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The Influence of Low Temperature on the Scots Pine Callus Culture

Korotaeva N.E.*, Shmakov V.N., Pyatrikas D.V.

[1] *Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences, Irkutsk 664033, Russia*

*E-Mail: knev73@yandex.ru

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The method of callus culture is a convenient tool for assessing of intracellular changes occurring in response to adverse effects, which reduces the experimental time and laboratory resources. Factors of intracellular resistance of coniferous trees to low positive temperatures have not been studied enough. The study of the sustainability mechanisms of the economically important species and forest-forming tree species, Scots pine, is relevant for the research region. The objective of the research was to assess the suitability of callus culture obtained from Scots pine (*Pinus sylvestris* L.) buds for studying the factors of bud cell resistance to low positive temperatures. Callus cultures obtained on bud explants were exposed to +4 or +1 °C for 7 days, after which the growth rate of the cultures, cell viability, and oxidative stress indicators (the content of hydrogen peroxide and superoxide anion; the activity of peroxidase and lipid peroxidation) were assessed. Before the exposure, callus cultures differed in growth rate, viability and in the development of oxidative stress, which is probably due to the genetic differences in the trees from which the buds were obtained, and is consistent with the data of other studies. Low positive temperatures suppress the growth of cultures, which indicates the restructuring of cell metabolism in the direction of protective activity. After exposure to low positive temperatures, all cultures were characterized by an increase in lipid peroxidation activity, indicating the development of oxidative stress. The absence of negative changes in cell viability and of an increasing in the content of hydrogen peroxide in all studied cultures indicates the ability of cells to withstand unfavorable changes. An increase in peroxidase activity found in all cultures indicates the activation of protective mechanisms. An increase in the content of superoxide anion may indicate the development of protective signaling. The study showed the ability of pine bud callus culture cells to withstand the used cold exposure, which makes the callus culture at researched conditions a useful test system for studying the mechanisms of cold resistance of Scots pine buds.

Key words: callus culture, cold stress, oxidative stress, Pinus sylvestris L.

Identification of the mechanisms of plant resistance to adverse abiotic effects remains an urgent task of plant physiology. One of the steps to work out this problem is the development of test systems that would allow us to identify physiological changes that occur in response to an external factor, but do not lead to plant death.

The using of callus cultures (or lines) in the study of the effects of adverse conditions on plant objects allows us to assess changes occurring at the cell level outside of control at the organism level. In the study of woody plants, the using of cell cultures also significantly reduces the time of the experiment and the scale of utilization of laboratory resources for research. The disadvantage of the using of cell cultures is an alteration in the activity of some genes that determine cell specialization (Chen *et al.,* 2024). However, it has been shown that pluripotent cells of the callus culture and specialized plant cells showed similar changes in response to the influence of external factors (Sacu *et al.,* 2023), in particular, for the accumulation of stress proteins (Tuchin 1998). Thus, subject to existing limitations, cell cultures are suitable for the assessing of intracellular changes in response to external influences.

Cooling is one of the common abiotic factors that negatively affect plants in their natural environment. One of the main negative consequences of cooling at the cellular level is the development of oxidative stress, which consists in the generation by mitochondria, chloroplasts and plasmalemma of toxic varieties of oxygen-containing chemically active molecules (reactive oxygen species, ROS) (Apel and Hirt 2004) and leads to disturbances in the structure of membranes and proteins (Apel and Hirt 2004; Czarnocka and Karpiński 2018). In particular, an indicator of a violation of the membrane structure is an increase in the activity of lipid peroxidation (Gill and Tuteja 2010). A change in the intensity and in the character of metabolism and a shift in its activity towards protective processes, which include the intensification of the activity of antioxidant enzymes (ROS utilization enzymes), gene expression and the formation of protective enzymatic and non-enzymatic metabolites are a response from cells (Gill and Tuteja 2010). A violation of the balance between the level of toxic molecules, as well as disturbed structures and the activity of regenerative systems leads to cell death and further death of the plant as a whole (Czarnocka and Karpiński 2018). However, if cells are able to maintain balance by utilizing ROS, removing toxic molecules and repairing damage, then cells adapt to adverse conditions, but cell death can occur when resources are exhausted.

Factors of intracellular resistance of coniferous trees to the action of low positive temperatures have not been studied sufficiently due to the complexity of such an object for research, although knowledge about such factors is an important link in breeding work with these plants and is of interest for the transfer of beneficial genes (Takata *et al.,* 2007; Lu *et al.,* 2022; Vuosku *et al.,* 2022). Scots pine (*Pinus sylvestris* L.) is an economically important species and one of the predominant forest-forming species of the Irkutsk region forest fund, which, after larch, occupies the second place in terms of area of growth and is included in reforestation measures (State report..., 2022). Thus, the study of the mechanisms of stability of this species is relevant for our region.

The purpose of this study was to evaluate whether it is possible to use a callus culture test system to assess the resistance of Scots pine cells to low positive temperatures. During the study, the effect of 4 or 1 °C temperatures on the indicators of physiological activity, viability and oxidative stress was evaluated.

MATERIALS AND METHODS

For callus culture production in the current study, five trees (No. 1-5) of Scots pine (*Pinus sylvestris* L.) were used, growing in experimental field of the institute at which the research was conducted. This field was established with annual seedlings obtained at the forest nursery of the Meget locality in the region of the location of the institute in 1985. The soil on the field is gray forest, non-podzolic loamy type. Groundwater lies at a considerable depth (11–50 m) and does not have a noticeable effect on the soil moisture regime. The age of the trees used at the time of the study was 38 years. To obtain callus cultures, samples were collected in

September-October 2023. The tops of branches from the lower third of the crown (approximately 3–4 cm) with needles and buds were taken as plant material for obtaining calli of Scots pine. The needles were removed prior to sterilization, and the buds with sections of bearing shoots were sterilized using sequentially a 3% solution of hydrogen peroxide (30 min) and a 0.1% solution of mercury chloride (10 min), then they were washed for 30 min in 3% hydrogen peroxide solution twice. To obtain explants, sterile buds were transferred to sterile filters (in Petri dishes). Transverse disks that were 2–3 mm thick were cut out from the middle parts of the buds, and the cuts were placed horizontally in a medium according to Murashige and Scoog (Murashige and Scoog 1962) with modifications. Half of the medium was composed of macro- and microsalts with the addition of 0.8 $mg \cdot L^{-1}$ thiamine, 0.4 $mg \cdot L^{-1}$ pyridoxine, 0.4 $mg \cdot L^{-1}$ nicotinic acid, 100 $mg \cdot L^{-1}$ inositol, 200 $mg \cdot L^{-1}$ casein hydrolyzate, and 20 $g \cdot L^{-1}$ sucrose; 2,4-dichlorophenoxyacetic acid (2 $mg \cdot L^{-1}$) and 6-benzylaminopurine (0.5 $mg \cdot L^{-1}$) were used as growth regulators. Explants and the induced calli were cultured in the dark at a constant temperature of 25 °C. The duration of one cultivation cycle of resulting callus was 28 days. Before the start of the experiment, the period for growing callus cultures was two cultivation cycles. The typical appearance of the obtained callus cultures is shown at Figure 1.

For low temperature conditions the calli were transplanted for 7 days (dark conditions) at cooled incubator (MIR-154, Panasonic (Sanyo), Japan) at +4 or +1 °C. For control the calli stayed at cultivation conditions for the same time. After the end of the low temperature treatment the first part of the samples were collected for viability and ROS content estimation, the second part of the samples were fixed by liquid nitrogen and stayed at–70 °C. Callus growth rate was calculated as the difference between the fresh weight of the callus before transfer to a low temperature conditions (or before the start of the experiment for control) and the fresh weight of the same callus after 7 days of cultivation, expressed as a percentage of the initial weight.

The viability of callus culture cells was determined using the reduction of 2,3,5-triphenyltetrazolium chloride (TTC, Dia-M, Russia), which is reduced to red water-insoluble formazan by the activity of dehydrogenases in a living cell (Towili and Mazur 1975). The resulting red formazan was extracted from cells by incubation with 95% ethanol at 65 °C for 15 min. The formazan ethanol solution absorbance was measured at 485 nm using BioRad SmartSpec Plus spectrophotometer. Extinction was calculated per 1 g of callus fresh weight.

Oxidative stress at the cell level accompanies all stressful effects. To identify whether intracellular oxidation occurs as a result of the action of the low temperature, the content of reactive oxygen species as well as the activity of lipid peroxidation and of antioxidant enzyme peroxidase were evaluated. To determine the content of superoxide anion 0.3 g of callus tissue was slightly loosened and incubated with NBT (Gerbu, Germany) solution (1 mg cm^{-3}) in 10 mM KH_2PO_4 (pH 7.8) in the dark in a water bath at 37 °C for 1 h, washed with distilled water, and dried on filter paper (Tarasenko *et al.,* 2012). The resulting darkblue formazan was extracted through grinding in a 2M KOH solution in DMSO (CDH, India) (1:1.167) according to (Myouga *et al.,* 2008). After centrifugation for 10 min at 12000 g, the absorbance of supernatant was measured at 700 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, USA). Extinction was calculated per 1 g of fresh weight. To determine the hydrogen peroxide content, 0.3 g of callus was incubated with 3,3′-diaminobenzidine (DAB, Sigma, USA) (2 mg cm^{-3}) in a 10 mM of Tris-acetate (pH 5.0) in the dark in a water bath at 37 °C for 5 h, according to (Ramel *et al.*, 2009). Subsequently, the callus was washed with distilled water and dried on filter paper. The resulting brown pigment was extracted with 0.2 M $HClO_4$. After centrifugation for 10 min at 12000 g, the absorbance of the supernatant was measured at 450 nm using a SmartSpec Plus Bio-Rad spectrophotometer. Hydrogen peroxide content was determined using a calibration curve method and assessed in relation to the fresh callus weight. To measure the activity of lipid peroxidation and the activity of peroxidase we used frozen callus samples.

To measure the activity of lipid peroxidation and the activity of peroxidase we used frozen callus samples. Lipid peroxidation activity was determined by assessing the content of products (MDA-eq.) resulting from the reaction with thiobarbituric acid (TBA), as described in (Qiu *et al.,* 2016). For this, 0.3 mg of callus was ground with 1.5 mL of 0.1% trichloroacetic acid (TCA, Panreac, Spain), and the mixture was centrifuged (12,000 g for 15 min). Then 2 mL of 0.5% TBA (Dia-M, Russia) in 20% TCA was added per 1 mL of the resulting supernatant, and the mixture was incubated in a boiling water bath for 30 min. The reaction was stopped by cooling, and the test tubes were placed on ice. The samples were centrifuged for 5 min at 12000 g, and the absorbance of supernatant was measured at 532 and 600 nm. The MDA-eq. content was calculated using the TBA extinction coefficient of 155 mM^{-1} cm^{-1} after subtracting the nonspecific absorbance measured at 600 nm and expressed as nM g^{-1} of fresh weight. To measure the enzyme activities, a frozen callus sample (0.5 g) was ground in liquid nitrogen with the addition of quarz sand and 2 mL of 0.2 M sodium-phosphate buffer (pH 7.0). After centrifugation, during the period of taking enzyme activity measurements, the supernatant was placed on ice in glass tubes and stored at +4 °C. The peroxidase activity was measured in 0.2 M sodium-phosphate buffer (pH 7.0) containing 10 mM hydrogen peroxide and 8 mM guaiacol (CDH, India) spectrophotometrically at 470 nm by the rate of formation of the reaction product tetraguaiacol using the extinction coefficient of tetraguaiacol ε_{470} =0.0266 $mM^{-1}\cdot cm^{-1}$. The peroxidase activity was expressed as the rate of tetraguaiacol formation per 1 mg fresh weight of the sample: $\mu M\cdot min^{-1}\cdot mg^{-1}$ fresh weight. Enzyme activity measurements were carried out at a constant temperature of +25 °C.

Measurements were carried out with at least three replicates, and the arithmetic mean and standard error of the mean were calculated. The reliability of the differences was assessed using the nonparametric Mann–Whitney test (p=0.05) by the Statistica soft.

RESULTS AND DISCUSSION

The level of viability differed in the cells of the callus cultures before exposure to temperature (Figure 2). It turned out to be higher in No. 3 compared with other cultures. After exposure to temperatures of 4 or 1 °C, there were no significant changes in the level of cell viability. Cell viability increased in cells of culture No. 4 after exposure to a temperature of 4 °C.

The growth rate of the studied cultures was the same before exposure to temperature, except for culture No. 2. The growth rate of this culture was lower than the growth rate of culture No. 3 (Figure 3). After the exposure to each of the temperatures, there was a significant decrease in the growth rate of all cultures except No. 4.

Before exposure to the temperature, the hydrogen peroxide content was highest in the cells of culture No. 2 (Figure 4). The hydrogen peroxide content was the same at No. 4 and No. 5 before exposure and did not change after the exposure. After exposure to a temperature of 1 °C, the content of hydrogen peroxide decreased in cells of callus cultures No. 1, No. 2 and No. 3. In No. 2 cells, such a decrease also occurred after the action of 4 °C.

Before the exposure the level of superoxide anion content was elevated for No. 1, No. 2 and No. 5 (Figure 5). An increase in its content occurred in cells of calli No. 3, No. 4 and No. 5 after the exposure to each of the temperatures. In cells of cultures No.1 and No.2, an increase in the content of the superoxide anion occurred only after exposure to 1 °C. The content of the superoxide anion decreased in No. 2 callus culture cells after exposure to a temperature of 4 °C.

Before the exposure the level of lipid peroxidation turned out to be the highest in the cells of culture No. 2 and the lowest in cells of culture No. 1 (Figure 6). After the exposure to each of the temperatures, there was a significant increase in lipid peroxidation activity in all studied cultures. In the cells of the cultures No. 3 and No. 4, the activity of lipid peroxidation was higher when exposed to 4 °C compared with exposure to 1 °C.

Before cold exposure the activity of peroxidase was increased in No. 3 and No. 4 cultures (Figure 7). After exposure, the activity of this enzyme was increased in all callus cultures: at No. 2 and No. 4 after 1 °C; at No. 1

after 4 °C. An increase in peroxidase activity occurred after the exposure to each of the temperatures at No. 3 and No. 5 cultures.

As can be seen, the growth rates of the cultures No. 2 and No. 3 differed before exposure to temperature (Figure 3). This fact is in good agreement with the data obtained in other studies aimed for the studying of callusogenesis in various species of higher plants. It is known that primary explants taken from plants of the same species and grown under the same conditions differ depending on the initial genotype (cultivars, lines) in the frequency of callus formation and in the intensity of cell culture growth (Schween, Schwenkel, 2003; Mostafa *et al.,* 2020). Such differences are usually due to the peculiarities of the genotypes of the trees used to produce callus cultures.

In this study, differences were also established between cultures obtained from different trees in the levels of viability, ROS content, lipid peroxidation and peroxidase activity, which indicates possible differences in stress resistance and is consistent with literature data (Lukatkin and Geras'kina 2003). The cultures No. 1 and No. 2 differed from the rest by reduced cell viability and peroxidase activity (Figure 2, 7). The growth rate of the No. 2 culture was also lower than that of other callus cultures (Figure 3). At the same time, the content of hydrogen peroxide and the activity of lipid peroxidation were increased in cells of culture No. 2 compared with cells of other cultures (Figure 4, 6). The culture No. 1 was also characterized by reduced viability and low peroxidase activity of the cells (Figure 2, 7). These facts suggest that the cells of the callus cultures No. 1 and No. 2 compared with cells of other cultures have a reduced ability to resist stress conditions. The visual appearance of callus cultures No. 1 and No. 2 is consistent with this assumption (Figure 1).

Areas of darkening (necrotization) of callus during the cultivation process were characteristic of the plant material of all five trees, however we found such areas to be especially typical for the cultures No. 1 and No. 2. Such darkening of tissues is a fairly common phenomenon in the *in vitro* system that indicates processes that often lead to death of the culture (Baimukhametova and Kuluev, 2020). It was shown that darkening is accompanied by a decrease in cell proliferation rate and a stop in developmental process, which is confirmed by an increase in the number of dying cells with increasing intensity of darkening (Laukkanen *et al.,* 1999). Such darkening is associated mainly with oxidation of phenolic compounds, which are represented by a large variety of compounds in plants, especially in coniferous species (Tang and Newton, 2004; Shimelis *et al.* 2015). As a result of the oxidation reaction of phenolic compounds, highly reactive chemical compounds – o-quinones – are formed, that are able to interact both with each other and with other cell compounds, such as amino acids or proteins, forming high-molecular compounds (melanins), which dye damaged surfaces mostly in brown, but also in red or black colors (Shengjun *et al.,* 2014). The complete loss of cell nucleus, which does not occur in living callus cells (Amineva *et al.,* 2017), evidence for death of dark-colored cells. The listed facts allow us to conclude that cells of callus culture No. 2 and No. 1, regardless of origin, are more susceptible to necrosis and early death compared to cells of other cultures.

On the other hand, the lower activity of lipid peroxidation was observed in cells of the cultures No. 3 and No. 4 at the influence of 1 °C compared with 4 °C (Figure 6), which allows us to assume the ability of cells of these cultures to adapt to exposure and their possible increased stress resistance. The data indicate the absence of growth suppression under the action of cooling and an increase in the viability of culture No. 4 cells under the action of 4 °C (Figure 2, 3), which is consistent with the assumption of greater stability of culture No. 4. Thus, the cultures studied were not the same in their characteristics and, perhaps, they differed in their resistance to external influences.

Our study shows that low temperatures suppress the growth of all studied callus cultures, except for the culture No. 4 (Figure 3). It is known that a decrease in the growth rate of plants under the action of an external stressor can be due to both passive (for example, with a lack of a particular resource under stress) and active factors (with the help of genetically fixed regulatory mechanisms). One of the main such mechanisms is regulation by kinases (Zhang et al., 2020). The reduction

in growth rates in most callus cultures indicates a decrease in the contribution of metabolites to growth processes and a restructuring of cell metabolism in the direction of protective activity. The conducted research does not allow to determ whether the growth suppression is passive, or it is due to active regulation or both factors act together. In any case, the suppression of growth indicates the presence of a response to the exposure.

After exposure to low positive temperatures, the changes in the studied stress indicators had a different direction. The direction of change of the indicators was typical for all or most of the studied cultures, although they initially differed in the values of these indicators. It has previously been suggested that crops may differ in their stress tolerance. All the studied callus cultures were characterized by an increase in the activity of lipid peroxidation (Figure 6). Such a change is a sign of adverse intracellular processes occurring in response to exposure. During the development of lipid peroxidation, membrane permeability and fluidity are impaired, and the formation of toxic reactive lipid-derived molecules increases, which trigger a chain reaction of destruction of lipid-containing intracellular structures, grows (Gill and Tuteja 2010). Of all the indicators of cold-induced oxidative stress investigated here, an increase in the level of lipid peroxidation is one of the most harmful consequences. At the same time, no decline in cell viability under the action of a stressor was determined (Figure 2), as well as the abcence of the increase of hydrogen peroxide content was detected (Figure 4), which speaks in favor of the ability of callus culture cells to withstand negative changes at the selected dose of exposure. The increase in peroxidase activity shown for all callus cultures (Figure 7) also indicates the activation of mechanisms of protection against the development of oxidative stress (Devi *et al.,* 2023). Peroxidases are one of the most active enzymatic antioxidants that react chemically with hydrogen peroxide. An increase in the activity of these enzymes under stressful conditions was noted for coniferous plants (Radotic *et al.,* 2000; Sudachkova. *et al.,* 2016; Korotaeva *et al.,* 2024).

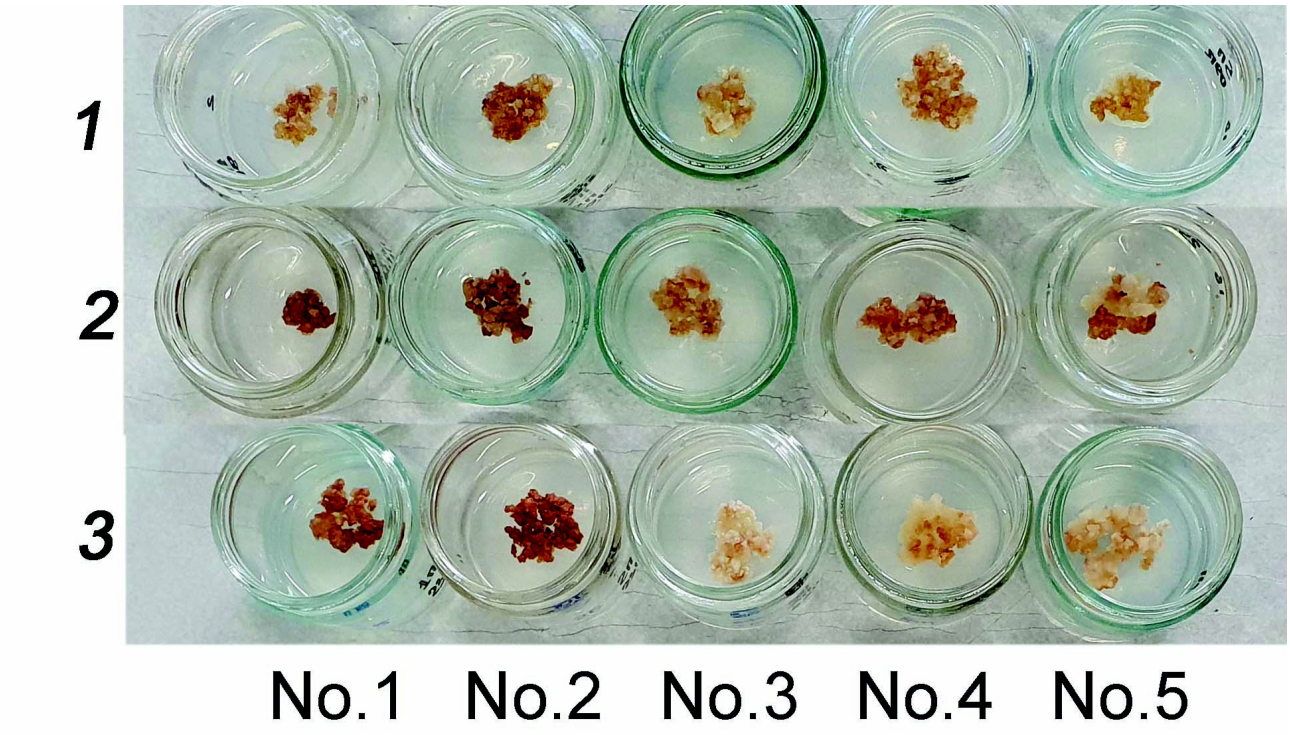

Figure 1. A typical visual appearance of callus cultures at control conditions. The numbers under the picture means the trees used to produce of the calli. The numbers on the left of the picture means the number of the repeats.

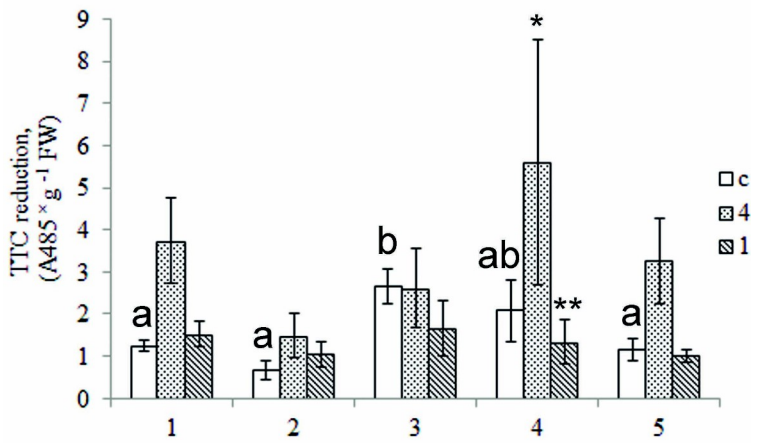

Figure 2. The influence of low temperatures on viability of callus cell cultures. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 4). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli. FW – fresh weight.

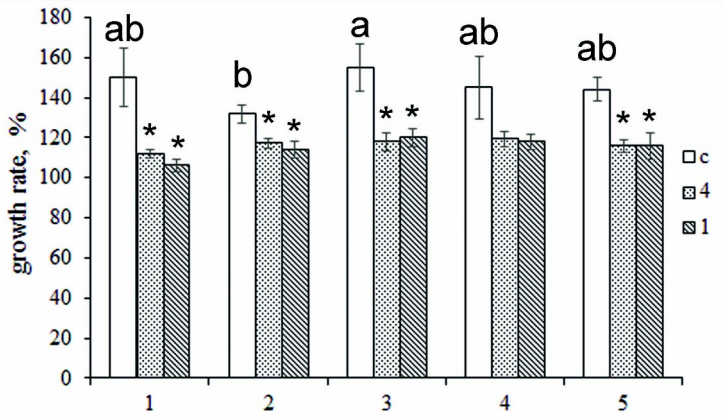

Figure 3. The influence of low temperatures on the callus growth rate. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 5-8). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli.

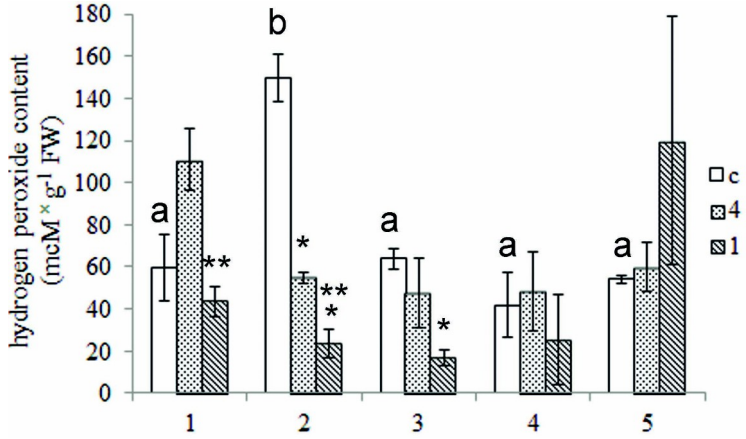

Figure 4. The influence of low temperatures on the hydrogen peroxide content. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 3-7). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli. FW – fresh weight.

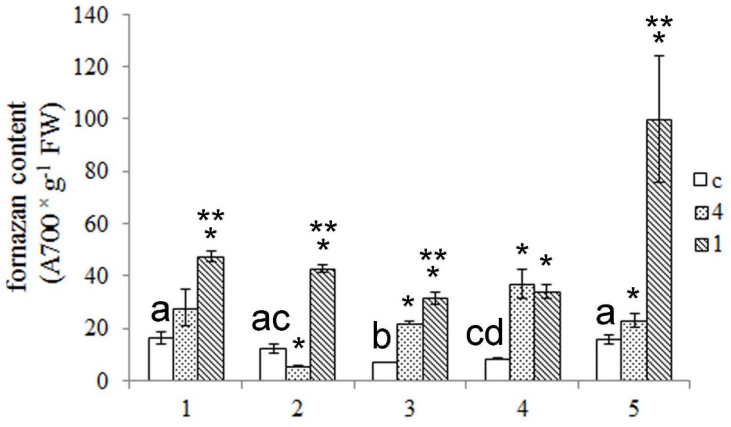

Figure 5. The influence of low temperatures on superoxide anion content. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 3-13). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli. FW – fresh weight.

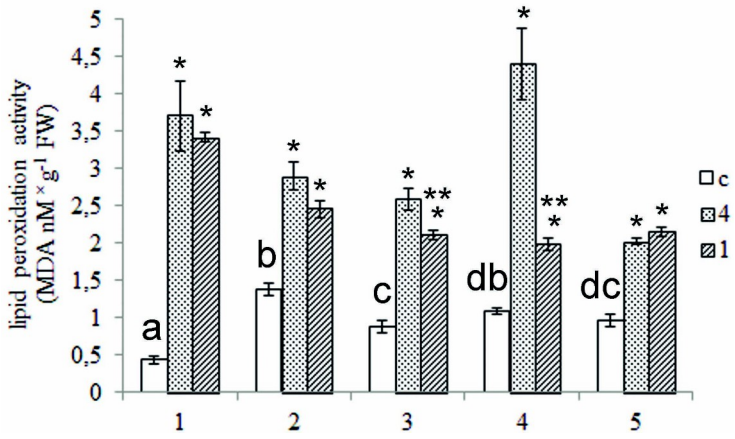

Figure 6. The influence of low temperatures on lipid peroxidation activity. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 4-10). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli. FW – fresh weight.

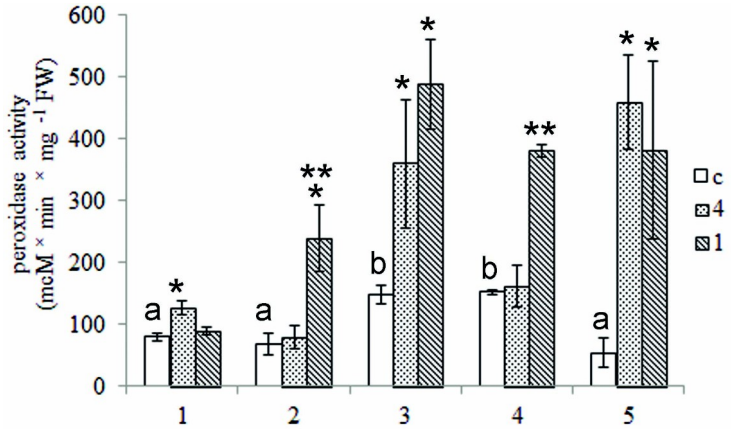

Figure 7. The influence of low temperatures on peroxidase activity. C – control; 4 – 4 °C; 1 – 1 °C. The means and standard errors of the mean are shown (n = 3-8). * indicates significant differences among control (c) and treatment; ** indicates significant differences among treatments ($p < 0.05$). Different letters indicate significant differences among calli obtained from different trees at control conditions ($p < 0.05$). The numbers means the trees used to produce of the calli. FW – fresh weight.

The increase in the content of the superoxide anion (Figure 5) may have two meanings. On the one hand, such an increase indicates the development of oxidative stress; on the other hand, the superoxide anion may be a factor in protective reactions and indicate the development of protective signaling (Mittler *et al.,* 2004; Czarnocka and Karpiński 2018). Since the increase in the content of the superoxide anion turned out to be small, and we did not record a decrease in the viability of callus cells, it can be assumed that the balance between the accumulation of the superoxide anion and its utilization under the action of the selected dose of exposure is not disturbed, and the cells manage to utilize the excess of this form of ROS.

As a result, the conducted study allows us to conclude about the ability of cells of callus cultures obtained from the buds of Scots pine to resist the action of the selected dose of low temperatures, despite the ongoing oxidation of lipids. Moreover, such a reaction turned out to be characteristic of callus cultures, the cells of which may have different stress resistance.

CONCLUSION

The selected dose of exposure to low positive temperatures (4 or 1 °C, 7 days) causes protective response changes in the cells of callus cultures (lines), which have differences in indicators of growth, viability and the development of intracellular oxidation under control conditions, and does not lead to cell death, although it is accompanied by lipid oxidation. Thus, this test system is suitable for analyzing the protective reactions of cells of callus lines obtained from the buds of Scots pine in response to low positive temperatures. In particular, this test system will be useful for studying the effect of oxidative stress accompanying the effect of low positive temperatures on the buds cells of Scots pine.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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