rapid bud growth and cambial development (Durzan 1969; Atkinson 1977). Arginine provides N for the synthesis of other amino acids some of which are precursors for growth hormones, polyamines, and nitric oxide (NO) (Durzan, Steward 1983; Durzan, Pedroso 2002). NO maintains metabolic homeostasis and protects against oxidative and nitrosative damage at high light intensities (Durzan 2002; Corpas et al. 2008).

In trees, the cessation of growth and bud set, induced by short days, is regulated by a (*CO/FT*) regulatory module for two genes, CONSTANS (*CO*) and FLOWERING LOCUS T (*FT*) (Böhlenius et al. 2006). The tracking of hours of sunshine and air temperature (Fig. 2) would comprise an integrated environmental signal for the *CO/FT* regulatory module to initiate enzymatic changes in N metabolism required for sapling habituation and over-winter survival. Spruce saplings in this study were juvenile and the transition of vegetative buds to male or female cones was not yet a factor for *FT* expression.

In Douglas-fir trees, arginine and guanidino compounds have been used as biomarkers to predict growth and the optimal time for adding fertilizers (Van Den Driessche, Webber 1977). Phloem was more useful than root analyses in determining the tree nutrient status. Arginine and guanidino compounds accumulated with the nitrate fertilizer treatment. In the next year, seed cone production was elevated 2 to 7 times (Ebell, McMullen 1970).

CONCLUSIONS

Light intensity, photoperiod, and temperature changes were tropistic factors contributing to the redistribution of amino acid N from needles to organs having meristems entering winter dormancy. More than a century after the discovery of arginine in conifers we now know that arginine N contributes to the seasonal and metabolic response to reduced light by a shade-tolerant spruce. Physiological changes in N metabolism are postulated as being under the genetic control of regulatory modules controlling the cessation of growth, and years later during maturity to flowering and viable seed production. Arginine-derived guanidino compounds as respiratory inhibitor respiratory inhibitors contributed to dormancy and increased with shading. The concentration of soluble N in arginine may spare photosynthates for the synthesis of carbon-rich secondary products which may protect against pathogens, insects, and frost damage. During the breaking of dormancy in spring, the removal of inhibitory guanidino compounds provides sources of N for the renewed synthesis of arginine. Arginine N and guanidino compounds may have utility as physiological biomarkers in tree improvement and breeding programs where soils are limited by the availability of N.

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