Use of organic biostimulant for growing Siberian spruce seedlings

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Abstract. This study aims to assess the possibility of using a biostimulant Verva-spruce based on spruce’s natural phenolic compounds to reduce the time of growing planting material with improved features. The targets were seeds and 1-4-year-old seedlings of Siberian spruce, untreated and treated with the biostimulant. The effect of the biostimulant on seed germination, seedlings growth, and the pigment’s content in needles were studied. Results shown that soaking seeds in biostimulant at a concentration of 0.00025% increased the germination energy and accelerated hypocotyl growth. Moreover, using the biostimulant significantly increased the growth rate of experimental seedlings and heightened the amount of green pigment chlorophyll a up to 2.5 times. In 2020, in order to study the dynamics of the qualitative characteristics of the plants grown using biostimulant, experimental forest plantations of 4-year-old Siberian spruce seedlings were planted in the Altai-Sayan mountain taiga area. The experimental plantation will be monitored at least until the closure of the canopy.

1. Introduction

The establishment of economically valuable forest plantations requires using high-quality planting material grown in a short time with minimal costs. Nowadays, reforestation is the highest priority area of forestry. Grown in a short time high-quality planting material determines the success of artificial reforestation. In Siberia, foresters traditionally use several tree species for reforestation with plantation methods. Siberian spruce has a long period of cultivation (3-4 years) due to its biological features. Siberian spruce is one of the main forest-forming species in Siberia and studying its morphological and physiological characteristics is crucial for effective planting material growing. Currently, a wide variety of cultivation methods can increase the quality and quantity of planting material and reduce growth time [1,2].
One proven method of obtaining high-quality planting material is using growth stimulants [3,4]. Growth stimulants have a wide range of positive effects on plants: they accelerate seed germination [5]; increase the intensity of growth, flowering, and ovaries formation [6,7], enhance the development of the root system [8]; and strengthen plant immunity [9]. There are various growth-stimulating agents: Epin, Zircon, Ecosil, Cresacin [10]; however, it is preferable to use organic agents, which are safer in terms of environmental health [11-14].

New commercial product Verva-spruce is a biological agent based on spruce needle extract with spruce flavonoids as active ingredients. Several research works studied its effect on the cultivation of Scots pine seedlings and some conifer species germs [15,16]. These studies have observed that at low concentrations, the agent positively affected the growth of the aboveground part of plants. Thus, they obtained two-year-old Scots pine seedlings with quality indicators meeting the requirements for planting material.

Our research aims to assess the possibility of using the biological agent Verva-spruce to reduce the time of growing the Siberian spruce planting material and obtain seedlings with improved quality features.

2. Material and methods
The material of research were the seeds and seedlings of Siberian spruce grown using the biological stimulant Versa-spruce (JOY AGB, Institute of Chemistry, Komi Scientific Centre UB RAS, Russia). The active ingredient was spruce flavonoids, 10g/l. A series of treatment solutions was prepared. To do this, 0.25, 0.5 and 1 ml of biological stimulant with an active substance concentration of 10 g/l were dissolved in 1L of distilled water. Solutions with concentrations of 0.00025%, 0.0005% and 0.001% were obtained. The control group of seeds was soaked in distilled water. After soaking, each experimental group were put into Petri dishes, 100 seeds per dish; each experimental variant was repeated three times. The seeds germination energy was determined; the hypocotyl length of all seeds was daily measured for nine days.

In order to obtain seedling of Siberian spruce treated with the biological stimulant, in 2016, the planting material treated was started growing planting in a nursery. The seeds were sowed in prepared beds of 0.9 m wide and 0.2 m high, in a row to a depth of 1 cm with a row spacing of 0.1 m. In the rows, in every variant, three segments with 100 seedlings in each one were distinguished. The aboveground parts of 30 plants (from the basal neck to the apical growth point) were measured on these segments. Siberian spruce seedlings’ seasonal height growth was observed every year during the growing season with a measurement interval of 14 days. At the end of the growing season in September, a row segment containing 30 seedlings was dug out along with the soil to measure the root system growth. The roots were washed in running water and unraveled. The main longest root was measured. Then, plants were divided into aboveground and underground parts and weighed.

Spectrophotometric method was applied to identify the pigment complex [17] in the needles of 4-year-old Siberian spruce seedlings. In the noon, in the second decade of August, ten seedlings from each row of experimental and control groups were selected. Under laboratory conditions, all living needles were removed from the seedlings, while scales and lignified particles were not considered. Weighed portions of 0.1 to 0.5 g were taken from each averaged sample, repeated three times for each experiment. The needles were chopped with scissors and ground in a mortar with sand (1/3 teaspoon of sand) to a homogeneous condition. 10 ml of alcohol (95-96%) was added to the weighed portion, ground in a mortar, and placed in a dark, dry place for five minutes; after that, it was filtered with crumpled filter paper. The sediment was washed with several portions of alcohol, rubbing for 30 seconds. It is necessary to wash the sample until the solution discolors and then pour the solution into a dark glass flask to avoid photosynthetic pigments destruction in the light. The optical density of obtained solution were measured with the spectrophotometer (PE-5400UF, Saint-Petersburg, Russia, EKROSKHIM LLC). For this, 3 ml of alcohol was poured into one cuvette (control); 3 ml of alcohol and 0.4 ml of the solution obtained from each experiment replication were poured into other cuvettes. The optical density of solutions was measured at the following
wavelengths: chlorophyll $a$ – 662 nm, chlorophyll $b$ – 645 nm, carotenoids – 440.5 nm. The experiment was repeated three times. The needles pigment concentration (mg/l) were calculated using the below formulas (formulas (1)-(3):

$$C_a = 9.784 \cdot D_{662} - 0.99 \cdot D_{645}$$

$$C_b = 21.426 \cdot D_{645} - 4.650 \cdot D_{662}$$

$$C_{car} = 4.695 \cdot D_{440.5} - 0.268(C_a + C_b)$$

where $C_a$ – chlorophyll $a$ concentration, mg/ml; $C_b$ – chlorophyll $b$ concentration, mg/ml; $C_{car}$ – carotenoids concentration, mg/ml; $D_{662}$, $D_{645}$, $D_{440.5}$ – optical density values of alcohol solution at corresponding wavelengths (662, 645, and 440.5 nm, respectively).

In April 2020, 4-year-old Siberian spruce seedlings grown with Verva-spruce were used to create an experimental plot of forest plantations under similar conditions to a nursery (located in the Altai-Sayan mountain taiga forest area). Seedlings were planted manually with a planting shovel (‘Kolesov sword’, Pushkino, Russia, Leskhoznab LLC) in rows with a planting step of 0.75 m and a row distance of 4 m. The total area of the forest plantation was 3 hectares. In September 2020, the first inventory of forest cultures was conducted, and the survival ability and adaptation of seedlings were assessed by measuring current linear growth.

The data were processed and analyzed with statistical methods and criteria such as analysis of variance, Mann-Whitney U test, Kruskal-Wallis H test, and Pearson’s goodness-of-fit test, based on checking samples for normal distribution and their scopes. To compare the two mean values (stem height in experimental group and control) an effect size (Cohen’s d) was calculated. A d value of 0.20 indicates a small effect, 0.50 indicates a mean effect, and 0.80 indicates a large effect. ‘Statistica’ software (Statistica 10, statsoft.com) was applied for statistical data processing.

3. Results and discussion

At the first stage of research, the germination capacity and germination energy of the seeds were determined and the seedlings’ development under laboratory conditions were observed. Results showed that seeds treated with the biological stimulant Verva-spruce at a concentration of 0.00025% started germinating on the second day. On the 5th day, the proportion of seed germination in this variant reached 100%, exceeding the control group result by 17% ($\chi^2=5.0$ at $\alpha<0.05$). In other experimental variants (0.0005% and 0.001%) the seeds started germinating on the 3rd and 4th day; and the share of germinated seeds in these variants and control group reached 100% only by the 7th day. The significance of difference within the ‘germination’ indicator in the variants with a concentration of 0.0005% and 0.001% comparing with the control variant was not considerable ($\chi^2=0.6$ at $\alpha>0.05$ and $\chi^2=0.1$ at $\alpha>0.05$, respectively). Considering all seeds in the experimental and control variants germinated quickly, results state that the germination energy in all cases was high. These results indicate the high quality of the seeds [18].

The further development of germs from treated seeds under stable laboratory conditions was characterized by active hypocotyl growth. The strongest response was in the variant treated with a stimulant at a concentration of 0.00025%: on the 9th day after seed germination, the average length was $6.47\pm0.28$ cm, which was 48% higher than the control group value of $4.37\pm0.38$ (r<0.05) (figure 1).
Figure 1. Hypocotyl growth kinetics.

The lowest concentration of the biostimulant in the solution had the most significant stimulating effect on the Siberian spruce growth processes while seeds germination. Thus, at the second stage of research, to prepare seeds for sowing in the nursery, a solution with a Verva-spruce concentration of 0.00025% were used. The first sprouts appeared 14 days after sowing outdoors, and on the 18th day, sprouts germinated massively. Systematic observations of the seedlings’ aboveground part growth during the growing season revealed two seasonal peaks of active development (figure 2). The first peak is small: it falls in the first half of June and represents a little burst of height growth activity. The second peak is prominent: it falls at the end of July – beginning of August, and during this period, spruce seedlings form the most considerable height growth (figure 2). This pattern of seedlings’ seasonal development is a feature of the experimental and control groups.

According to the existing state requirements for planting material for the Altai-Sayan mountain-taiga forest area, 4-years old seedlings must have a stem height of at least 12 cm [19]. In our study, by the fourth year, the seedlings’ height in the control group reached the value of 14±0.4 cm, which corresponds to the standard.

Figure 2. Seasonal growth rate of the stem.

In the experimental group (concentration of 0.00025%), the linear annual growth had a high rate and exceeded the control group rate by 60.1% on average (figure 3). In the third year, the experimental variant’s seedlings (grown with the biostimulant) exceeded the planting material’s height
requirements. By the end of the fourth year, the average height of the seedlings aboveground part in the experimental variant reached 22.9±1.3 cm, which was 60% higher than the average seedlings size in the control variant (figure 3). The significance of difference within the ‘stem height’ indicator between the control and experimental variant was statistically significant (at r<0.05). The effect’s size between mean values is substantial (d=1.0) (figure 4). The biomasses of the compared spruce seedlings variants at the age of three years had a significant difference as well, seedlings of the experimental group exceeded the control by 128% (23.41±3.11 g (Verva-spruce) versus 10.29±2.47 g (control) (r<0.05)).

![Figure 3. Annual growth dynamics of Siberian spruce seedlings.](image)

![Figure 4. Diagram of the aboveground part height span and general appearance of 4-year-old Siberian spruce seedlings.](image)

When implementing the experiment, we noticed that the seedlings’ needles in the experimental group had much dark green color than in the control group. In this regard, in the fourth year of cultivation, the pigment complex of needles was studied. A spectrophotometric method (see Material and methods section) to determine the content of photosynthetic pigments were applied.
In the needle of seedlings grown with the biological agent, the chlorophyll \(a\) content reached 5.51±0.02 mg/g of needles wet weight, and it exceeded the results of the control variant (2.26±0.07 mg/g) by more than 100%. The chlorophyll \(b\) content in the experiment (Verva-spruce) reached 2.31±0.01 mg/g, which exceeded the control by 96% (1.23±0.06 mg/g). The excess of the carotenoids content was 167%: 2.41±0.01 mg/g (Verva-spruce) versus 0.92±0.03 mg/g (control). The increased concentration of photosynthetic pigments in the autumn and pre-autumn periods refers to adaptive traits. This protective mechanism provides the resistance of the spruce assimilation apparatus to adverse conditions [20]. The spruce needles are susceptible to late spring frost [21]. According to this, it is reasonable to sow spruce on a period that excludes the risk of frost occurrence. The use of the biostimulant Verva-spruce could promote Siberian spruce seedlings' resistance to low temperatures and thereby shift the period of spruce physiological activity. To check this hypothesis, it is necessary to conduct additional studies of growth and biochemical processes during spring awakening and the onset of autumn dormancy.

In 2020, experimental planting material were used when creating an experimental plot of forest plantations. The autumn inventory results showed that the planting survival rate was 100%, and most of the plants successfully adapted to the new conditions. Almost all seedlings had growth (Verva-spruce – 100% adaptation; control – 96% adaptation; \(\chi^2=3.1\) at \(r>0.05\)). According to the Mann-Whitney test, the average growth in the compared groups (Verva-spruce – 4.43±1.87 cm; control – 3.21±1.15 cm) has a significant difference (\(r<0.05\)).

4. Conclusion
Treating Siberian spruce seeds with a water solution of Verva-spruce at a concentration of 0.00025% while germinating under laboratory conditions, had a stimulating effect on seeds’ germination energy further kinetics of seedling development.

Using seeds treated with a water solution of Verva-spruce at a concentration of 0.00025% while cultivating seedlings in the open ground, high-quality planting material were obtained, which exceeded standard requirements. It is notable that by the end of the second year of growth, the stem height of seedlings reached the value required for planting material of the Altai-Sayan mountain taiga forest area [19]. By the end of the third year of growth, this value was two times exceeded.

Two bursts of seasonal height growth activity of seedlings were determined. The primary height growth occurred in late July - early August. Knowing the seasonal height growth rate of seedlings is crucial for planning agrotechnical methods of reforestation work [19].

According to the results, the needles of seedlings grown from seeds treated with Verva-spruce by the end of the growing season contained an increased pigment count. This indicates the plant resistance to adverse environmental conditions, in particular low temperatures, which is a limiting factor for spruce in the taiga zone.

Thus, the use of organic biological stimulant Verva-spruce allows reducing the period of growing Siberian spruce seedlings in the nursery by one year. The experimental planting material will continue to be examined with the aim of studying the effect of the stimulant application deeply.

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