підсаджування сіянців. При цьому потрібно залишати деяку частку, навіть всохлих дерев, бо внаслідок суцільної вирубки дерев відбувається швидке заболочування території.

Подяки. Щиро дякуємо за сприяння та допомогу в дослідженнях директору ДП "Новоград-Волинське ДМЛГ Степану Антоновичу Нусбаум та головному лісничому Георгію Арсентійовичу Юзвінському. Також велика подяка за надані матеріали та цінні поради колегам із Житомирської гідрогеолого-меліоративної експедиції та Новоград-Волинського міжрайонного управління водного господарства.

Висновки

1. Депресії радіального приросту ясена та захворювання дерев кореневою гниллю виникли внаслідок збільшення кількості посух у липні та серпні; незначного збільшення кількості опадів взимку на фоні підвищення зимових та ранньовесняних температур, що спричинило підняття рівня ґрунтових вод, на яке також вплинув незадовільний стан вторинних меліоративних каналів, які не виконують свої функції повною мірою.
2. Вплив температур та опадів на радіальний приріст дерев збільшився у 1985-2014 рр., порівняно з 1956-1984 рр., про що свідчить зменшення стійкості ясеневого насадження. Цей деревостан почав всихати надзвичайно високими темпами у 2011-2014 рр.

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Коваль И.М. **Биоиндикация состояния насаждения ясеня обыкновенного Западной Лесостепи на примере древостоя Ярунского лесничества ГП "Новоград-Волынское ГЛОХ"**

Представлены результаты дендроклиматических и дендроиндикационных исследований ясеневых насаждений в Новоград-Волынском физико-географическом районе. Выявлено увеличение чувствительности радиального прироста ясеня обыкновенного в 1986-2014 гг. в сравнении с 1956-1985 гг. к климатическим факторам, что свидетельствует об уменьшении стойкости насаждений вследствие изменений климата.

Депрессии радиального прироста вызваны повышением температуры в июле-августе и повышением уровня грунтовых вод. Вследствие этого ясеневые насаждения страдают от корневой гнили и массово усыхают.

Ключевые слова: дендроклиматические и дендроиндикационные исследования, динамика радиального прироста деревьев, *Fraxinus excelsior* L., изменения климата, уровень грунтовых вод, корневые гнили.

Koval I.M. **Biological Indication of the State of European Ash Stands in West Forest-Steppe Zone on the Example Stand of Jarunske Forestry of GE 'Novograd-Volynsky GFHF'**

Some results of dendroclimatic and dendroindicative research of ash stand in Novograd-Volynsky physiographic region are presented. In 1986-2014 sensitivity of ash radial growth to climatic factors comparing with 1956-1985 was detected that indicates decrease of stands resistance caused by climatic changes. Depressions of radial growth were caused by increase of temperature in July, August and increase of water table. Consequently ash stands are damaged by root rot and large-scale drying occurs.

Keywords: dendroclimatological and dendroindication researches, dynamic of tree radial growth, *Fraxinus excelsior* L., climatic changes, water table, root rot.

УДК 630*232.3

EXPRESSION OF THE PEROXIDASE GENE FROM *PINUS SYLVESTRIS* L. IN SEEDLINGS UNDER ABIOTIC STRESS

V.A. Kovaleva[1], N.I. Hrunyk[2], Yu.M. Yusypovych[3], R.T. Gout[4]

Abiotic factors such as cold, salt, drought, flooding, and heavy metal pollutants cause production of reactive oxygen species in different intracellular and extracellular compartments in plants. Peroxidases play a key role in the control of cellular H_2O_2 level. Extracellular peroxidases are involved in a wide range of physiological processes such as lignification, suberization, cross-linking of cell wall proteins, stress tolerance, and defense against phytopathogenic attacks. The activity of extracellular peroxidases in roots of seven-day-old Scots pine seedlings was detected. Expression of the Scots pine *peroxidase* gene was up-regulated by copper, cobalt, zinc, sodium chloride, flooding and hydrogen peroxide treatments. High temperature and drought suppressed expression of the gene

Keywords: abiotic stress, gene expression, peroxidase, Scots pine

Introduction. Stressful environmental conditions such as cold, salt, drought, pathogenic attacks, and heavy metal pollutants cause production of reactive oxygen species (ROS) in different intracellular and extracellular compartments in plants. In order to cope with these stresses, plants have evolved the counteract effects to ROS

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with a versatile and cooperative antioxidant system that modulates intracellular ROS concentrations and sets the redox status of the cell [1, 2]. Peroxidases are the antioxidative enzymes playing key roles in the control of cellular H_2O_2 levels. "Classical" plant peroxidases forming class III plant heme peroxidases (POXs, EC 1.11.1.7) are a large family of plant secretory enzymes, able to catalyze oxidoreduction between a variety of phenolic substrates and hydrogen peroxide [3]. POXs are encoded by a large number of genes, and products of their expression, due to the presence of signal sequence are secreted from plant cells or transported into vacuoles via the endoplasmic reticulum. Since these enzymes exist in multiple isoforms with different substrate specificities, they have been involved in a wide range of physiological processes such as lignification, suberization, cross-linking of cell wall proteins, auxin metabolism, stress tolerance, defense against phytopathogenic attacks and growth regulation [4].

Peroxidases show tissue-specific and developmentally regulated expression profiles. In *Arabidopsis thaliana*, the tissue-specific expression of 73 peroxidase genes was studied by microarray assays [5, 6]. The expression level of peroxidase genes is regulated by biotic and abiotic environmental factors such as wounding, ethylene, pathogen infection, drought, low-temperature, iron deficiency, light and plant growth regulators [7-9]. Do *et al.* found that the expression of a peroxidase gene *CAPO1* was highly unregulated by copper stress and this gene may be involved in pepper defense against pathogen attack [10]. More recently, overexpression of *CaPO*2 in transgenic *Arabidopsis thaliana* plants conferred enhanced tolerance to high salt, drought, and oxidative stress, while enhancing resistance to infection by the necrotrophic fungal pathogen *Alternaria brassicicola* [11]. Overexpression of sweet potato (*Ipomoea batatas*) *swpa*4 peroxidase significantly increased the salt and drought stress-tolerance of tobacco plants. Expression of poplar peroxidase gene *PoPOD1* is down-regulated in response to environmental stresses, including metals, NaCl, methyl viologen and polyethylene glycol, and plant growth regulators such as jasmonic and gibberellic acids [7].

Expression of peroxidase genes in response to stressful environmental factors have been well documented in angiosperms. Only a few peroxidases from Scots pine, Norway spruce and ginkgo have been described in gymnosperms [12-14]. The coniferous trees are among the main producers of biomass on Earth in the form of lignified wood; they are important source of bioenergy, timber and pulp production. Therefore, the study of the enzymes involved in monolignol biosynthesis and lignin polymerization is important task. Recently Marjamaa et al. cloned the cDNAs of three xylem-expressed class III peroxidase-encoding genes (*px1, px2* and *px3*) from Norway spruce (*Picea abies*) and showed that mRNA, codifying PX1 and PX2, accumulated in the cytoplasm of young, developing tracheids within the current growth ring, where lignification occurs [15].

In this study, in order to better understand antioxidant defence mechanisms in Scots pine plants against abiotic stress, we investigated expression patterns of a peroxidase gene, homologous to the *px2* coding sequence from the *Pinus taeda* EST library, under abiotic stress conditions such as salt, cold, HCl, drought, hydrogen peroxide, heat shock and water.

Materials and methods. *Plant material*. Seeds of Scots pine (*Pinus sylvestris* L.) were obtained from Lviv forest breeding seed center (Lviv region, Ukraine). Surface-sterilized seeds were germinated on Whatman paper soaked with distilled water in Petri dishes at 26 °C in the thermostat. After 7 days, the seedlings were used for treatments.

Detection of extracellular peroxidase activity in Scots pine seedlings. Diaminobenzidine (DAB) is a substrate for peroxidases, the addition of DAB with H_2O_2 and the formation of the colored precipitate is considered to be indicative of peroxidase activity. Three milliliters of the reagent containing 0.05 M Tris-HCl buffer, pH 7.6, $1.4x10^{-3}$ M DAB, 0.05 % H_2O_2 were added to the Petri dish with the seven-day-old pine seedlings. The staining was developed for 3-20 min. Images were examined using conventional light microscopy.

Stress Treatments. On 7th day, seedlings were transferred onto sterile filter paper in Petri dish, soaked with solutions of 10 mM HCl (acid stress), 250 mM NaCl (salt stress), 100 mM mannitol (drought stress), 10 mM hydrogen peroxide (oxidative stress), $2.5x10^{-4}$ M Cu^{2+}, $5x10^{-6}$ M Cd^{2+}, 5×10^{-4} M Cr^{3+}, 5×10^{-4} M Co^{2+} and 1.7 10^{-3} Zn^{2+} (heavy metal stress). Control plants were treated with sterile ddH_2O. For cold and heat stress treatments, pine plants were placed at 4 °C or 37 °C, respectively. For water-stress 7-day-old seedlings were fully submerged in distilled water for 24 h. Mock and stress-treated seedlings were collected at 24 h after treatment, immediately frozen in liquid nitrogen and kept at −80 °C. All experimental plates were placed in a thermostat at 24 °C (except cold and heat stress) in dark with 60 % relative humidity. All treatments were performed and analyzed in triplicate in separate experiments.

Semi-quantitative RT- PCR analysis of peroxidase gene expression. Total RNA was obtained using modified method of lithium-chloride precipitation by Chang [17]. First strand cDNA was synthesized using reverse transcriptase Revert Aid Premium (Fermentas). The obtained cDNA was used for semi-quantitative analysis of the expression levels of *Pinus sylvestris* peroxidase gene homologous to the *px2* from Norway spruce. The specific primers GCTCTAGCGGCTAAAGAGT (forward) and GAGGTCTGTGACGTTGAGA (reverse) were designed against cDNA sequence (GenBank Acc. No. CO165441) from *Pinus taeda* cDNA library 015903. The housekeeping gene – *60S ribosomal protein L44* (RPL44; GenBank Acc. No. EL342388) was used as a standard control in semi-qPCR [18]. PCR was run for 30 cycles in a thermal cycler using the program: 95 ºC, 45 s; 54 ºC, 45 s; 72 ºC, 45 s. The PCR products were separated by electrophoresis on a 2.0 % agarose gel, visualized by ethidium bromide staining, and photographed. Densitometric analysis was run with Software GelProAnalyzed 4.0.

Results and discussion. Peroxidases are found in all terrestrial plants and have many diverse functions, including H_2O_2 detoxification and degradation of indole acetic acid, lignin biosynthesis, and the formation of ROS. Plant peroxidases are present in all organs and almost all tissues. They play a key role in the formation of ROS, which are implicated in promoting or inhibition of cell growth and elongation. Elongation and meristematic zones are rich in $O_2^{\bullet -}$, which in combination with peroxidases, can produce $OH^{\bullet}$ necessary for cell wall loosening, cell growth and elongation, while in the differentiation zone, where cell elongation ceases and root hairs are formed, the predominating ROS is H_2O_2 [18]. Main processes, which occurs on early stages of postembryonal development in pine, are related with growth and elongation.

To detect extracellular peroxidase activity in young seedlings we used diaminobenzidine – a known substrate for peroxidase activity, which is oxidized with hydrogen peroxide resulting in a reddish-brown precipitate. With the presence of hydrogen peroxide in the reaction mixture and on the surface of the root cap, we observed the formation of brown precipitate (Fig. 1, C), indicating the presence of extracellular peroxidases associated with cell wall and which may be involved in the lignification processes. When added to Petri dishes DAB without hydrogen peroxide, a light-brown precipitate formed after 20 minutes of incubation, indicating the presence of endogenous hydrogen peroxide on the root cap of seedlings (Fig. 1, B).

Fig. 1. Detection of extracellular peroxidase activity and endogenous H_2O_2 in the 7-day-old Scots pine seedlings. A- non-treated seedlings; B – seedlings stained with diaminobenzidine (DAB) without H_2O_2; B – seedlings stained with DAB and H_2O_2

Class III peroxidases are antioxidative enzymes involved in several physiological processes including plant development, cell wall lignification and oxidative stress. Due to their reactive cycles, these proteins are involved in both – production and detoxification of ROS and are affected by several stress conditions. The expression levels of peroxidases from angiosperms are changing during different biotic and abiotic stimuli **[**5-11**]**. Among conifers, in *Picea abies* were identified 17 peroxidase genes (*PabPRX*), 11 of which are expressed in lignifying xylem of adult trees, but the highest expression in all lignin-forming materials (lignifying tissue-culture line, mature xylem, young vertical xylem, and young compression wood) was related to *PabPrx02, PabPrx03, PabPrx08, PabPrx13,* and *PabPrx14* genes **[**19**]**. Koutaniemi et al. showed that only *PabPrx02* and *PabPrx03* have a general stress induced function **[**19**]**. However, PabPrx02 transcripts were found inside the differentiating tracheids, but not in the mature tracheids, ray cells or cambium **[**15**]**. To find an orthologous gene in Scots pine, we used EST database from *P. taeda.* We found a large number of peroxidase-like clones in the cDNA libraries from embryos, seedling roots, stem and differentiating xylem.

To evaluate the possible roles of the *Pinus sylvestris* peroxidase (*PsPrx02*), the ortholog to *PabPrx02*, in the defense response in pine against various abiotic stimuli, the *PsPrx02* expression analysis was carried out by semi-qRT-PCR using gene-specific primers designed to a cDNA clone (GenBank Acc. No. CO165441) from *Pinus taeda.*

Heavy metal stress. To determine the expression of *PsPrx02* in response to metals, seven-day-old seedlings were treated with high concentrations of various metals. *PsPrx02* expression response varied greatly with the metal supplied. The presence of heavy metal in toxic concentration can result in the formation of ROS, which can be initiated directly or indirectly by heavy metals. The levels of *PsPrx02* transcripts were decreased in chromium- and cadmium-treated pine seedlings suggesting that this gene is not responsible for broad-scale scavenging of ROS in pine. Cadmium and chromium are environmental pollutants which are toxic to many plant species at low concentrations. Cd toxicity was attributed to the formation of reactive oxygen species and uncontrolled cell death when the antioxidative capacity of the cells was overwhelmed **[**20**]**. Cadmium and chromium overloads have been found to increase the peroxidase activity in the cell wall of *Pisum sativum* L. **[**21**]**. Sharma and Sharma **[**22**]** found that the application of 0.5 mM Cr in wheat cultivar cv. UP2003 decreased the activities of peroxidase. At present, however, it is difficult to explain the effects of cadmium and chromium on the *PsPrx02* down-regulation.

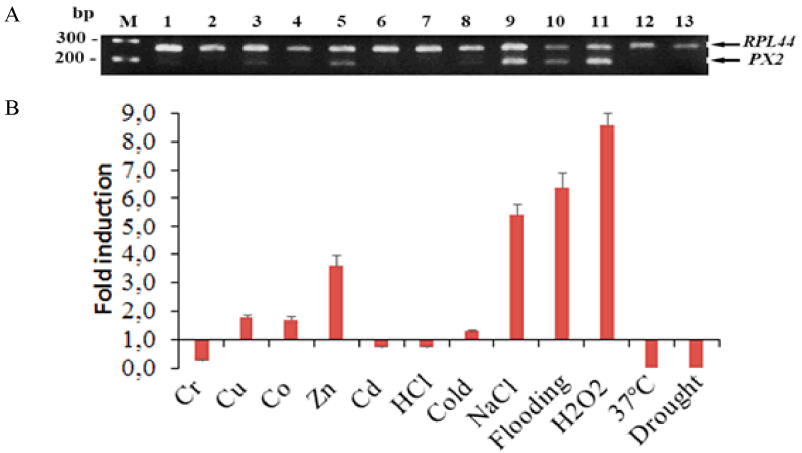

Fig. 2. Analysis of the peroxidase gene PsPrx02 expression in Scots pine seedlings after different abiotic stress treatments. *A) electrophoregram of the PCR products obtained from RNA of pine seedlings after treatments: lines 2-6 – heavy metal stress (Cr, Cu, Co, Zn, Cd, respectively); line 7 – acid stress, line 8 – cold stress; line 9 – salinity stress; line 10 – water stress; line 11 – oxidative stress; 12 – heat stress, 13 – drought stress. Line 1 – non-treated pine seedlings. Line M - GeneRuler 100 bp Plus DNA Ladder (Fermentas). Right lines indicate the PCR-products: PsPrx02 and "housekeeping" gene RPL44. B) The values of the expression level of PsPrx02 calculated relative to RPL44. The expression level in control seedlings was set to one*

Copper, cobalt and zinc up-regulated the *PsPrx02* expression levels 0.8-, 0.7- and 3.5-fold, respectively (Fig. 2, B). Little information is available about the relationship between heavy metal toxicity and responses of plant peroxidases. Copper is a major redox metal causing free radical formation and, hence, oxidative damage in plants. Roots of *Raphanus sativus*, grown in the presence of Cu (1-10 mM $CuSO_4$), showed higher levels of cationic and anionic POX activities and higher levels of lignin content in comparison with plants grown in the absence of this element and was regulated in dose-dependent manner **[**23**]**. *PoPOD1* gene expression was slightly increased by zinc, and significantly decreased by copper **[**24**]**. Cobalt and zinc inhibited peroxidase activity in tubers from Jerusalem artichoke **[**25**]**. Thus, although the evidence indicates that plant peroxidases are involved in heavy metal stress response, the precise mechanisms remain unknown.

Temperature stress. The results showed diverse expression patterns in response to cold and heat stresses (Fig. 2 A, lines 8, 12). It is well established, that temperature stress elevates the levels of ROS in plants and would be expected to induce various kinds of antioxidant enzymes, including peroxidases **[**26**]**. In our study, the *PsPrx02* transcription levels were increased 0.3-fold at 24 h of cold stress. Similar up-regulation by 0.7 times was observed in pepper *CanPOD* on mRNA level, at 24 h of cold treatment, comparing with the control **[**27**]**. On the contrary, Choi found that cold stress strongly induced the $CaPO_2$ gene expression in pepper leaves up to 25 h after treatment **[**28**]**. Park reported that six peroxidase genes showed different expression levels in response to chilling, and only *swpa*4 gene was strongly expressed at 4 °C, while the expression level of *swpb*3 was decreased **[**8**]**. These results suggest that there are differences in peroxidase gene expression patterns from various plants in response to cold stress.

In contrast to the results obtained from cold stress treatment, the *PsPrx02* gene expression was suppressed under heat-shock condition. It is known, that heat stress (HS) affects the stability of membranes, proteins, and enzymatic reactions, which subsequently disrupts the metabolic balance that causes the accumulation of ROS. Wang et al. demonstrated that ROS including H_2O_2 are considered to be the first signaling components produced by HS **[**29**]**, which is involved in the downstream signaling pathways leading to the production of heat stress transcription factors and heat shock proteins. Thus, genes, which encode proteins with hydrogen peroxide reduction activity, such as *PsPrx02,* could be suppressed.

Water stress. Drought and flooding are two different forms of water stress that adversely affect the growth and development of pine plant, in particular at early stages of development. Flooding stress was imposed by fully submerging of 7-day-old seedlings in distilled water and drought stress was caused by treatment with 100 mM mannitol. Previously it was demonstrated upregulation of *POX* genes in response to drought stress, and transgenic plants expressing exogenous *POX* genes showed high levels of drought-stress tolerance **[**30**]**. In our study, expression of *PsPrx02* was suppressed in response to drought stress (Fig. 2). Our results correlate well with the results obtained from poplar $PoPOD_1$ gene expression which was down-regulated by PEG that induced the generation of ROS in plant cells **[**27**]**. Kravic et al. also showed that changes in root peroxidase activities ranged from approximately 40 % reduction to 20 % stimulation, depending on the genotype, under drought stress **[**31**]**. These results suggest that the Scots pine peroxidase gene may be suppressed during the drought stress.

As known flooding provokes hypoxia (reduction of oxygen below optimal levels) in plant roots. The generation of ROS is characteristic for hypoxia. Expression of genes encoding some enzymes involved in ROS scavenging system can be significantly changed by the flooding stress. Qi et al. demonstrated that expression of gene encoding POX in cucumber increased 25-fold at 24 h after waterlogging treatment **[**32**]**. The *PsPrx02* gene expression levels were significantly enhanced in pine seedlings under flooding 5.4 – fold.

Salinity stress. Salt stress is known to induce the accumulation of ROS, which could play as the mobile signals, able to elicit ROS scavengers and other protective mechanisms, in addition to acting as damaging agents that contribute to stress injury

in plants **[**33**]**. Salt stress causes an alteration of water transport as a consequence of increasing the amount of lignin and suberin in the roots **[**34**]**. Treatment of pine seedlings with 250 mM NaCl enhanced the expression level of peroxidase gene 4.4-fold comparing with non-treated plants (Fig. 2, A). Up-regulation of genes encoding extracellular peroxidase by salinity stress was reported in pepper leaves, rice, foxtail millet seedlings and sweet potato **[**27, 30, 35, 36**]**. Therefore, we conclude that the *PsPrx02* gene involves in pepper plant resistance to salt stress.

Oxidative stress. Hydrogen peroxide is constantly generated from various sources during normal metabolism in plant cells. An oxidative burst, with rapid H_2O_2 synthesis, is a common response to pathogens, elicitors, wounding, heat, ultraviolet light and ozone **[**37**]**. H_2O_2 is a signaling molecule in plants, which mediates various physiological, and biochemical processes. We treated pine seedlings with exogenous H_2O_2 to investigate its effect on pine peroxidase expression. Compared to the controls, exogenous 10 mM H_2O_2 significantly increased the *PsPrx02* gene expression level (7.6-fold). These findings suggest that peroxidase *PsPrx02* are able to remove H_2O_2 via oxidation of the co-substrate guaiacol, which is involved in lignin formation **[**38**]**.

Conclusions. In conclusion, we revealed the activity of extracellular peroxidases in seven-day-old Scots pine seedlings. We studied the expression patterns of Scots pine peroxidase gene, the ortholog to *PabPrx02* gene from *Picea abies,* in the defense response of pine seedlings against various abiotic stresses. The results indicated that *PsPrx02* was significantly up-regulated by zinc, NaCl, flooding and H_2O_2. Cooper, cobalt, and cold treatments slightly enhanced the expression levels of pine peroxidase. The *PsPrx02* gene was not evidently expressed under chromium, cadmium, acid, and cold stresses comparing to the other abiotic stresses. In addition, heat and drought suppressed the expression of this gene. Taken together, these results suggest that *PsPrx02* is involved in defense response of Scots pine to abiotic stress.

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Ковальова В.А., Груник Н.І., Юсипович Ю.М., Гут Р.Т. **Експресія гена пероксидази у проростках сосни звичайної (*Pinus sylvestris* L.) за абіотичного стресу**

Абіотичні фактори, такі як холод, засолення, посуха, затоплення та важкі метали, спричиняють утворення активних форм кисню в різних внутрішньоклітинних і позаклітинних компартментах рослин. Пероксидази відіграють ключову роль у контролі рівня H_2O_2 у клітині. Позаклітинні пероксидази залучені до широкого спектра фізіологічних процесів, таких як лігніфікація, здерев'яніння, зшивання протеїнів клітинної стінки, стійкість до стресу, захист від фітопатогенних атак. Виявлено активність позаклітинної пероксидази в коренях семиденних проростків сосни звичайної. Рівень експресії гена пероксидази сосни звичайної підвищується під час обробляння сіянців міддю, кобальтом, цинком, хлоридом натрію, пероксидом водню та в разі повного занурення їх у воду. Висока температура і посуха пригнічують експресію цього гена.

Ключові слова: абіотичний стрес, експресія гена, пероксидаза, сосна звичайна.

Ковалева В.А., Грунык Н.И., Юсипович Ю.М., Гут Р.Т. **Экспрессия гена пероксидазы в проростках сосны обыкновенной (*Pinus sylvestris* L.) при абиотическом стрессе**

Абиотические факторы, такие как холод, засоление, засуха, затопление и тяжелые металлы, вызывают образование активных форм кислорода в различных внутриклеточных и внеклеточных компартментах растений. Пероксидазы играют ключевую роль в контроле уровня H_2O_2 в клетке. Внеклеточные пероксидазы задействованы в широком спектре физиологических процессов, таких как лигнификация, одревеснение, сшивка протеинов клеточной стенки, устойчивость к стрессу и защита от фитопатогенных атак. Обнаружена активность внеклеточной пероксидазы в корнях семидневных проростков сосны обыкновенной. Уровень экспрессии гена пероксидазы сосны обыкновенной повышается при обработке сеянцев медью, кобальтом, цинком, хлоридом натрия, пероксидом водорода и при полном погружении их в воду. Высокая температура и засуха подавляют экспрессию этого гена.

Ключевые слова: абиотический стресс, экспрессия гена, пероксидаза, сосна обыкновенная.