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The Effect of Low Positive Temperatures on the Formation of Secondary Metabolites in *Rhodiola quadrifida* (Pall.) Fisch. et C.A. Mey. In Vitro Cultures

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Abstract: Global warming is one of the most serious problems leading to changes in the distribution areas of species and biodiversity. *Rhodiola quadrifida* is a rare plant with adaptogenic properties and grows in the highlands in a narrow temperature range of 2–15 °C. The aim of our work was to study the growth and content of the main metabolites in two in vitro cultures of *Rhodiola quadrifida* at temperatures of 5, 15 and 25 °C. Hairy roots and calli were cultivated on agar medium for 28 days. The maximum values of the growth index were observed at 25 °C (2.32 and 2.12 for calli and hairy roots, respectively). HPLC-MS showed the absence of tyrosol and rosarin in both cultures, and rosin in the root culture. The content of salidroside changed slightly in calli and roots. Cultivation at 5 °C significantly stimulated the formation of rosin in calli. Only a residual amount of rosavin was noted in the roots, regardless of temperature. The content of rosin was higher in calli at 15 °C with a maximum content at the end of the cultivation cycle 25 µg/g DW. Thus, *Rhodiola quadrifida* will be able to grow with an increase in temperature by 10 °C but this will be accompanied by a significant reduction in its medicinal value

Keywords: *Rhodiola quadrifida*; callus; hairy roots; salidroside; rosavin; rosarin; rosin; tyrosol

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1. Introduction

Significant climate change (first of all, its warming) is currently taking place, which is considered one of the most serious problems facing mankind. According to the IPCC estimations based on the analysis of observational data over 100 years (1907–2006), the increase in the average annual temperature was 0.75 °C [1]. These climatic changes lead to increased abiotic stresses, such as drought, flooding, salinization, extreme temperatures and unpredictable rainfall. Global warming also affects ecosystems, changing the boundaries of biocenoses. Therefore, an important consequence of increasing temperature is a change in the area distribution of species [2] and biodiversity.

The increase in temperature is observed throughout the globe and it is most significant in high latitudes and mountainous areas [3]. The ecosystems of these regions are especially susceptible to climate change. Representatives of the genus *Rhodiola* belong to mountainous and alpine plants growing at an altitude of 500–5920 m above sea level [2]. For most representatives of this genus, a rise in temperature contributes to the expansion of the range, as new territories previously unsuitable for life due to too low temperatures appear. However, for some highland *Rhodiola* species growing in narrow temperature ranges, areas with suitable conditions may disappear, this is the so-called "nowhere to go" hypothesis [4]. Such species include *Rhodiola quadrifida*, a rare plant with adaptogenic properties [5,6]. It is used in traditional medicine in China and Mongolia, and in Russiaas a dietary supplement [7]. The habitat of *R. quadrifida* is located at an altitude of 1800–5000 m above sea level with fluctuations in positive temperatures from 2 to 15 °C in summer months. *R. quadrifida*

is found in the highlands of Russia (Altai, Sayan Mountains), in the mountainous regions of China and the highland regions of Mongolia [6]. The limiting factors for the growth of *R. quadrifida* include poor ecological plasticity and low competitiveness of the species [8].

The Qinghai–Tibetan plateau (habitat of *R. quadrifida*) is one of the most sensitive ecosystems to climate change [2]. On the one hand, this can lead to a reduction in its area, and on the other hand, to a possible change in the synthesis of secondary metabolites [9]. It should be noted that an increased temperature usually intensifies the secondary metabolism of plants, and the qualitative content of metabolites may also change [9,10]. However, since *R. quadrifida* is an alpine plant growing at low temperatures, changes in secondary metabolism may be of a different pattern than in plants whose habitat conditions are not so extreme. Thus, the species needs not only to be preserved, but also to assess the potential risk of temperature changes on the synthesis of secondary metabolites.

The roots and rhizomes of *Rhodiola* spp. contain a variety of chemical compounds: the most valuable of them are phenylethanoids—tyrosol and salidroside; and phenylpropanoids—rosin, rosavin androsarin (Figure 1). They are formed as a result of the phenylpropanoid pathway through the biosynthesis of aromatic L-amino acids—phenylalanine and tyrosine [11]. Nevertheless, the pathway of tyrosol biosynthesis and its regulation are not fully understood. Salidroside is formed from tyrosol by the addition of a sugar residue. Rosin is the simplest glycoside of cinnamic alcohol and is formed by the transfer of one glucose. Rosin is transformed into rosavin by the addition of the arabinopyranose unit and into rosarin by the addition of the arabinofuranose unit [12].

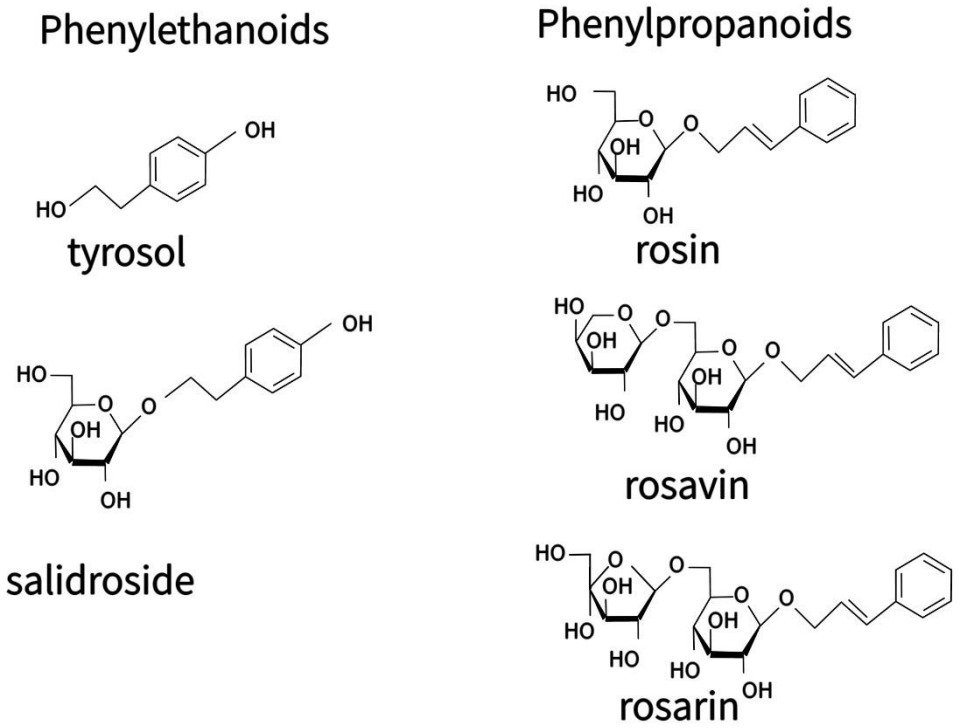

Figure 1. The general metabolites of *Rhodiola* spp.

The use of biotechnological processes and methods is promising for medicine and environmental protection [13–16]. The cultivation of plant material under in vitro conditions is possible either as undifferentiated cultures, individual organs, or sterile plants. As observations of *Rhodiola quadrifida* in nature are difficult due to the inaccessibility of its habitat, the study of cultures obtained from it in vitro is advisable. The use of cultures with different degrees of organization will allow a more complete characterization of the changes occurring in different types of tissues with temperature changes.

Thus, the aim of our work was to study the content of the main metabolites in the calli and hairy roots of *Rhodiola quadrifida* at different temperatures: 5, 15 and 25 °C. In this

regard, the average temperature was chosen on the basis of the summer climatic conditions of *Rhodiola quadrifida* growing region. The average July temperature in one of the large habitats of these plants in the Republic of Tyva is 14.6 °C, while temperatures of 5 and 25 °C are close to the lower and upper limits of the optimum zone at which normal plant growth is possible.

2. Materials and Methods

2.1. Plant Material

Previously obtained cultures of hairy roots and calli were used as objects of research [17]. Hairy roots were grown on solid Murashige and Skoog medium (MS) [18] without hormones (Figure S1). Calli were cultured in the same nutrient medium supplemented with 3 mg/L6-Benzylaminopurine (BAP) and 0.1 mg/L 1-Naphthaleneacetic acid (NAA) [17] (Figure S1). The cultivation of plant material was carried out in the dark at constant temperatures of 5, 15 and 25 °C for 28 days.

Callus cultures and the hairy roots were cultivated for 28 days in the dark at constant temperatures of 5, 15 and 25 °C.

2.2. Estimation of Growth Characteristics of Hairy Roots and Callus Culture

The growth index (I), calculated by the following formula, was used to evaluate the growth of calli and hairy roots:

$$I = (m_{max} - m_0)/m_0$$

where m_{max} and m_0 are the raw weights (g) of calliand hairy roots at the beginning and on days 3, 7, 14 and 28 of cultivation. The initial weight of hairy roots and calliwas 0.5 g.

The graph shows the arithmetic mean values of growth parameters from three or four biological replicates for each variant.

2.3. Extraction and Preparation of Samples for HPLC-MS/MS Analysis of Flavones

The determination of salidroside, rosavin, rosin, rosarin and tyrosol was performed by HPLC in the samples taken on days 3, 7, 14 and 28 of cultivation. The plant material was lyophilically dried. The extraction of extractive substances was carried out by three-fold extraction with 75% ethanol at a ratio of raw material:extractant equal to 1:100, according to the previously described method [17]. We used 75% ethanol, since several authors have shown that this concentration is optimal for the extraction of rhodiola metabolites. Extraction was performed in an ultrasonic bath (Fisher Scientific, Hampton, NH, USA) for 90 min. Afterwards, 1 mL of the extract was taken and centrifuged for 15 min at 8000 rpm. A 0.5 mL amount of supernatant was transferred to a pure Eppendorf tube, 1.5 mL, and used for HPLC.

2.4. HPLC-MS/MS Conditions

The content of salidroside, rosavin, rosarin, rosin andtyrosol in extracts of hairy roots and calliof *Rhodiola quadrifida* was determined by high performance liquid chromatography with mass-spectrometric detection. A total of 150 µL of deionized water was added to 50 µL of the extract in order to avoid erosion of chromatographic peaks. After dilution, 5 µL of the extract was injected into a liquid chromatograph Sciex Exion LC with mass-spectrometer «5500 QTRAP» (AB SCIEX, Toronto, ON, Canada), and a 150 × 2 mm Shim-pack XR-ODS II column (Shimadzu, Kyoto, Japan) with a particle size of 2.2 µm was used for separation. Separation was carried out in a gradient mode, deionized water was used as phase A, methanol was used as phase B. The separation was carried out with constant flow at 0.2 mL/min in a gradient mode: 32% B was supplied for 0.2 min, from 32% to 40% B from 0.2 min to 3.5 min, then to 45% B from 3.5 to 4.5 min, then to 70% B from 4.5 to 8 min and to 100% B from 8 to 10 min, to keeping 100% B from 10 to 13 min, then equilibration at 68% A until 19.5 min. The column thermostat temperature was set at 35 °C. The signal of all analytes was recorded using a 5500 QTRAP

triple quadrupole mass-spectrometer in the electrospray ionization mode (Sciex, USA), registration of negatively charged ions, multiple reaction monitoring (MRM).

For each substance, two MRM transitions were set at $m/z299.1 \rightarrow 119.0$, $299.0 \rightarrow 89$ for salidroside, $m/z137 \rightarrow 119$, $137 \rightarrow 106$ for tyrosol, $m/z295 \rightarrow 161$, $295 \rightarrow 101$ for rosin and $m/z427 \rightarrow 293$, $427 \rightarrow 149$ for rosavin and rosarin. Rosarin and rosavin have the same MRMs, but in the selected conditions both analytes were separated. Linear calibration curves ranging from 1 to 50 ng/mL for tyrosol, from 1 to 100 ng/mL for rosin and from 1 to 200 ng/mL for salidroside, rosarin and rosavin were constructed by analyzing salidroside (98%, Sigma-Aldrich, St. Louis, MS, USA), tyrosol (99.5%, Sigma-Aldrich), rosarin (98%, Sigma-Aldrich), rosin (95%, Sigma-Aldrich) and rosavin (98%, Sigma) standard solutions in a water–methanol mixture (1:3).

The method was validated using spiked extracts, and the following parameters were determined: linear range, limit of detection (LoD), limit of quantification (LoQ), recovery, matrix effect, within-run precision, between-run precision across 3 runs, long-term stability at −20 °C and stability of samples in autosampler at 4 °C. The parameters of the analytical method are presented in Supplemental Data (Table S1).

2.5. Statistical Processing

Statistical analysis was performed using a one-way ANOVA test. All the experiments were performed in triplicate with at least three independent runs. The data are presented in the figures as means ± SD. Different symbols show significantly different values. Mean values were considered significantly different at $p < 0.05$.

3. Results

3.1. The Effect of Temperature on the Growth of Calli and Hairy Roots

Root and calli growth index analysis showed a correlation between biomass growth and cultivation temperature (Figure 2). The lowest growth index was observed at 5 °C; it was 0.34 for callus and 0.36 for root culture. The maximum values of the growth index were observed at 25 °C (2.32 and 2.12 for callus and hairy roots, respectively). The growth index at 15 °C at the end of the cultivation cycle was 1.9 and 1.36 for callus and roots, respectively. Thus, both cultures slightly differed from each other in terms of growth index at the same temperature.

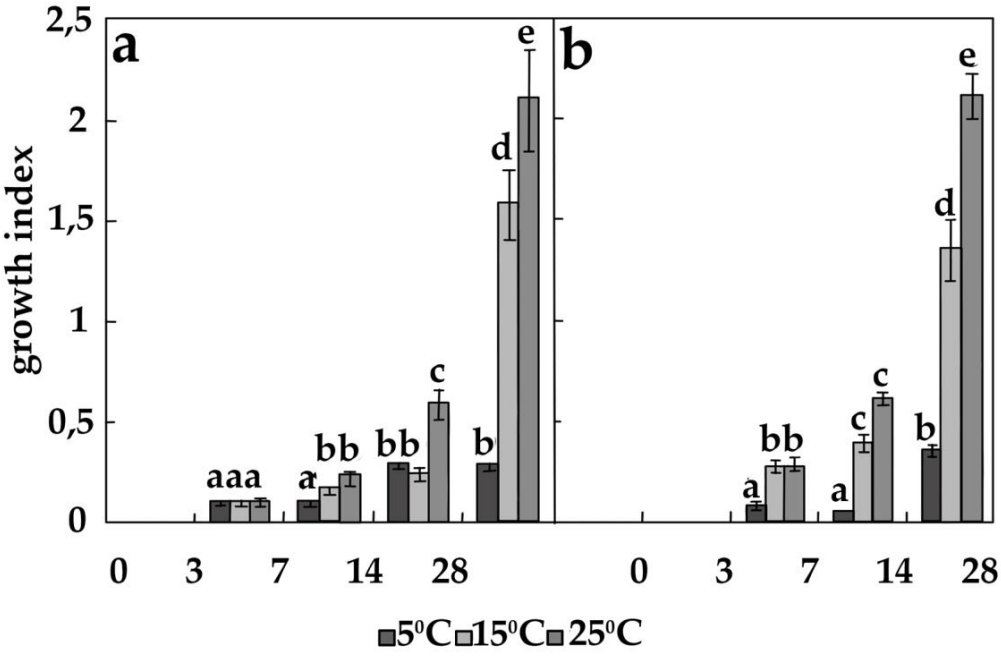

Figure 2. (**a**) Growth index of calli, (**b**) growth index of hairy roots of *Rhodiola quadrifida*.

The cultivation temperature significantly changed the shape of the callus growth curve during the cultivation cycle. In calli grown at temperatures of 15 and 25 °C, all the main phases of growth and the maximum increase in biomass were observed on days 21–28. In calli cultured at 5 °C, the growth phases were smoothed out (Figure 2a). In this variant, the growth was the weakest; a small increase was observed by day 21 of cultivation, after which it remained unchanged.

For the first week, the temperature had no effect on root growth. Differences appeared in the second week: in the roots grown at 15 and 25 °C, the growth was 3 times higher compared to the roots grown at 5 °C. The greatest increase in root mass was noted in all variants in the period of 14–28 days (Figure 2b).

3.2. The Content of the Main Phenolic Compounds in Calli and Hairy Roots

The absence of tyrosol and rosarin was shown in the study of the content of the main phenolic compounds of *Rhodiola quadrifida* in all samples of the studied cultures, and the absence of rosinin the root culture. The total content of the main substances studied was low and amounted to a maximum of 0.051 mg/g for calli and 0.00274 mg/g for roots.

The content of salidroside in calli of all the variants was similar during the first three days of cultivation (Figure 3). Then, at 15 and 25 °C, the content of salidroside gradually decreased by the end of the cultivation cycle and was 23% less compared to 5 °C. Although the content of salidroside in calli at 5 °C changed slightly during the entire cultivation cycle, on the 14th day it increased by 25% and was the highest in the whole experiment. The content of salidroside in the roots was an order of magnitude lower than in the calli, and when cultivated at different temperatures it did not change reliably for 28 days.

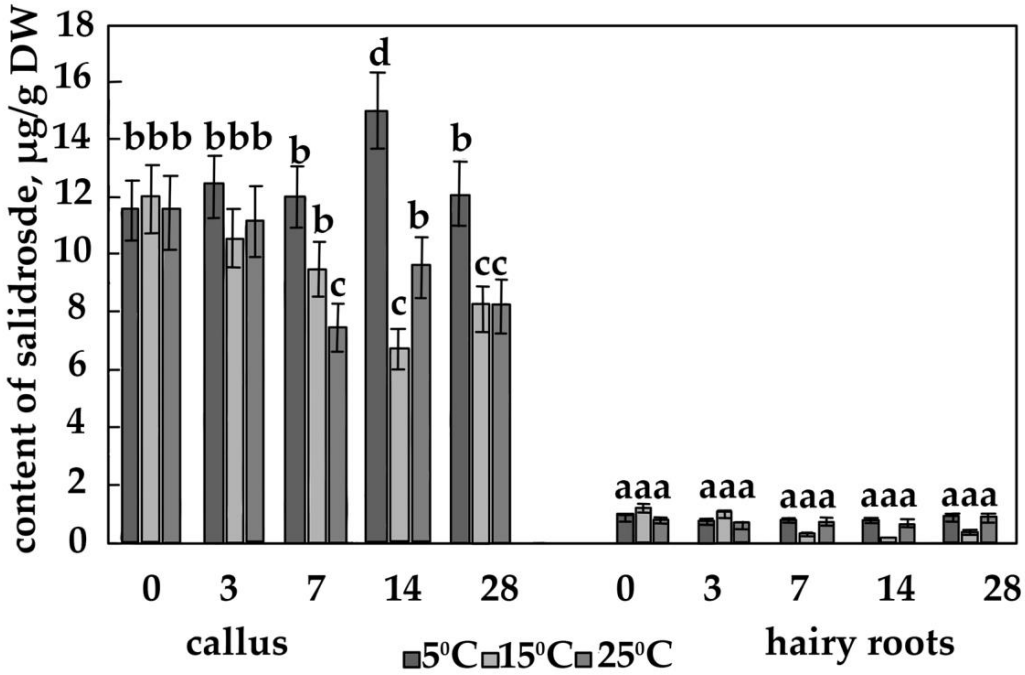

Figure 3. Salidroside content in calli and hairy roots of *Rhodiola quadrifida*.

Cultivation at 5 °C stimulated the formation of rosavin in calli significantly and it almost did not decrease during the cultivation cycle. On the third day of the experiment, the rosavin content in calli growing at 15 °C increased sharply and did not differ significantly from the variant of cultivation at 5 °C. Afterwards, however, it decreased rapidly and on day 7 was only 10.5% of the value at 5 °C. Rosavin content, in general, was significantly lower at 25 °C than at other temperatures (Figure 4). By the end of the cultivation cycle, the content of rosavin at 15 and 25 °C was 11.1 and 17.5% of its concentration in calli grown at

5 °C. Only a residual amount of rosavin was noted in the roots throughout the cultivation cycle, regardless of temperature.

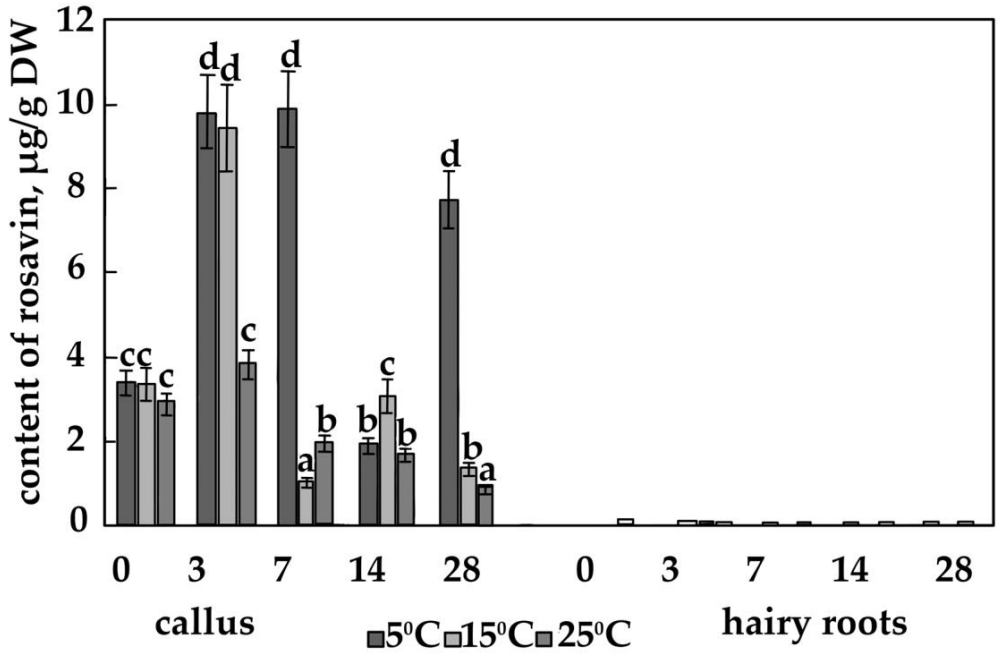

Figure 4. Rosavin content in calli and hairy roots of *R. quadrifida*.

The cultivation temperature also had an effect on the rosin content during the cultivation cycle (Figure 5). For example, on the third day, it was higher in calli growing at 5 °C, but after a week of cultivation it decreased and was 46% of the maximum value by the end of the cycle. An increase in rosin content was observed at 15 °C in the first week, then a decrease in the second week and a rise again by day 28. In general, the rosin content was higher in calli growing at 15 °C. It should be pointed out that its maximum content was observed at the end of the cultivation cycle (0.025 mg/g DW). No rosin was detected in the root culture throughout all variants.

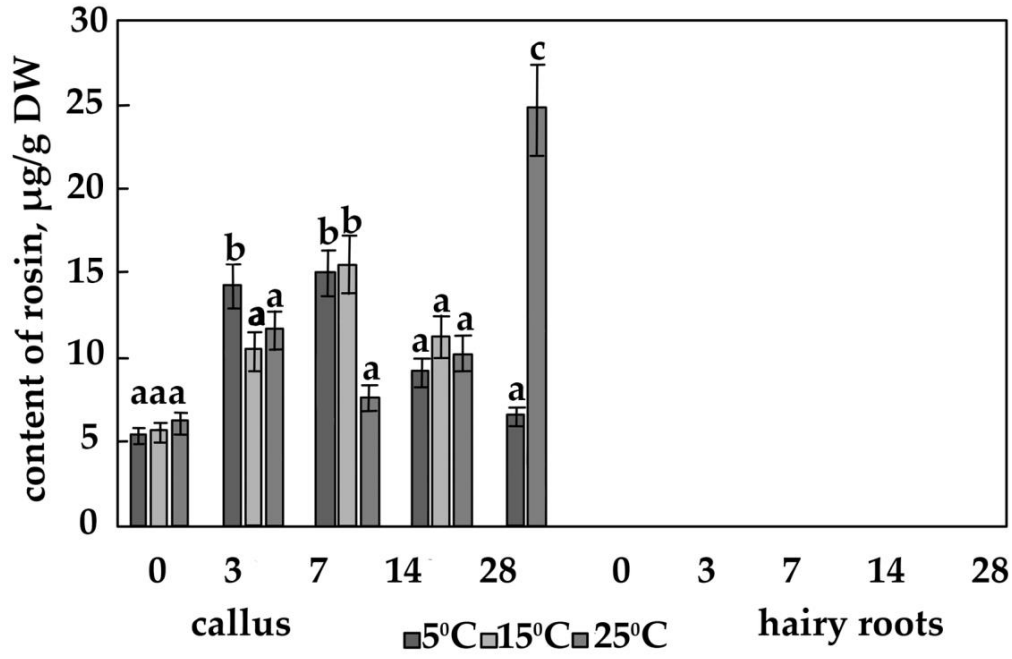

Figure 5. Rosin content in calli and hairy roots of *R. quadrifida*.

4. Discussion

4.1. Features of In Vitro Growth of Rhodiola Quadrifida Cultures and Its Effect on the Content of Secondary Metabolites

Rhodiola quadrifida grows in high mountain regions at low temperatures. There are no data on the growth rate of *Rhodiola quadrifida* under natural conditions, but the slow growth may be related to biological characteristics of plants of this genus. For example, insignificant increase in growth during the cultivation cycle was observed in the calli of other *Rhodiola* spp.—*Rhodiola rosea* and *Rhodiola imbricata* [19,20]. It is known that slow-growing callus is formed from plants that grow slowly in nature. So, for the suspension-cultured calli of the slow-growing, frost-resistant orchid *Bletilla striata*, the growth index was about 3 on 36th day of cultivation [21].

Most works on secondary metabolites have noted the effect of growth on their formation. The synthesis was usually associated with a stationary growth phase. Detailed work carried out on *Rhodiola imbricata* shows that the synthesis of *Rhodiola* phenolic compounds is confined to the stationary phase of growth [22]. In our case, due to the low growth intensity, no relationship between growth and metabolite synthesis was found for eithertypes of cultures.

4.2. The Effect of Culture Type on the Content of Secondary Metabolites

It should be pointed out that under natural conditions the content of the main metabolites in the roots of *Rhodiola quadrifida* plants is low, compared with other representatives of *Rhodiola* spp. [23]. It is probable this trend can also be seen in in vitro cultures. A lower content of the studied substances in the roots in vivo compared to in vitro suspension (undifferentiated) cultures is shown in the work carried out on *Rhodiola crenaluta* [24].

Despite the fact that hairy roots are currently considered one of the promising technologies for obtaining secondary metabolites, it has repeatedly been shown that their substance content could be lower than that of other in vitro cultures [25–27]. In our study, the content of the tested metabolites was minimal in the hairy root culture. We have previously suggested that the low total salidroside content in *Rhodiola quadrifida* may be related to the type of synthesizing tissue [17]. Growing roots are known to contain less salidroside than storage rhizomes [28]. The storing parts of the plant are represented mainly by parenchymatous cells. Calli are composed of cells of different types; however, there are more parenchymatous cells.Therefore, the higher content of salidroside in calli compared to roots is understandable.

A common feature of our cultures is the absence of tyrosol in all samples. According to the literature, the content of tyrosol in *Rhodiola* plants is usually less than that of other metabolites [29]. The works of other researchers, as well as ours, lacked tyrosol in the samples. In the paper [30], the authors conclude that low concentrations of tyrosol suggest that it is an intermediate metabolite and has no significant function for the plant, being metabolized to salidroside. In our case, probably due to the initially low content of secondary substances, tyrosol was quickly converted to salidroside, so it was not detected in our samples.

A residual amount of rosavin was observed in the roots. Węglarz et al. showed that the rosavin content in roots was half as much as in rhizomes [31]. Overall, it should be noted that very few metabolites of the phenylpropanoid pathway were present in the bearded roots, namely, rosin and rosarin were absent, and rosavin was present only in small amounts. This is probably due to the fact that the synthesis of precursors of this group of metabolites was minimal in the roots, and further synthesis quickly passed through the synthesis of rosavin.

4.3. The Effect of Environmental Factors on the Content of Secondary Metabolites in R. quadrifida

At present, the influence of individual environmental factors and habitat conditions (a set of environmental factors) on the qualitative and quantitative composition of secondary metabolites has been well studied. Of all the representatives of *Rhodiola* spp., the greatest number of studies has been conducted on *Rhodiola rosea*. For example, it has been shown that

phenylethanoid and phenylpropanoid content in *Rhodiola rosea* depend on the geographical place of growth of the plants [23,29], harvesting time [28,32,33], place of raw material harvesting (natural stock of raw material or commercial cultivation) [29,34–37], its age [33,36] and plant gender [29,33]. The content of the main substances also depends on the plant part (stem, leaves, roots, rhizome) from which the plant material was taken [31], as well as on the methods of cultivation under in vitro conditions [38–40].

However, there are certain contradictions in the works on the study of the content of the main metabolites depending on the geography of distribution. For instance, a low content of secondary metabolites was found in the roots of plants from natural populations of *Rhodiola rosea* in Russia and Mongolia, but in the roots of *Rhodiola* from other regions it was an order of magnitude higher [6,41]. The authors suggested that this may be due to unfavorable climatic conditions [6]. In some studies, salidroside and rosavin were not found in the roots of introduced species, although they are the main metabolites [29,42]. In this case, the authors linked this phenomenon to genetic mechanisms.

4.4. The Effect of Temperature on the Content of Salidroside, Rosin and Rosavin in Calli of R. quadrifida

The most frequently studied factors influencing the synthesis of substances are the effects of temperature. Most studies have shown increased formation of secondary metabolites as a result of exposure to elevated temperatures. However, this intensification may be indirect. For example, Jochum et al. showed that elevated temperatures increase leaf senescence and root secondary metabolite concentration in the understory herb *Panax quinquefolius* [43]. It was shown that increasing the cultivation temperature by 5 °C led to an increase in the concentration of ginsenosides in *Panax quinquefolius* roots by 49%, but this was accompanied by a general inhibition of growth and a decrease in root mass by 33% [43].

Relatively few works have been devoted to the effect of low temperatures on the secondary metabolism of plants and their results are contradictory [44]. Some studies have shown that lowering the temperature reduces the synthesis of secondary metabolites, but in others lowering the temperature, on the contrary, leads to an increase in their content. In a culture of *Hypericum perforatum* adventitious roots, for example, hypericin synthesis was shown to increase when they were incubated for a week at −4 °C, and rising the temperature to 25 °C reduced its level sharply [45]. The authors concluded that since St. John's wort is a frost-resistant plant and its hypericin content increases only with a severe decrease in temperature, it is a protective response to stress. This assumption was confirmed by other work that demonstrated that the *Hyp-1* gene involved in hypericin biosynthesis is activated at 15 °C, the lowest of the three studied [46]. The accumulation of anthocyanins after exposure of *Mikania micrantha* to a low positive temperature of 4 °C (12 h) was considered as an adaptation to low winter temperatures [47]. We also showed on *Rhodiola* calli that there was the lowest content of salidroside and rosavin at 25 °C. The results of our work thus support the hypothesis that a decrease in temperature is necessary for cold-tolerant plants to increase the content of secondary metabolites.

5. Conclusions

The process of increasing the average annual temperature is an extremely serious environmental problem. According to our research, an elevation of the temperature by 10 °C increases the biomass growth of in vitro cultures of *Rhodiola quadrifida* but reduces the content of secondary metabolites. This suggests the possibility that *Rhodiola quadrifida* will be able to grow under new temperature conditions caused by global warming; however, it may be accompanied with a decrease in its medicinal value.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11010028/s1. Figure S1. In vitro cultures of Rhodiola quadrifida: hairy roots (left), calli (right); Table S1. Parameters of analytical method.

Author Contributions: A.Y.S. developed the concept; A.Y.S., A.I.S., E.A.G. and D.V.T. designed the experiments; A.Y.S., A.I.S., E.A.G. and D.V.T. carried out the experimental work and analyzed the data; A.Y.S., A.I.S., E.A.G. and D.V.T. wrote the article; R.N.S. and E.N.G. carried out the HPLC. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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