

Article

Nitrogen and Boron Dosage Effects on Arginine Accumulation in Scots Pine Needles

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Abstract: Free arginine (Arg) content was observed to multiply when the level of nitrogen (N) nutrition was high, and additional fertilization with boron (B) potentiated this effect. Owing to this feature, conifers can be suggested for use as bioproductors of Arg. Concentrations of Arg in relation to N and B fertilization needed to be better understood. The effect of soil fertilization with N and B on accumulation of these elements and free Arg in one-year-old needles of 16-year-old Scots pine (*Pinus sylvestris* L.) trees was determined in this study. Plantations were fertilized with doses of N from 0 to 1000 kg ha^{−1} and B from 0 to 6 kg ha^{−1}. Fertilization with 3 kg ha^{−1} B at N doses of 200–500 kg ha^{−1} stimulated the accumulation of N in needles of up to 3.1–3.6% dry weight (DW). The level of Arg in needles increased from 74.7 to 175.9 μmol g^{−1} DW at these levels of N and B.

Keywords: *Pinus sylvestris* L.; needles; nitrogen; boron; fertilization doses; Arg; enrichment technology



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1. Introduction

Boreal coniferous forests usually grow on acidic podzolic forest soils that are deficient in mineral nutrients, first of all in nitrogen (N). Boron (B) is deficient for the growth of conifers under the conditions of Fennoscandia [1–7]. Excessive competition between trees for nutrients, light, and water is prevented by precommercial cuts (thinning) with retention of a sufficient number of trees to secure high productivity of the site [8], while the supply of nutrients to trees is optimized by fertilization [9–11]. Alongside many other factors, the effect of fertilization on tree species depends on the content of nutrients in soil and on fertilizer form and dosage [6,8,12–25].

Precommercial thinning results in an increased output of lumber, but the question of improving financial performance remains open [26]. Increasing the profitability of silvicultural actions (thinning, pruning, and fertilization) in young and middle-aged stands can be augmented by processing and utilizing the removed green tissue, thereby reducing the costs. Data on the accumulation of aboveground biomass in coniferous species are necessary to assess the reserves of tree greenery suitable for harvesting plant materials enriched with biologically active substances (BAS) [27–30].

The growing demand for BAS and exhaustion of the traditional sources of plant material necessitates the search for new resources. A topical task is finding new sources of BAS of high practical value. Yet, large-scale exploitation of natural sources of BAS and their massive harvesting is fraught with environmental problems. The use of foliage contributes to solving environmental problems [30].

Tree foliage contains various organic compounds used in the manufacturing of nutritional and pharmaceutical products. Ethnobotanical and ethnomedicine literature is used to search for new sources of raw materials in feed production and pharmaceuticals [29].

The water-soluble fraction of coniferous greenery contains free amino acids, in particular L-arginine (arginine). A hypothesis has been proposed about the nutrient and synergistic role of arginine and its metabolites, which supplement the role of vitamin C [29]. It is an ingredient of many drugs and antiviral agents. Being a source of nitrogen oxide—a potent vasodilator and neuromediator—it is applied in medicine [31,32]. The demand for Arg is high in carnivores, fish, and poultry [33,34]. Arg boosts the growth of animals, their productivity, and their immune response [35,36].

The development of technologies for modifying the biochemical composition of coniferous woody greens to obtain plant raw materials enriched with BAS necessary for practical purposes is promising for the purposeful use of new raw materials.

Different plant species have very different needs for Arg. In the organs of one plant species, Arg can accumulate in a wide range of values, depending on the phenophase and growing conditions [37–39].

Arg content in needles was seen to multiply where N nutrition was elevated as a result of economic activities or in experiments with mineral nutrition of conifers [5,39–44]. Fertilization of B significantly increased the effect. The finding can be used to produce Arg-enriched coniferous tree foliage [30]. A novel protocol for supplying coniferous plants with N and B has been designed to cause plant material to be used as feedstock for manufacturing Arg-rich coniferous products—conifer meal and aqueous conifer extract [35,36]. Positive results were obtained in the trials of Arg-rich coniferous extract in fur animal farming, specifically in American mink (*Mustela vison* Shr.) [36]. The extract improved their immune status and survival rates. Administered as a feed supplement, conifer meal made of Arg-enriched needles promoted animals growth and productivity [35].

Arg-rich woody green tissue can be obtained during thinning in young stands, when relieving linear facilities from woody vegetation, or from forest crops planted specifically for woody green tissue cultivation [30]. The potential of the resource was estimated by studying the distribution of Arg in crowns of 10-year-old Scots pine (*Pinus sylvestris* L.) trees in an experiment with modulation of N and B supply [30]. The intake, distribution, and consumption of N in conifers is known to depend on the timing of fertilizer placement in soil, and the composition of N compounds in conifers' organs and tissues varies among phenological phases over the annual cycle [5,39]. The seasonal dynamics of Arg content in conifers in relation to the timing of N and B application to soil was studied [45]. N and B placement under Scots pine at any time during the growing season was found to be effective for producing Arg-rich needles throughout the annual cycle. The greatest effect was observed when fertilization happened in June, and Arg accumulation reached a maximum in one-year-old needles in September the same year and February–March next year [45]. The most effective and economically expedient fertilization doses need to be determined to optimize the technique of Arg enrichment of coniferous plants. The aim of this study was to identify the effect of soil fertilization with N in a wide range of doses in combination with three fixed levels of B on Arg accumulation in one-year-old Scots pine needles.

2. Materials and Methods

2.1. Study Area

The area is situated in the northwest of European Russia (61°56' N, 34°21' E), in the middle taiga subzone. The region belongs to the zone of cold and wet. The average annual air temperature is +3.1 °C, and the average annual amount of atmospheric precipitation is 611 mm. The duration of the frost-free period is up to 120–130 days, and the active growing season is around 100 days or more. Studies were carried out in 16-year-old Scots pine (*Pinus sylvestris* L.) crops planted in a sand quarry in 1998 using containerized seedlings (Figure 1). The research site is almost flat with a slope of up to 1° orientated towards the southwest.

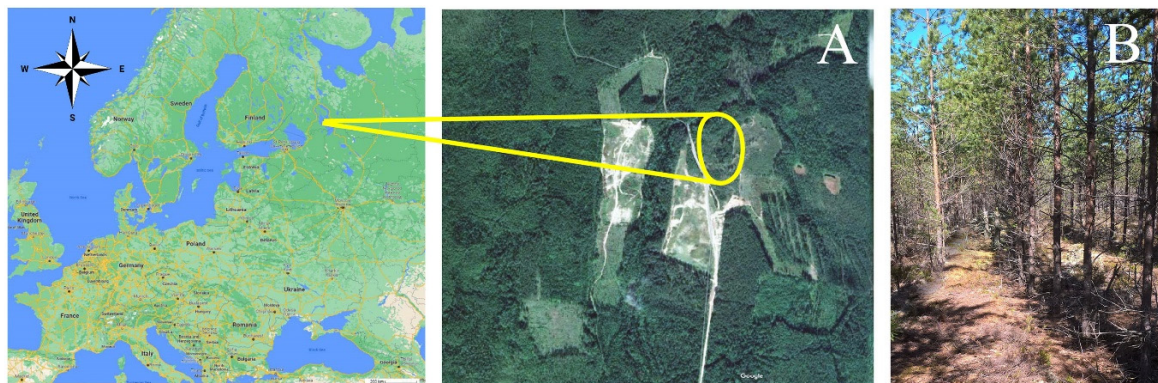


Figure 1. Geographic location of the study sites in the Karelia Republic, Russian Federation (A); 16-year-old Scots pine crops (B).

2.2. Experimental Site

2.2.1. Silvicultural Survey Methods

A forestry-geobotanical description of the site of study was made. A continuous count of trees was carried out in accordance with the methods generally accepted in silviculture. The diameter at breast height (DBH) of each counted tree was measured. The height and diameter of ten trees of the site of study was measured, and a statistical regression model describing the relationship between height and diameter for stand was constructed, in order to calculate the mean height of the stand.

Total basal area and volume of the stand were found in regional reference sources made for Karelia [46]. The quality class was located on the commonly used scale according to the genesis, age, and mean height of the stand. The relative basal area (BA_r) was calculated by the formula:

$$BA_r = \sum BA_{\partial} / \sum BA_H$$

where $\sum BA_{\partial}$ is the basal area of the valuated tree stand, m², and $\sum BA_H$ is the basal area of the standard tree stand, m².

2.2.2. Silvicultural Characteristics of the Site

The planting was characterized as average by the value of the relative completeness (Table 1), calculated from the sum of the cross-sectional areas of the trunks at a height of 1.3 m with a density of forest cultures of 3100 pcs. per 1 ha, Bonitet class of II, 5. Forest type was heather pine forest.

Table 1. Descriptive statistics of the main forest inventory variables of the site of study and the content of nitrogen and boron in the needles of the year before exposure (May 2014).

Variable	Mean	±SE	V _{min}	V _{max}
Stocking rate, 1 k st.—1000 stems	3.10	-	-	-
Diameter at breast height, D	6.20	0.154	5.00	8.30
Tree height, h	5.53	0.116	4.40	6.45
Stand basal area all trees, m ² ·ha ⁻¹ (BA)	9.35	0.655	6.08	16.76
Relative basal area all trees, (BA _r)	0.64	0.045	0.42	1.15
Volume, m ³ ·ha ⁻¹ (V)	34.00	2.30	-	-
Quality class, Q	II, 5	-	-	-
Tree age, years	16	-	-	-
Nitrogen, % DW needles	1.3	0.1	1.2	1.4
Boron, µg g ⁻¹ DW needles	16.0	0.2	15.9	16.2

±SE—Standard Deviation; V_{min}—Minimum Value; V_{max}—Maximum Value.

The ground cover in the area was poor, represented chiefly by *Cladonia*, true mosses (Bryales), heather (*Calluna vulgaris*), and occasional cowberry plants (*Vaccinium vitis-idaea*) (Figure 2).

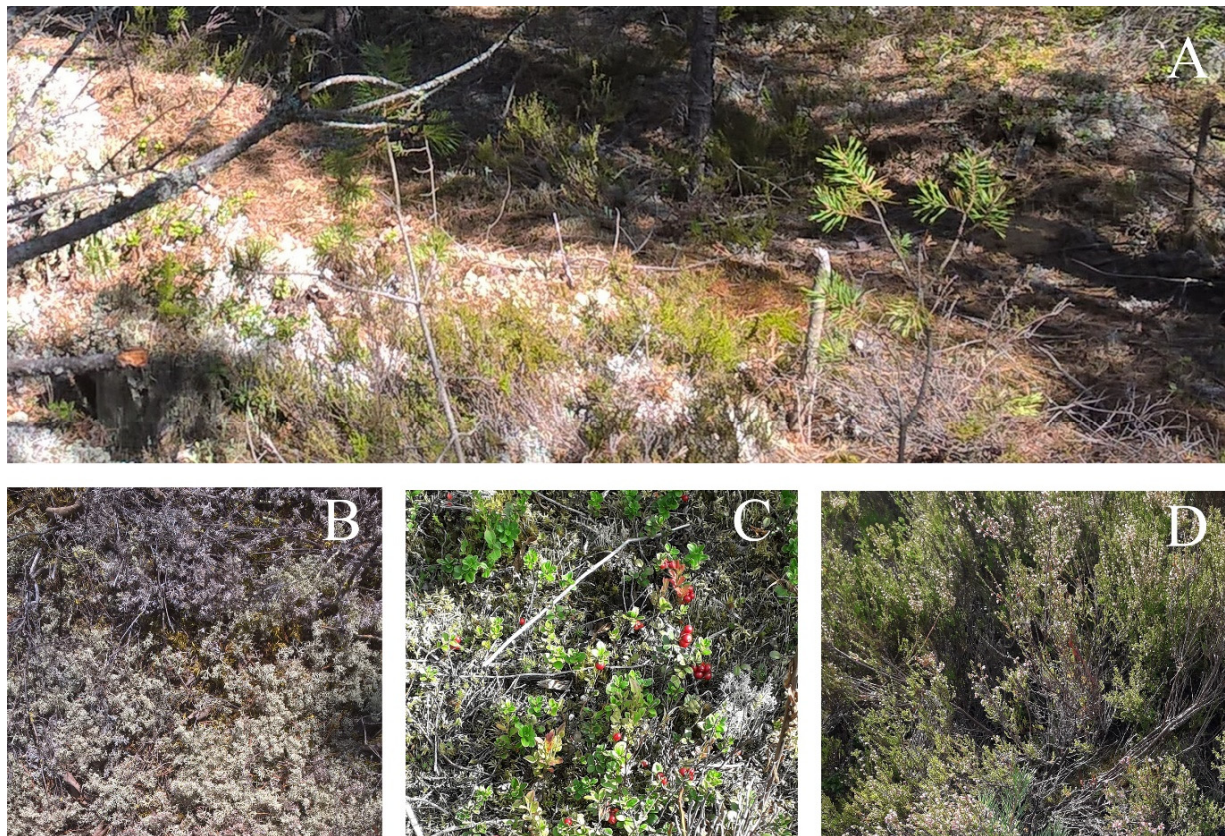


Figure 2. Ground cover of the study area: Overview image (A); *Cladonia rangiferina* (B); *Vaccinium vitis-idaea* (C); *Calluna vulgaris* (D).

2.2.3. Characteristics of Soil Conditions

The soil cover was a combination of sandy Albic Podzol and Entic Podzol [47]. The soil surface was lined with a shallow (ca. 1 cm) forest floor composed of litter (needles, bark, pine cones, and some dwarf-shrub leaves) in different stages of decay, with a substantial proportion (ca. 30–50%) of mineral sand particles. The upper soil layer was a disturbed, turbated horizon [E + BFH]tur 10–12 cm thick.

Soils in the experimental and control plots had an acid reaction (Table 2). Acidity was the highest in the top organic horizon, pH was 3.7, and the values in mineral horizons gradually moved towards weakly acidic, to pH was 4.8–4.9. Base saturation of the soils was low, reaching a maximum of 30–40% in the lower horizons.

Table 2. Physicochemical soil properties of the plots before exposure.

H	D, cm	pH _{KCl}		V *		C Org *		N *		K ₂ O **		P ₂ O ₅ **		B ***	
		M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
O	0–1	3.7	0.1	0		10.7	0.3	0.29	0.05	52.6	11.4	10.34	2.6	0.3	0.04
EBHtur	1–10	3.9	0.1	0		2.5	0.3	0.04	0.01	1.7	0.03	5.2	0.6	0.3	0.02
BF	10–25	4.6	0.1	0		0.7	0.1	0.1	0.05	1.3	0.2	15.2	1.3	0.2	0.01
BC	25–88	4.9	0.1	27.2	7.5	0.3	0.1	0.03	0.01	1.3	0.2	14.9	2.6	<0.01	
C	>88	4.8	0.2	31.5	10.3	0.32	0.03	0.1	0.05	1.8	0.6	17.5	3.6	<0.01	

H—horizon; D—depth; V—base saturation; C Org—organic carbon content; N—nitrogen content; K₂O—content of mobile K₂O; P₂O₅—content of mobile P₂O₅; B—content of boron; M—mean; SE—standard error. *—% oven-dry soil; **—mg 100 g^{−1} oven-dry soil; ***—mg kg^{−1} oven-dry soil.

The content of organic carbon (C) and total N in soil was low. Their concentrations were the highest in the upper horizons, with 2.5% C and 0.04% N in the [E + BFH]tur

horizon underlying the forest floor, and declined significantly down the profile to 0.3% C and 0.01% N. Carbon content in the forest floor was rather low—around 10%, because the floor was young, just beginning to form. Labile potassium (K) content in mineral horizons of the soil was very low—below 2 mg per 100 g^{−1} soil. This macroelement typically resides in the top organic horizon—the forest floor, where its content was the highest—40–70 mg 100 g^{−1}. Labile phosphorus (P) content was somewhat elevated, or even high in some horizons, which is typical of Karelian soils [48]. Labile B content in the soil was low, declining with depth—from 0.3–0.4 mg kg^{−1} in upper horizons to below the detection limit in horizons BC and C. According to the gradation of labile B content, the soils in our study belonged to the “poor” and “very poor” category [49]. This is typical of coarse-textured acidic soils [50].

2.3. Experimental Design

The field study consisted of a randomised block design with three replicates of 11 N fertiliser treatments, where N treatments were randomised within each block. Blocks, which were geographically defined, were confounded with B treatments. Plots were marked out, and trees measured in May 2014 when trees were 16 years old. Treatments of B (blocks) were: (B0) without boron fertilizers, (B3) application of boron fertilizers in the form of aqueous boric acid solution at a dose of 3 kg ha^{−1}, and (B6) the same application at a dose of 6 kg ha^{−1}. N fertilizers were applied in dry ammonium nitrate form in 0 to 1000 kg ha^{−1} doses with a 100 kg ha^{−1} step (Table 3).

Table 3. Indexation of N and B doses.

	N0	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10
B0	0/0	0/100	0/200	0/300	0/400	0/500	0/600	0/700	0/800	0/900	0/1000
B3	3/0	3/100	3/200	3/300	3/400	3/500	3/600	3/700	3/800	3/900	3/1000
B6	6/0	6/100	6/200	6/300	6/400	6/500	6/700	6/700	6/800	6/900	6/1000

Doses of B is in the numerator (kg ha^{−1}); doses of N in the denominator (kg ha^{−1}).

The soil in the experimental plots was fertilized on a single occasion in the first third of June 2014. B doses of 3 and 6 kg/ha were selected as optimal and excessive for Arg accumulation, respectively. For the study, we used 3 trees with a height of 5.53 ± 0.116 m from each plot. The content of N and B in the one-year-old needles was determined before fertilization in May 2014 (Table 1). The needles of the current year were chosen because they accumulate Arg most efficiently. Samples of current year needles were collected for N, B, and Arg analyses from the third whorls off the tree tops on clear days (at 2–3 p.m.) in the last thirds of September 2014 and March 2015. The experiment lasted 10 months, from early June 2014 to late March 2015. The dates of determination of Arg in needles are due to the fact that a high level of Arg in needles is observed during the period from September to April with maxima in September and February–March [45]. The content of nitrogen and boron in the needles was determined once in September because it remains stable during the autumn-winter-spring period [5,15].

2.4. Analysis Methods

2.4.1. Needle Analysis Methods

The needles were fixed by lyophilization. Amino acids were extracted from the needles with hot water (60 °C) for 30 min. Arg content in needles was determined by the Sakaguchi test, customized for assaying the amino acid in conifers [51], with a control test at reference points using automatic amino acid analyzer AAA-339 (MIKROTECHNA, Czech Republic).

The N content in the needles was determined by the Kjeldahl method, and the B content was analyzed spectrophotometrically using azomethine-N. The results of the analysis of N, B, and Arg in needles are presented in the corresponding units on dry weight

(DW). DW was determined after drying the needle samples in a thermostat at 70 °C for 24 h.

2.4.2. Soil Chemical and Physical Analysis

The following agrochemical parameters were determined in soil samples using conventional techniques: pH in salt suspension potentiometrically (pH-meter Hanna 2210, HANNA Instruments Deutschland GmbH, Vöhringen, Germany); total C and N content by CHNS analyzer (Perkin Elmer's 2400 Series II CHNS/O, Perkin Elmer, Norwalk, CT, USA); labile P and K in 0.2 N HCL extract using Kirsanov's method; P by color reaction with ammonium molybdate completed by spectrophotometry (OKB Spectr SF-2000, St. Petersburg, Russia); K by the atomic emission technique (Shimadzu AA 7000, Shimadzu, Kyoto, Japan); labile B using the method of Berger and Truog by colorimetric assay with azomethine-N.

2.4.3. Data Analysis

A statistical regression model describing the relationship between height and diameter for stand was constructed in order to calculate tree mean height in the plots.

Regression analyses were performed to evaluate the effects of N and B fertilizers on Arg content in one-year-old needles. Arg concentrations were used as dependent variables and N fertilizers as independent variables. Polynomial relationships were used, and the coefficients of determination (R^2) were determined for each treatment variant.

Analysis with two-sample t-test with equal variances was applied to estimate significance of post hoc differences between treatments of different doses of B fertilized at the same level of N (Tukey's test at a significance level of $\alpha = 0.05$).

Regression analysis was used to reveal the relationship between the content of N and Arg in pine needles at different levels of B provision based on the results of the experiment on the application of N and B fertilizers. All data shown in figures and tables are original, with untransformed mean values ± 1 standard error (SE). Statistical analysis was conducted using a Microsoft Office Excel 2016 spreadsheet.

Analyses of Arg, N, and B content in the needles and B content in the soil were carried out with equipment of the Core Facility Analytical Laboratory of the Forest Research Institute KarRC RAS. Agrochemical analyses of the soil were performed at the Forest Soil Science Laboratory of the Forest Research Institute KarRC RAS.

3. Results

3.1. Nitrogen Content in Needles

N content in one-year-old needles was 1.2% in the control plot in the last third of September 2014 (Figure 3). It rose to 2.1% after application of N fertilizers to the soil at dose 100 kg ha⁻¹ in all variants of B supply.

N levels in needles in the treatments with nitrogen at 200–400 kg ha⁻¹ were the same as in the variants with boron deficit and its surplus, rising to 2.8%.

As N dosage was increased in B-deficient plots, N levels in needles rose (to 3.2%) until the N dose was 800 kg ha⁻¹, and then, as N doses were elevated further, the level declined to 2.5%. In the B-excessive variant, N content in needles changed little between fertilization at 400 kg ha⁻¹ and the highest dose. In the variant with optimal B supply (3 kg ha⁻¹), N content in needles increased more significantly with elevation of fertilization doses to 400 kg ha⁻¹ (to 3.6%) than in the plots with B deficit and surplus, but starting at 600 kg N ha⁻¹ and higher, it declined to the same level as in the other two variants of B supply. Thus, the optimal dose of B, as opposed to its deficit and surplus, augmented N levels to the maximum in needles where N fertilization doses were 200–500 kg ha⁻¹. Its levels became equal (up to 2.5%) in the needles of trees of three variants of B supply at the nitrogen dose 1000 kg ha⁻¹.

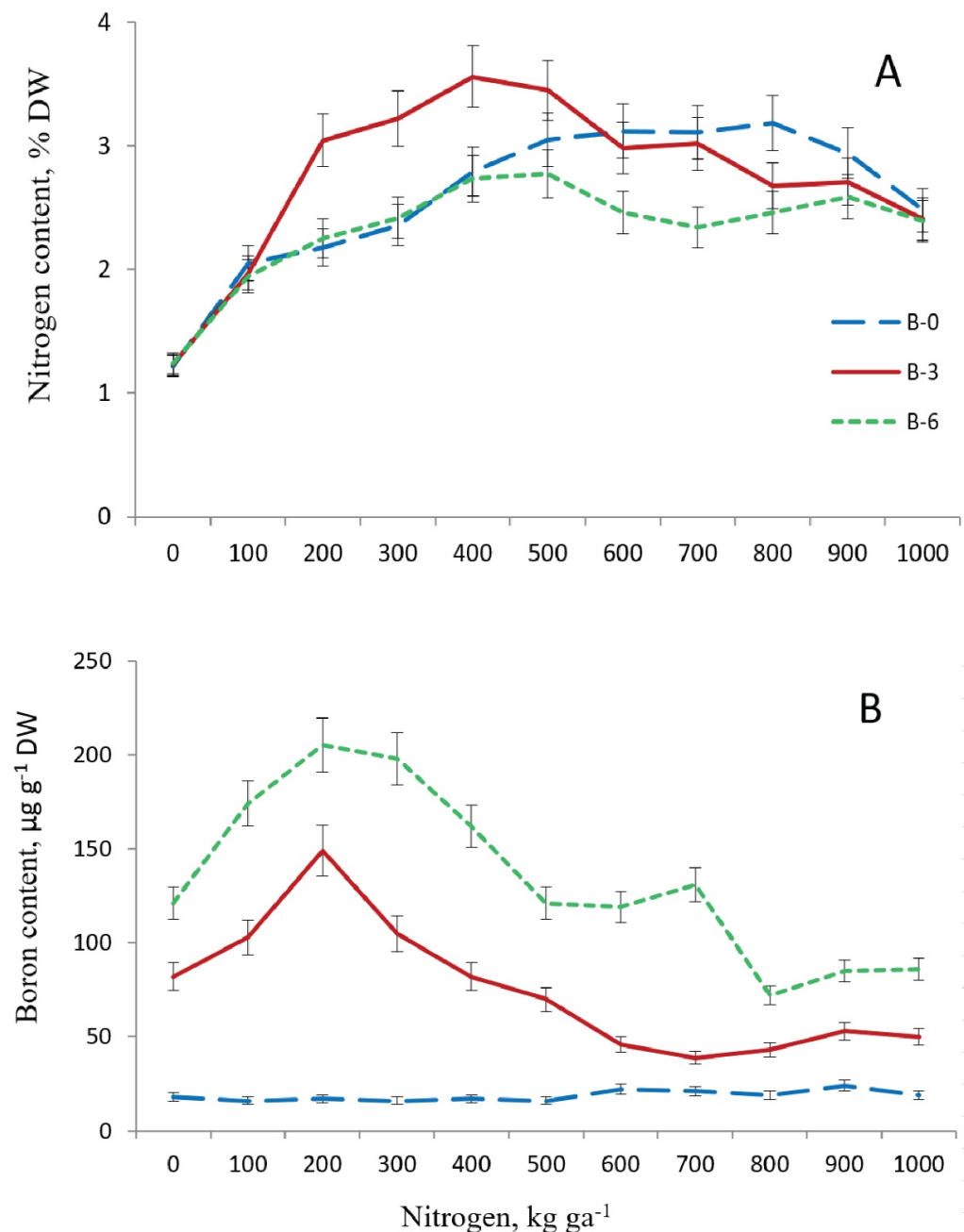


Figure 3. Nitrogen (A) and boron (B) content in one-year-old needles of 16-year old *Pinus sylvestris* L. depending on the doses of their application to soil. B0, B3, B6—boron doses of 0, 3.0, 6.0 kg ha⁻¹, respectively. September 2014 (observations per plot n = 3).

3.2. Boron Content in Needles

B content in one-year-old needles of pine trees from the control plot in September 2014 was low and amounted to 17 μg g⁻¹. In B-deficient settings, the element's level in needles changed insignificantly when different N doses were applied. In B-fertilized plots, foliar B content in pine trees was higher than in B-deficient plots in all N dosage variants. In the variants with optimal and excessive B supply, its foliar content was more significantly augmented by application of the second dose of N compared to the first dose, i.e., from 103 and 174 μg g⁻¹ to 149 and 205 μg g⁻¹, respectively. As N doses were further raised, foliar B levels declined, starting with 300 kg N ha⁻¹ in B-optimal plots and starting with 400 kg N ha⁻¹ in B-excessive plots, yet remaining higher in the variants with B boron surplus compared to boron optimum. The decline in foliar B content continued until the

nitrogen dose of 600 kg ha⁻¹ in B-optimal plots and until 800 kg N ha⁻¹ in B-excessive plots. When even higher N doses were applied, B content in needles changed little in the two B supply variants. In treatments with the highest N dose, B content in needles was 50 and 86 µg mg⁻¹ in B-optimal and B-excessive plots, respectively. Thus, application of the second N dose coupled with optimal and excessive B supply promoted foliar B content more significantly than the first N dose. Elevation of N doses to higher levels, from 300 to 600 kg ha⁻¹ (for B optimum) or from 400 to 800 kg ha⁻¹ (for B surplus), entailed a decline in the B content in needles.

3.3. Arginine Content in Needles

The treatments of N fertilizers alone and combined with B significantly increased the content of Arg in one-year-old needles of pine trees (Figure 4, Table 4). Differences in tree needle Arg concentration between the 11 treatments of N levels at the three treatments of B levels (B0, B3, and B6), analyzed with a two-sample t-test with equal variances, was significant ($p = 0.05$) in September in all variants except N0 (Table 5). In March, no significant differences were observed in variants N0, N4, and N10. Followed for pairwise comparison, when the analysis of variance showed significance ($p = 0.05$), it was possible to reveal that the accumulation of Arg in needles significantly differed in three B levels in variants N3–N5 in September and N3 and N5 in March. Regression analysis results between Arg concentration and treatments of N levels are shown in Figure 4 and Table 4. In the last third of September 2014, foliar Arg levels in plots with soil B deficiency (B0) were similar to the control in the treatments with the first three N doses (N1–N3) and then increased to 145.0 µmol g⁻¹ in the treatments with N at 600 kg ha⁻¹, and they showed little change at higher N doses.

The amount of Arg in the needles of trees in the control variant (B0N0) during the study period did not exceed 8.6 µmol g⁻¹. Optimal B availability to pine, unlike its shortage and surplus, helped significantly augment foliar Arg content (to 102.3 µmol g⁻¹) when coupled with the placement of the second and third N doses (N2 and N3). In the variants with B deficit or surplus coupled with the same N doses, on the other hand, the level of amino acid in needles was lower—17.2 and 46.0 µmol g⁻¹, respectively. In treatments with N doses above 500 kg ha⁻¹, differences in amino acid content between the B deficit and B optimum variants became less significant (Table 5), while the Arg level in the variant with B surplus was lower than in the other two variants.

In the last third of March 2015, as well as in September 2014, Arginine content in one-year-old pine needles in B-deficient plots was similar to that of the control when the first two N doses were applied; as N doses were raised to 500 kg ha⁻¹, the amino acid content rose to 107.5 µmol g⁻¹, and when the N dose was increased further, the change in Arg was insignificant. In plots with optimal B supply in March as well as in September, the first N dose had no significant effect on Arg content in needles; the second and further N doses, up to 500 kg ha⁻¹ inclusively, significantly promoted the Arg level to 175.9 µmol g⁻¹. Foliar Arg level in March, as compared to September, was promoted even more significantly by the application of N starting with the second dose in the B-optimal variant and with the third dose where B was in surplus. The level of the amino acid in needles in the variant with B deficiency, on the contrary, was lower in March compared to September in treatments with N in doses above 500 kg ha⁻¹ and was also lower in the N treatments of 300 kg ha⁻¹ and higher compared to trees from B-fertilized plots.

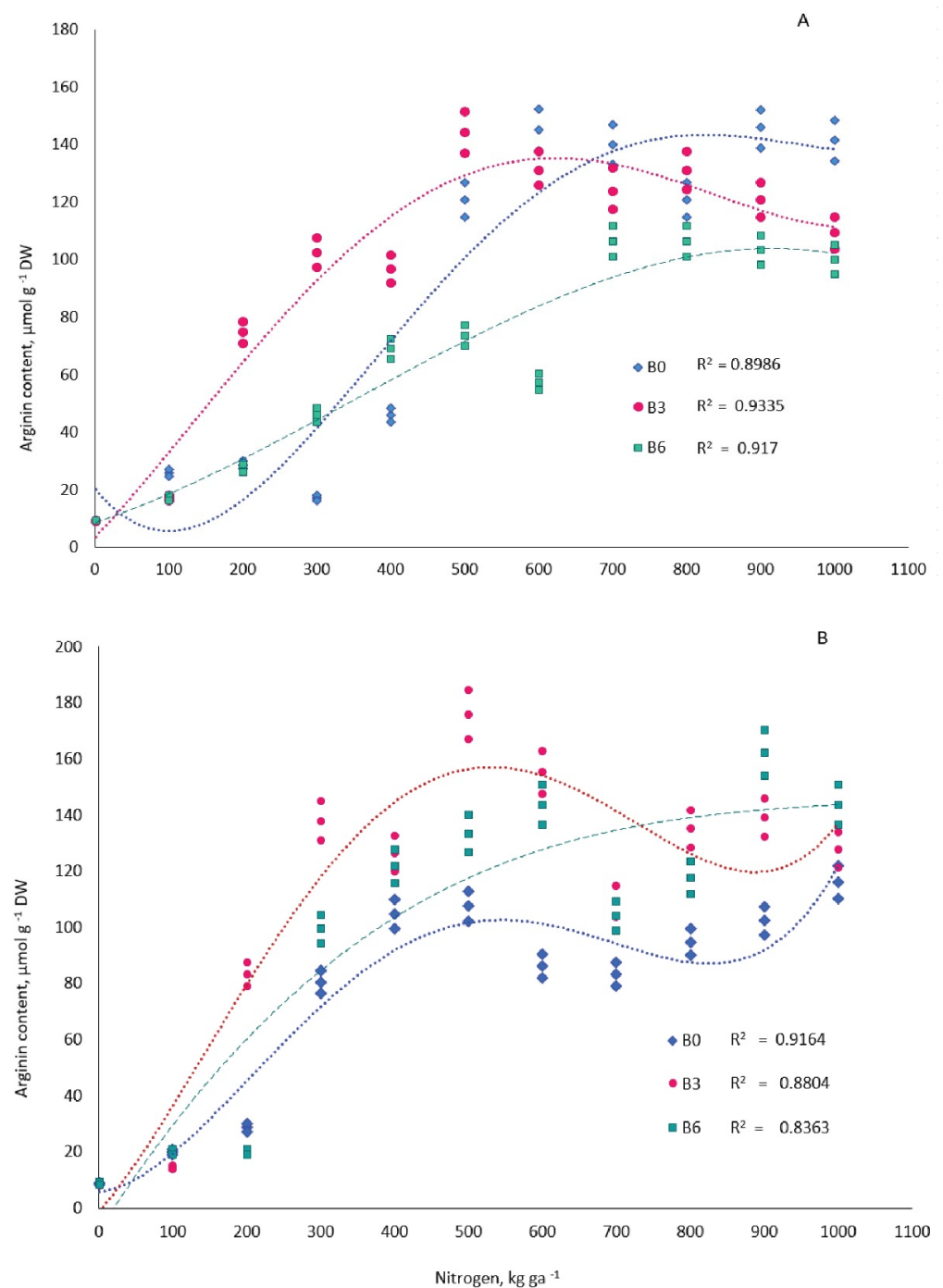


Figure 4. Dependence of the accumulation of arginine in the one-year-old needles of 16-year old *Pinus sylvestris* L. on the nitrogen fertilizers on three backgrounds of boron supply. B0, B3, B6—boron doses of 0, 3.0, 6.0 kg ha⁻¹, respectively. (A) September 2014; (B) March 2015 (for regressive equation, see Table 4).

3.4. Relationship between the Content of N, B, and Arg in Pine Needles

Arg accumulation in needles in September positively correlated with their N content in all B supply variants (Figure 5).

Table 4. Regression analyses were performed using arginine concentration ($\mu\text{mol g}^{-1}$ DW) in the one-year-old needles of 16-year-old *Pinus sylvestris* L. as dependent variables (y) and N fertilizers (kg ga^{-1}) as independent variables (x). Polynomial relationships and the coefficients of determination (R^2) were determined for each variant of boron treatment.

Treatment	Regressive Equation	R^2
September 2014		
B0	$y = 9 \times 10^{-10}x^4 - 2 \times 10^{-6}x^3 + 0.0019x^2 - 0.3177x + 20.476$	0.8986
B3	$y = 6 \times 10^{-10}x^4 - 1 \times 10^{-6}x^3 + 0.0004x^2 + 0.2673x + 3.4668$	0.8986
B6	$y = -1 \times 10^{-7}x^3 + 0.00026x^2 + 8.5814$	0.8986
March 2015		
B0	$y = 2 \times 10^{-9}x^4 - 3 \times 10^{-6}x^3 + 0.0013x^2 + 0.034x + 5.780$	0.9164
B3	$y = 2 \times 10^{-9}x^4 - 3 \times 10^{-6}x^3 + 0.001x^2 + 0.3066x - 1.3232$	0.8804
B6	$y = 1 \times 10^{-7}x^3 + 0.0004x^2 + 0.4149x + 8.1520$	0.8363

Table 5. Two-sample *t*-test analysis with equal variances ($\alpha = 0.05$) was applied to estimate significance of post hoc differences between treatments of different B-fertilized doses at the same levels of N on arginine content ($\mu\text{mol g}^{-1}$) in one-year-old needles of 16-year old *Pinus sylvestris* L. in September 2014 and March 2015. Doses of N were from 0 to 1000 kg ha^{-1} with a 100 kg ha^{-1} step (N0, N1, . . . N10) and B doses were 0 (B0), 3.0 (B3), and 6.0 (B6) kg ha^{-1} . Different lower case letters (a, b, or c) beside means indicate significant post hoc differences between treatments (Tukey's test). ($p = 0.05$).

Needle Arg Concentration, $\mu\text{mol g}^{-1}$ DW							t-Statistic		
N, kg ga^{-1}	B0		B3		B6		p-Value B0–B3	p-Value B0–B6	p-Value B3–B6
	M	$\pm\text{SE}$	M	$\pm\text{SE}$	M	$\pm\text{SE}$			
September 2014									
0	9.2 a	0.64	9.1 a	0.64	9.3 a	0.65		0.802	
100	25.9 a	1.81	17.2 b	1.20	17.2 b	1.20	0.001	0.001	0.947
200	28.7 a	2.01	74.7 b	5.23	27.6 a	1.93	<<0.05	0.393	<<0.05
300	17.2 a	1.20	102.3 b	7.16	46 c	3.22	<<0.05	<<0.05	<<0.05
400	46 a	3.22	96.6 b	6.76	69 c	4.83	0.001	0.001	0.001
500	120.7 a	8.45	144.3 b	10.10	73.6 c	5.15	0.044	0.000	0.000
600	145 a	10.15	131 a	9.17	57.5 b	4.03	0.202	0.000	0.001
700	140 a	9.80	123.6 ac	8.65	106.3 c	7.44	0.137	0.003	0.021
800	120.7 a	8.45	131 a	9.17	106.3 b	7.44	0.264	0.036	0.007
900	146 a	10.22	120.7 a	8.45	103.4 b	7.24	0.050	0.001	0.020
1000	141.4 a	9.90	109.2 b	7.64	100 b	7.00	0.021	0.001	0.098
March 2015									
0	8.6 a	0.60	8.5 a	0.60	8.7 a	0.61		0.893	
100	20.1 a	1.41	14.4 b	1.01	20.2 a	1.41	0.016	0.954	0.015
200	28.7 a	2.01	83.3 b	5.83	20.1 c	1.41	0.000	0.013	0.000
300	80.5 a	5.64	137.9 b	9.65	99.4 c	6.96	0.003	0.023	0.017
400	104.6 a	7.32	126.4 a	8.85	121.8 a	8.53		0.083	
500	107.5 a	7.53	175.9 b	12.31	133.3 c	9.33	0.005	0.049	0.029
600	86.2 a	6.03	155.2 b	10.86	143.7 b	10.06	0.003	0.004	0.400
700	83.3 a	5.83	109.2 bc	7.64	104 ac	7.28	0.031	0.055	0.583

Table 5. Cont.

N, kg ga ⁻¹	Needle Arg Concentration, $\mu\text{mol g}^{-1}$ DW						t-Statistic		
	B0		B3		B6		p-Value B0–B3	p-Value B0–B6	p-Value B3–B6
	M	$\pm\text{SE}$	M	$\pm\text{SE}$	M	$\pm\text{SE}$			
800	94.8 a	6.64	135.1 bc	9.46	117.8 ac	8.25	0.013	0.058	0.170
900	102.3 a	7.16	139.1 b	9.74	162.1 b	11.35	0.021	0.006	0.136
1000	116.1 a	8.13	127.6 a	8.93	143.7 a	10.06		0.313	

N—nitrogen dose; M—average value needle arginine concentration ($\mu\text{mol g}^{-1}$); $\pm\text{SE}$ —standard error based on within-plot error (n = 3), a, b, c beside means indicate significant post hoc differences between treatments.

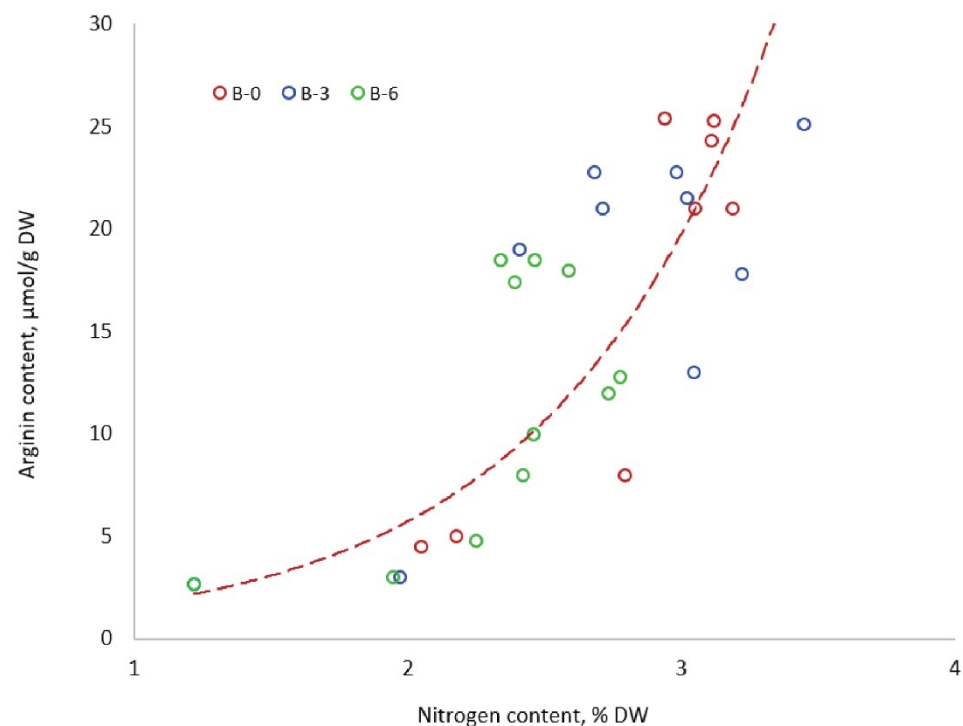


Figure 5. Dependence of the accumulation of arginine in the needles of *Pinus sylvestris* L. on the nitrogen content on three backgrounds of boron supply. B0, B3, B6—boron doses of 0, 3.0, 6.0 kg ha⁻¹, respectively. September 2014.

To quantify the relationship between the content of N, B, and Arg in pine needles under the influence of the studied treatments, a nonlinear exponential regression was fitted written as $y = \alpha e^{\beta x} + \varepsilon$, where independent variable (x) is nitrogen content in pine needles (CN), dependent variable (y) is arginine content in needles (CArg), ε is error, and R^2 is the coefficient of determination (Table 6).

Table 6. The estimations of parameters (α and β) of the exponential function for the three levels of boron supply (B0, B3, and B6) and for the entire dataset (SUM), written as $y = \alpha e^{\beta x} + \varepsilon$, where independent variable (x) is nitrogen content in pine needles (CN), dependent variable (y) is arginine content in needles (CArg), ε is error, and R^2 is the coefficient of determination.

Variant of Boron Level	α	β	R^2
B0	1.3853	1.4469	0.9334
B3	4.8085	1.0469	0.4979
B6	0.8969	1.7335	0.4399
SUM	2.3642	1.2992	0.6985

4. Discussion

4.1. Efficiency of Nitrogen Fertilizers in Forestry

Many experiments have been carried out on the effect of nitrogen and boron fertilizers on the growth of conifers because N and B are elements that plant growth in forest ecosystems—in many ecosystems of Fennoscandia [1–7,14–16,18,19,39,44,52,53]. Doses of N and B application were tested in a wide range to increase the growth rate of coniferous trees.

Since the purpose of this experiment was to identify the most effective doses of nitrogen and boron for the accumulation of arginine in the needles of Scots pine, tests on the introduction of a wide range of nitrogen doses into the soil, from 100 to 1000 kg ha^{−1}, and two variants of boron doses, 3 and 6 kg ha^{−1}, were held.

One of the most obvious effects of N fertilization is elevation of N content in needles for several years after treatment [16]. In this experiment, the high foliar N level (3.1–3.6%) in 16-year-old Scots pine growing on N- and B-poor sandy soil fertilized with 200–500 kg N ha^{−1} in the variant with optimal B supply versus its deficit and surplus was due to the stimulatory effect of both the said doses of N and the optimal B dosage on N intake by coniferous plants. The stimulating effect of B on N (¹⁵N) supply in Scots pine was shown [5]. This study revealed the levels of N and B supply to 16-year-old pine that generate this effect, namely, N doses of 200–500 kg ha^{−1} with foliar N content of 3.1 to 3.6% and the B dose of 3 kg ha^{−1} with foliar B content of 70–149 µg g^{−1}.

The reduction in foliar N content when its application to soil was raised to 600 kg ha^{−1} and greater in the optimal and especially excessive B supply variants and to 900–1000 kg ha^{−1} in the variant with B deficit proved these N doses were excessive and inhibited N intake by coniferous plants. Excessive N nutrition in Scots pine trees reduced CO₂ assimilation and slowed down the transport of ¹⁴C assimilates from needles, causing malformation of the leading bud and disrupting the development of needles, reducing the number of root tips, and inhibiting the formation of new clavate and furcate roots as the most physiologically active types, which are the first to respond to changes in soil nutrients [53]. Examples are known of tree growth inhibited and die-back promoted by fertilization with high N doses [7]. High nitrogen dosage can affect the environment [7,19,52,54]. Environmental regulations worldwide have exhibited a high degree of efficacy with respect to decreasing emissions of pollutants, including and especially N compounds. As a result, atmospheric deposition of N has declined over the past few decades on the global scale [8,24,40,55–59].

4.2. Mutual Influence of Nitrogen and Boron Fertilizers

N-supplementation to plants can change the balance of other nutrients [44]. The positive effect of N fertilization on growth in conifers was found to be reduced when combined with liming [14]. This was supposedly due to B deficiency, since its typical morphological signs (apical growth impairments or top dieback) and reduced B concentration in pine needles were observed in liming experiments [14]. Signs of B deficiency in liming treatments were detected in Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) in Scandinavia, in radiata pine (*Pinus radiata* D. Don) in New Zealand, and in other coniferous species in different regions [6,7,12,13]. They became less pronounced in these species when the sites were fertilized with B alone or in combination with N. Fertilization with N reduces B content in coniferous plants [6,60]. The foliar B level of 12 µg g^{−1} DM is considered to be the lowest acceptable threshold for B content in needles of a stand in N fertilization decision making assessments. B placements at 1.5–3.0 kg ha^{−1} to sites with B-deficient soils were shown to be sufficient for maintaining a favorable B status and active growth of trees over a long term, without the risk of growth impairment and terminal shoot death, which are typical in B-deficient conditions [6].

The characteristics of the boron status in coniferous plants are presented in [15,60–62]. The accumulation of boron in the needles occurs mainly due to its intake from the soil. In pine and spruce, boron recycling is practically absent. Most of the boron is localized in the needles, where its content changes under the influence of the supply of boron to plants to the greatest extent compared to other organs.

In our experiment, not only did B supply influence N intake by needles, but N supplementation, too, influenced foliar B accumulation in the cases of optimal and excessive B levels in soil. The concentration of B in the needles decreased with increasing doses of nitrogen application, from 300 and 400 kg ha⁻¹ to 700 and 800 kg ha⁻¹, respectively, at an optimum and an excess of B, but this was not due to the dilution effect that occurs with an increase in the weight of needles in the case of intensification growth. This experiment in September showed no significant differences in either mass or length of current-year needles depending on the N and B doses applied to soil (unreported data). According to previous studies, after fertilization in June with 120 kg ha⁻¹ N (¹⁵N), 15-year-old Scots pine trees chiefly stored N in their organs in the first growing season, while the next year, the N was utilized to stimulate growth [5]. In addition, N-induced foliar biomass gain in *Pinus sylvestris* and *Picea abies* mainly happened in the second and third growing seasons after fertilization [14]. The most pronounced effect of N fertilization on pine growth was observed in the first five to ten years after the treatment, but the effect lasted even longer on a minor scale [18,19].

B content declines in pine needles after fertilization with B intake, as observed in [1]. It is remarked that an important role in B availability to woody plants belongs to the edaphic conditions—elevated content of NH₄⁺, Fe³⁺, and Al³⁺ ions in soil—and its pH elevation by liming promote B fixation by soil particles.

4.3. Influence of Nitrogen and Boron Supply on Arg Content

N and B nutrition is a significant factor for the content of free amino acids in conifers [5,30,39,45]. Where N content in Scots pine needles exceeds 1.9% dry matter, it would be stored in the soluble nonprotein fraction, mainly in the free amino acid pool [42]. Alteration of the free amino acid profile in tissues of conifers can be triggered by a misbalance in mineral nutrition or by other adverse environmental conditions [41,42,63]. Many plant species were shown to accumulate N-rich amino acids in response to high N availability and low phosphorus availability [64]. The free amino acid pool in conifers growing on N-rich soils is usually dominated by Arg and has a high content of glutamine, ornithine, and lysine [39,40,63]. Featuring the highest N/C ratio among proteinogenic amino acids, Arg is the most suitable form for organic N storage in plants. Its metabolism is of key importance in N distribution and utilization in plants [65]. Arg synthesis can be stimulated by a shortage of other macro- and microelements and appears to be a general response to the stresses that inhibit growth in conifers [39], e.g., excessive N deposition (up to 35 kg ha⁻¹), which caused damage or death of young shoots in 10–20-year-old Scots pine, multiplied foliar Arg content up to 103-fold, raised the concentration of soluble proteins, but inhibited the accumulation of certain polypeptides believed to be important for tree resistance to adverse winter conditions [40]. A proposition regarding Rubisco is that apart from its role in C assimilation, this protein is a compound storing surplus N [66].

Our earlier study of the annual dynamics of the content of Arg in the needles of 10-year-old Scotch pine at different times of applying N at a high dose (300 kg ha⁻¹) and B at the optimal dose (3 kg ha⁻¹) into the soil showed that the greatest effect was observed in one-year-old needles when fertilizing in June [45]. The amino acid content in one-year-old needles exceeded its level in two-year-old needles by a maximum of 4.0 times. The maximum amino acid level in annual needles in annual dynamics was noted in September and February–March, with a high level until April. Further, the content of Arg in one-year-old and two-year-old needles decreased, which can be explained by the reutilization of Arg N for growth processes that actively proceed in a coniferous plant under conditions of Karelia, starting from May [5]. On 15-year-old Scotch pine in experiments with ¹⁵N at a dose of 120 kg ha⁻¹, we showed an increase in the intensity of tree growth in the second and third years after exposure to N [5]. As a result of N reutilization for growth, the content of Arg in the needles of both the first and second years in the following year was lower than in the year of fertilization (according to our unpublished data).

The significant rise in the Arg content of one-year-old pine needles (up to $30.6 \mu\text{g mg}^{-1}$) that we observed in our experiment when foliar N level increased (to 3.6%) in all variants of B supply testifies to the dominant role of N in the accumulation of this amino acid. An increase in foliar Arg level in pine needles in September, and especially in March, with an increase in N doses from 200 to 500 kg ha^{-1} under conditions of B optimum (dose 3 kg ha^{-1}), in contrast to the variants of B deficiency, indicates the effect of B on the accumulation of amino acids. Where N supply to a coniferous plant is high compared to other nutrients, organs and tissues are unable to utilize all of the N for protein synthesis and store it in the form of N-rich amino acids, which, in Scots pine, is mainly Arg [30,40,43,45]. Activating N intake by roots, B promotes Arg synthesis in needles [5]. At the same time, stimulating carbohydrate transport in plants and thus eliminating the shortage of sugars—a precondition for the activity of the enzymes arginase, urease, and argininedecarboxylase—B can indirectly inhibit Arg catabolism [67]. As a result, B apparently stimulates the synthesis and inhibits the catabolism of Arg, thus facilitating its accumulation in needles, as corroborated by experimental data [45]. This experiment revealed the ranges of the N and B supply to pine in which these mechanisms can be implemented. Namely, B nutrition optimization for coniferous plants with B fertilization at 3 kg ha^{-1} and its foliar content of $70\text{--}149 \mu\text{g g}^{-1}$ coupled with high foliar N content of 3.1–3.6% and $200\text{--}500 \text{ kg ha}^{-1}$ N fertilization generated a maximal accumulation of the amino acid in needles in September.

The even higher (compared to September) foliar Arg accumulation stimulating effect of the optimal B dose coupled with certain doses of N observed the next March was probably due to the action of B on various metabolic processes [68,69] activated in needles before the onset of the growing season. In March and April, protein hydrolysis takes place in one-year-old needles, and pools of free amino acids, amides, and other N compounds to be transported to growing buds are formed [5]. In conifers, Arg and its derivatives are directly involved in the processes of transition to dormancy and preparation for bud break in spring [40,43]. Arg is a key element of the urea cycle in woody plants, especially at the onset of their growth in spring. To better understand the contribution of Arg to the efficiency of N utilization by plants, researchers investigate the regulatory mechanisms responsible for its content in organs and tissues for the prevalence of N storing or metabolizing processes and the generation of N oxide as a signaling agent for growth, reproduction, or stress defense [70].

This experimental study produced interesting results regarding the effect of B availability to pine on Arg retention in needles in the period from September to March. Where B was in deficit and N doses applied were above 500 kg ha^{-1} , the amino acid level in needles in March was lower than in September. Where pine was additionally supplemented with B, on the other hand, foliar Arg content did not decline over this period. These findings support the assumption that B has an inhibiting effect on Arg catabolism in needles. The mechanism for the suppression of the activity of Arg catabolism enzymes by B possibly involves both elimination of carbohydrate deficiency as well as stimulation of the inhibitors of these enzymes. More studies are needed to identify the putative role of arginase inhibitors, especially, which can be expected to exhibit high activity with such a high level of Arg accumulation in needles. Further research of the biochemical and physiological characteristics of the enzymes mediating Arg metabolism will provide a new insight into its role in the efficiency of N use by plants [70].

The content of Arg in one-year-old needles was studied because earlier, when studying the distribution of Arg in the pine crown, it was shown that one-year-old needles make the greatest contribution to its accumulation [71]. In continuation of this experiment on the experimental plots, we plan to study the effect of various doses of N and B fertilizers on the biochemical composition of needles as well as on tree growth indicators—height, DHB, annual ring width, and cell wall thickness of early and late wood—8 years after exposure.

Knowledge of the key mediators and triggers of N storage in Arg form and its remobilization are needed in developing technology for producing plant material enriched in Arg and inhibitors of its catabolism. The technology for targeted modification of the chemical

composition and pharmacological properties of the biomass derived from woody plants will enable utilization of new sources of feedstock for BAC. More efficient application of N and B will help improve conifer cultivation conditions and avoid the negative economic and environmental effects from overuse of nitrous fertilizers in forestry. It is interesting to investigate the possibilities for producing environment-friendly, high-performance, organic fertilizers with high N content from Arg-enriched coniferous foliage. Arg-rich needles can be obtained through a specially designed sequence of silvicultural operations.

5. Conclusions

Seeking to optimize the process of enriching coniferous foliage with Arg, the levels of N, B, and Arg in one-year-old needles were determined in 16-year-old Scots pine in relation to the N and B doses applied to soil originally deficient in N and B.

Foliar N content reached a peak of 3.6% when the N dose rose to 500 kg ha⁻¹ and the B dose was 3 kg ha⁻¹. B stimulated N intake by needles in the fertilization treatments with N at 200–500 kg ha⁻¹ and B at 3 kg ha⁻¹. The lack of stimulatory effect of B fertilization, even in the optimal dose, on N intake by needles in fertilization treatments with N at 600 kg ha⁻¹ and greater may be due to the negative effect of N surplus on the functional activity of the tree organism, especially the root system.

The elevated accumulation of B (up to 149 and 205 µg g⁻¹ in the optimal and excessive B supply variants, respectively) in N fertilization treatments at 200 kg N ha⁻¹ should apparently be attributed to the stimulation of functional activity, probably including B transport from roots to needles, in Scots pine at this level of N supply. The decline in foliar B content as N dosage increased, starting at 300 and 400 kg N ha⁻¹, for the optimal and surplus B variants, respectively, may be due to B fixation in soil in an ammonium-rich environment.

Arg content in needles rose to a maximum of 144.3 µmol g⁻¹ in September and 175.9 µmol g⁻¹ in March when N was applied in doses of 200 to 500 kg ha⁻¹ and the B dose was 3 kg ha⁻¹. With these N and B doses, the content of the elements in needles in September was, respectively, 3.1–3.6% and 70–149 µg g⁻¹. In the treatments with higher N doses coupled with B optimum, foliar Arg content was lower than the maximum, and where the lowest N dose was applied, Arg content was near the level in the control.

The comparative analysis of the effect of different B supply options on Arg accumulation in pine needles showed that in the treatments with high N (200–500 kg ha⁻¹) and optimal B (3 kg ha⁻¹) supplementation, foliar Arg content in September and March was higher than that of where B was in deficit. This fact, as well as the elevated foliar Arg level in March in the treatments with 300 kg ha⁻¹ or more of N and additional B supplementation compared with the B deficit variants, speaks in favor of our previous hypothesis that B stimulates Arg synthesis and inhibits its catabolism in conifers.

The results of the study can help optimize the process of enriching Scots pine needles with Arg through dose regulation in N and B fertilization. Although Arg accumulation in needles was maximized by N and B doses of 500 and 3 kg ha⁻¹, respectively, N dosage can be lowered to 300 kg ha⁻¹ to still reach a significant increase in foliar Arg content while saving on fertilizer costs and minimizing the environmental impact of nitrous fertilizers.

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