https://doi.org/10.15407/ukrbotj79.03.184

RESEARCH ARTICLE

Effect of low-temperature stress on the growth of plants of *Secale cereale* (*Poaceae*) and endogenous cytokinin content in roots and shoots

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Abstract. Phytohormones play a key role in the regulation of plant acclimation to low temperature. To elucidate the role of cytokinins in rye plant response to chilling, we studied the dynamics of these hormones in shoots and roots under short-term and prolonged cold stress. The 7-day-old plants were exposed to cold stress (2 °C) for 2 h (alarm phase of response) or for 6 h for two days (acclimation phase of response). Endogenous content of cytokinins was analyzed by HPLC-MS method. Low temperature had a differential effect on the content of individual cytokinins and their localization in rye plants. During the short-term stress, a decrease in the content of active cytokinins (*trans*-zeatin and *trans*-zeatin riboside) in the roots and an increase in the shoots were shown. Prolonged low-temperature stress declined the amount of cytokinins except *trans*-zeatin riboside, which was detected in both roots and shoots. Significant rise in *trans*-zeatin riboside content in roots and shoots in this period evidenced an important role of this cytokinin during cold acclimation of rye plants.

Keywords: adaptation, cytokinins, growth, low temperature, *Secale cereale*, stress

Article history. Submitted 28 March 2022. Revised 6 June 2022. Published 29 June 2022

Citation. Vedenicheva N., Shcherbatyuk M., Kosakivska I. 2022. Effect of low-temperature stress on the growth of plants of *Secale cereale* (*Poaceae*) and endogenous cytokinin content in roots and shoots. *Ukrainian Botanical Journal*, 79(3): 184–192. https://doi.org/10.15407/ukrbotj79.03.184

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Introduction

Plants are highly vulnerable to fluctuations in ambient temperature. One of the signs of global climate change on the planet is general warming accompanied by a short-term cold snaps, which negatively affect the growth and productivity of crops and cause significant losses of agricultural yield (Kolupaev et al., 2018). Low-temperature stress induces both plant growth retardation and a wide range of damages at the biochemical and ultrastructural levels, including inhibition of photosynthesis and alteration of membrane integrity (Ritonga, Chen, 2020). Plants differ in their sensitivity to low temperature. Some crops, such as winter cereals, are cold tolerant; they even need a prolonged exposure to chilling (vernalization) for their transition to the reproductive period (Deng et al., 2015). Nevertheless, cold stress is very dangerous at the early stages of seedling development through the cascade of negative changes, such as decrease in membranes fluidity and ion leakage, disruption of photosynthetic activity and respiration rate, reduction in enzymatic activity, disrupted water relations, nutrient uptake, carbohydrate metabolism, translocation of assimilates, oxidative stress, etc. (Hassan et al., 2021). Phytohormones play a key role in regulation of plant response to low temperature and further acclimation (Eremina et al., 2016; Zhao et al., 2021). In particular, one of the major groups of phytohormones are cytokinins, which are involved in the control of all vital processes, including adaptation to abiotic stresses (Cortleven et al., 2019). The primary cold sensor in plant cell is thought to be the membrane, since the altered composition and ratio of lipids to proteins trigger the autophosphorylation of the membrane histidine kinases (Shi et al., 2015), some of which are known to be cytokinin receptors (Romanov et al., 2018). *Arabidopsis* Heynh. (*Brassicaceae*) mutants with repressed genes encoding the cytokinin receptors *ahk2, ahk3, ahk4* demonstrate enhanced freezing resistance. Cold stress did not influence the expression of these genes, but induces the activity of genes encoding *Arabidopsis* response regulators (*ARRs*), which are part of the two-component system of the cytokinin signaling pathway (Jeon et al., 2010; Jeon, Kim, 2013). Effect of cold stress on cytokinin signaling has been reported in cereals as well. Thus, cytokinin biosynthesis and signaling is inhibited in rice under chilling (Maruyama et al., 2014). Moreover, this study showed the difference between regulatory mechanisms of rice and *Arabidopsis* responses to low temperature. Cold stress provokes rapid down-regulation of active cytokinins in leaves of winter wheat, but the level of these hormones increases after 21 d of acclimation (Vanková et al., 2014). Rapid decline in bioactive cytokinin content in the leaves of winter wheat has been established in the alarm phase of cold stress (1 d), whereas in spring wheat, this response is weaker and slower (Kosová et al., 2012). Comparison of wheat genotypes with different frost resistance showes that the decrease in the level of active cytokinins in the leaves and roots after cold stress is observed only in frost-resistant plants. However, the expression of the *ARR6* gene, which encodes a negative regulator of the cytokinin response, increases in the leaves and roots of both varieties under chilling stress (Kalapos et al., 2017). Cytokinin levels could be reduced at low temperatures due to conjugation, as it has been demonstrated in maize (Li et al., 2000). Nevetheless, the involvement of active cytokinin (zeatin) in plant response to cold stress could not be identified in experiments with rice (Maruyama et al., 2014) and sweet corn (Xiang et al., 2021). Moreover, in rice, the chilling results in an increase of isopentenyladenine level (Maruyama et al., 2014). Unexpected is the up-regulation of *IPT8* gene, encoding the key cytokinin biosynthetic enzyme isopentenyl transferase, in wheat after cold stress (Kalapos et al., 2017). The above contradictory results indicate the complexity of the regulatory mechanism of plant adaptation to low temperatures and involvement of cytokinins in this process.

Little is known about the phytohormonal control of cold acclimation of cereals, such as rye. Rye plants are more stress-tolerant and less demanding of soil nutrition compared to other crops. Winter rye grows and goes through the tillering stage at low temperatures. The seedlings are able to emerge at 5 °C, but the optimum temperature for growth is 16–22 °C (White et al., 1990). Even the short treatment with low temperatures is very harmful for development of nonhardened rye seedlings (Kolupaev et al., 2019). Recently, we have shown that hyperthermia has a differential effect on the content of individual cytokinins and their localization in the shoots and roots of rye plants, which depend on stress duration (Vedenicheva et al., 2021). We were not able to find reports of such results on the response of the hormonal system of rye plants to cold stress. At the same time, investigation of 96 rye genotypes reveals that winter survival of plants is associated with the number of leaf initials produced at shoot apical meristem during cold acclimation, which is regulated by phytohormone levels (Bahrani et al., 2021).

Considering the above, in the current study, we investigated the effect of short-term and prolonged cold stress on the growth of *Secale cereale* L. plants, dynamics and localization of cytokinins in shoots and roots at the early stages of plant development.

Materials and Methods

Plant material and chilling conditions

Rye seeds (*Secale cereale* 'Boguslavka') were sterilized with 80% ethanol, then washed with purified water and soaked in water for 3 h. After imbibition, water-soaked seeds were germinated on wet filter paper in Petri dishes in a climate chamber Vötsch (Balingen, Germany) at a temperature of 16 °C, a photoperiod of 16/8 h, day/night (light intensity 190 μmol/m^2·s) and humidity of 60%. Than the seeds were transplanted in plastic pots filled with 1.5 L of sterile river sand and grown under the same conditions. Sowing density was 100 seeds per pot. Watering was carried out daily with 50 mL of Knop solution, which contained in 1 L of purified water $Ca(NO_3)_2$ 1 g, KH_2PO_4 0.25 g, $MgSO_4$ 0.125 g, KNO_3 0.25 g, and $FeCl_3$ traces. When seedlings reached the age of seven days, pots were divided into four divisions. In the first phase of experiment, the first part of pots was placed into a refrigerator at the temperature 2 °C, humidity of 60%, in the darkness for 2 h. The second part of pots remained in climate chamber at a temperature of 16 °C for 2 h in darkness (control). In 2 h, seedlings from the first and second part of pots were rinsed from sand with purified water, the roots were separated from the shoots, samples (10 g) were fixed in liquid nitrogen and stored at –20 °C. In the second phase of experiment, the third part of pots with the seven-day-old seedlings were placed into refrigerator at a temperature 2 °C, humidity of 60%, in the darkness for 6 h. Then they were returned into the climate chamber for 2 h in the darkness and 16 h in the light. Next day, these pots were placed into a refrigerator at the temperature 2 °C for 6 h in the darkness and after that experiment was completed. Overall, in the second phase of theexperiment, the seedlings were stressed twice for 6 h during two days. The fourth part of pots remained in the climate chamber in control conditions all this time (9 d). The samples from the third and fourth parts of pots were taken in the same way as in the first phase of the experiment. All stress manipulations were carried out in the dark time of photoperiod. All experiments were carried out in three biological and five to seven technical replicates.

Cytokinin analysis

Cytokinins were extracted from homogenized samples with 80% methanol solution. Crude extracts were purified by centrifugation (pH 2.5, 15000 g, 4 °C) for 30 min in the K 24 centrifuge (Janetzky, Germany). Supernatant was adjusted with water to volume 50 ml and fractionated with the same volume of n-butanol at pH 8. The butanolic fraction was evaporated *in vacuo* to dryness. Dry residue was dissolved in 0.1 M HCl and passed through the column of ion-exchange resin Dowex 50Wx8 (Serva, Germany) in H^+ form. After washing with H_2O, the column was eluted with 0.1 N ammonia. Obtained fraction was dried and further purified by thin layer chromatography on Silicagel 60 F_{254} plates (Merck, Darmstadt, Germany), run in isopropanol : ammonia : water (10 : 1 : 1 by volume) solvent. Methods of purification are described in more details by Vedenicheva et al. (2016).

Detection and quantification of cytokinins were performed using the high-performance liquid chromatography–mass spectrometry (HPLC-MS) system (Agilent 1200, Santa Clara, CA, USA). The purified extracts were dissolved in 200 µL of mobile phase and a 5 µL aliquot was injected into Agilent Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm). Elution was performed in a stepwise gradient system of solvents methanol: water: acetic acid according to the following scheme: 0 min – CH_3OH/0.5% solution of CH_3COOH in deionized water (37/63) → 25 min: CH_3OH/0.5% solution of CH_3COOH (70/30) → 35 min: CH_3OH/0.5% solution of CH_3COOH (100/0) with a constant flow rate of 0.5 mL/min at a column temperature of 30 °C. The effluent was passed through an ultraviolet-diode array detector (wave length 269 nm, bandwidth 16 nm, spectrum scanning range 207–400 nm) and the mass spectrometer (Agilent 6120 Quadrupole LC/MS) in a combined regime ‘multi mode’ (electrospray and chemical ionization at atmosphere pressure) of positive ionization. Data were analyzed and processed using the Agilent ChemStation software version B.03.01 online. Standard solutions of *trans*-zeatin, *trans*-zeatin riboside, isopentenyladenine, isopentenyladenosine, and *trans*-zeatin-*O*-glucoside (Sigma-Aldrich, USA) were used as markers during all chromatographic procedures.

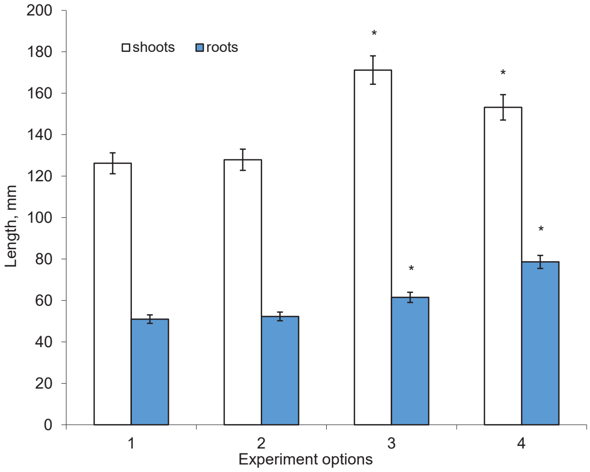

Fig. 1. Changes in morphological parameters of *Secale cereale* plants exposed to cold stress:

1 – control 7-day-old plants; 2 – plants after 2 h of cold stress (2 °C); 3 – control 9-day-old plants; 4 – 9-day-old plants after double exposure to cold stress (2 d for 6 h at 2 °C).

Data are the mean ± SEM. * $p < 0.05$ as compared to the control plants

Statistical analysis

The results are expressed as the mean ± SEM of three independent experiments. Experimental data were analysed using program Statistix 10.0 (Analytical Software, Tallahassee, FL, USA). The accepted level of significance was set at $P < 0.05$.

Results

The first section of our experiment focused on elucidation of the short-term cold stress influence on the growth and cytokinin levels in young rye plants. No visually noticeable changes in the morphological parameters were detected in both control and experimental 7-day-old seedlings after 2 h of exposure to 2 °C (Fig. 1). Simultaniously, the differences in cytokinin status in shoots and roots was essential. In shoots, the content of *trans*-zeatin and *trans*-zeatin riboside increased seven-fold and three-fold, respectively, after stress (Fig. 2). The ratio between these phytohormones also changed. The level of isopentenyl-type cytokinins and zeatin-*O*-glucoside decreased but the ratio between them remained the same (Fig. 2). In contrast, under short cold stress *trans*-zeatin and *trans*-zeatin riboside disappeared in roots, while isopentenyladenosine and isopentenyladenine levels declined about three times (Fig. 3). Amount of zeatin-*O*-glucoside was twice reduced (Fig. 3).

The second section of current investigation was to explore the effect of prolonged cold stress on rye plants. In the 48 h experiment, the shoots of control rye plants grew by 34% and roots – by 17%. At the same time, the morphological parameters of experimental plants changed differentially: the shoots grew by 20% and roots – by 50%. Thus, after chilling, the proportion between the plant shoot and root size significantly changed. In the control plants, the ratio between the shoot and root length was 2.8, then after the cold stress – 1.9 (Fig. 1). Hence, low temperature had a negative impact on the shoot growth, but the elongation of the root was stimulated.

In 9-day-old rye plants, the constitutive level of free cytokinins changed in comparison with 7-day-old plants. In particular, significant accumulation of *trans*-zeatin riboside and two-fold decrease in the isopentenyl-type cytokinin level was detected in shoots (Fig. 4). Changes in cytokinin status of roots were similar, but more pronounced. Sharp rise in the *trans*-zeatin riboside content was found, whereas the concentration of isopentenyladenosine, isopenteniladenine and zeatin-*O*-glucoside declined about three times (Fig. 5). As a result of the prolonged cold stress, the level of *trans*-zeatin, isopentenyladenosine and isopenteniladenine in shoots dropped to minimal values, the content of *trans*-zeatin riboside increased by 87%, and the level of zeatin-*O*-glucoside declined by 47% (Fig. 4). Similar alterations in cytokinin homeostasis were detected in roots under low temperature: *trans*-zeatin disapeared and isopentenyladenosine content was close to minimum, *trans*-zeatin riboside amount enhanced about 60%, zeatin-*O*-glucoside amount reduced twice, but the isopentenyladenine level remained the same (Fig. 5).

Thus, the hormonal response of young rye plants to low temperature depended on the stress duration and part of the plant studied. On the other hand, different changes in separate cytokinin isoforms indicated organ-specifity of cytokinin metabolism under cold stress.

Discussion

Plant response to short-term stress, which is considered as the alarm phase, includes the activation of signaling systems and changes in the expression of proteins (Kosová et al., 2015). We did not record changes in the growth activity of rye plants during this period, but essential alterations in cytokinin balance were noted after 2 h of low temperature exposure. Rise in the content of *trans*-zeatin and *trans*-zeatin riboside in the shoots, and its synchronous decline in the roots should be emphasized. Zeatin-type cytokinins are known to be among the main regulators of plant growth processes. In shoots, they promote cell division and apical meristem development, whereas in roots, they act as growth inhibitors by stimulating cell differentiation in the root apical meristem (Kieber, Schaller, 2018). Thus, *trans*-zeatin and *trans*-zeatin riboside dynamics in rye seedlings at the alarm phase of cold stress response is obviously directed to the maintaining of intensive growth under short exposure to unfarourable conditions. Similar changes in the active cytokinin content in the leaves and roots have been observed in adult *Arabidopsis* plants during the first 30 min at a temperature of 40 °C, but after 2 h of stress, a reduced cytokinin level was detected (Dobrá et al., 2015). A small decrease in bioactive cytokinins after 2 h of heat shock is found in tobacco leaves (Macková et al., 2013). There are a few reports about phytohormone changes at the alarm phase of plant response to cold stress. Root cooling (6 °C) of 7-day-old wheat seedlings for 15 min has been associated with a sharp decline in the cytokinin level in leaves due to the increased activity of cytokinin oxidase (Veselova et al., 2005). Xiang et al. (2021) did not find changes in zeatin amount in 4-day-old maize seedlings after 15 and 30 h at 10 °C. In the leaves of winter wheat, 24 h cold stress leads to a decrease in *trans*-zeatin, isopentenyladenine and dihydrozeatin concentration, while spring wheat exhibits strong increase in *cis*-zeatin and dihydrozeatin content (Kalapos et al., 2016, 2017). Sharp decline in the active cytokinin pool after 1 d of cold stress

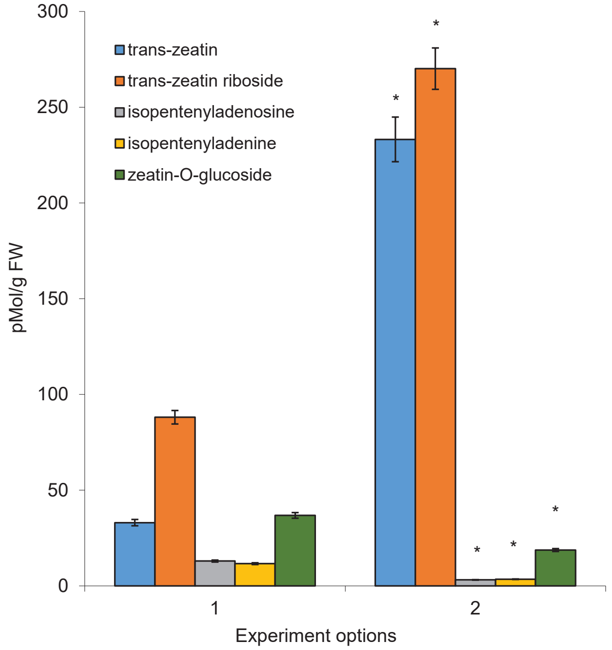

Fig. 2. Cytokinin content in the shoots of 7-day-old *Secale cereale* plants after 2 h of cold stress:

1 – control plants; 2 – plants after 2 h of cold stress (2 °C). Data are the mean ± SEM. * $p < 0.05$ as compared to the control plants

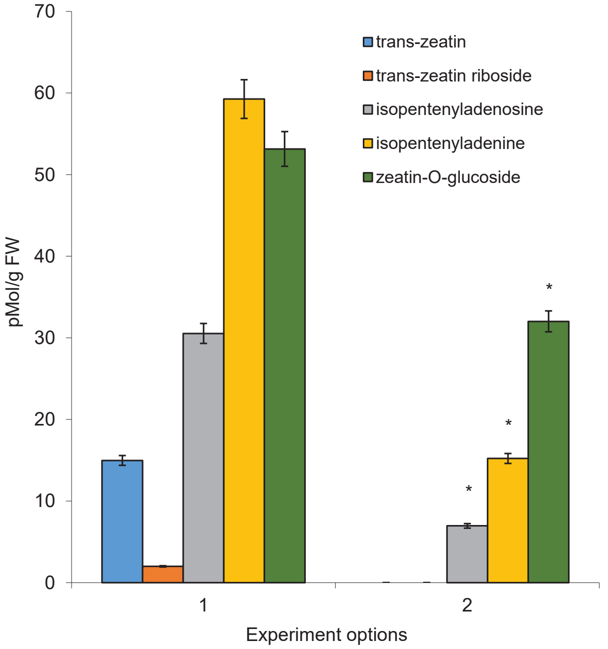

Fig. 3. Cytokinin content in the roots of 7-day-old *Secale cereale* plants after 2 h of cold stress:

1 – control plants; 2 – plants after 2 h of cold stress (2 °C). Data are the mean ± SEM. *, $p < 0.05$ as compared to the control plants

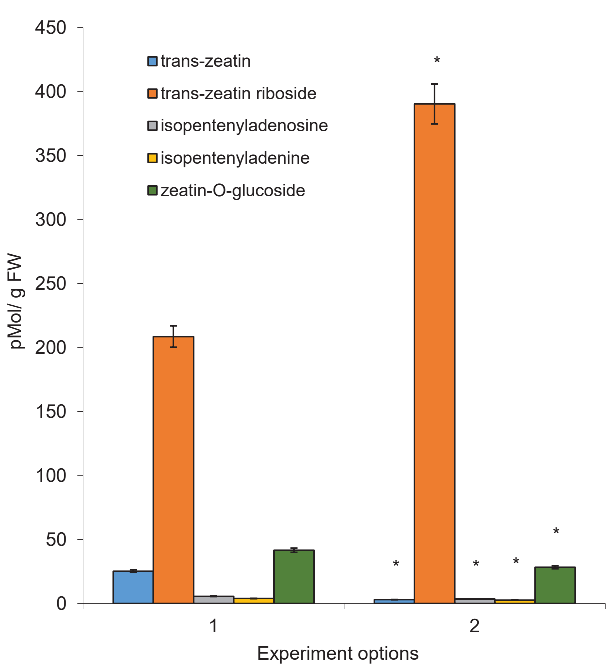

Fig. 4. Cytokinin content in the shoots of 9-day-old *Secale cereale* plants after 2 h of cold stress:

1 – control plants; 2 – plants after double exposure to cold stress (2 d for 6 h at 2 °C). Data are the mean ± SEM. * $p < 0.05$ as compared to the control plants

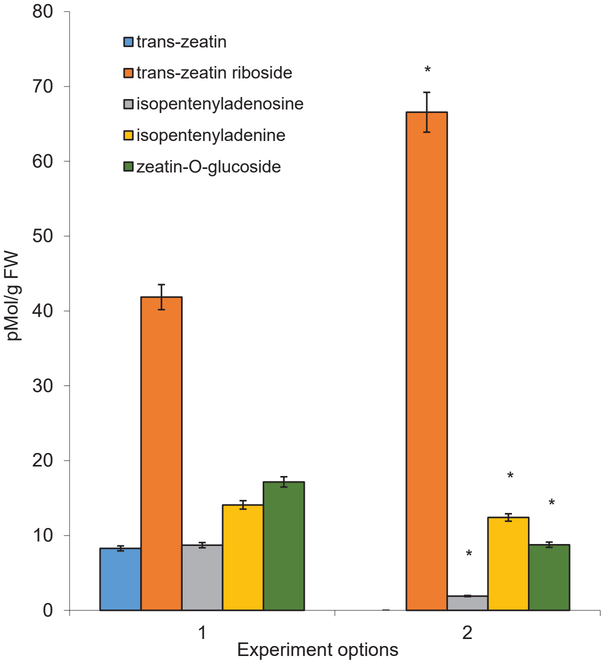

Fig. 5. Cytokinin content in the roots of 9-day-old *Secale cereale* plants after 2 h of cold stress:

1 – control plants; 2 – plants after double exposure to cold stress (2 d for 6 h at 2 °C). Data are the mean ± SEM. * $p < 0.05$ as compared to the control plants

(4 °C) has been observed in the leaves of winter wheat, but not in the roots (Vanková et al., 2014). Moreover, significant enhancement in zeatin-*O*-glucoside level was observed in similar experiment (Kosová et al., 2012). Cold stress (4 °C) during 1^{st} d results in a decrease of the *trans*-zeatin and isopentenyladenine level in the aerial parts of *Arabidopsis* plants; nevertheless, in the aerial parts of rice, it causes decline in the *trans*-zeatin riboside-5′-phosphate content, while the isopentenyladenine level is significantly higher (Maruyama et al., 2014). These authors also revealed down-regulation of *CYP735A3* and *CYP735A4* genes, encoding cytokinin transhydroxylases, which control active cytokinin biosynthesis. In our experiment, we detected a decrease in the content of isopentenyl-type cytokinins that are the primary products of cytokinin biosynthesis. It can be assumed that cytokinin biosynthesis in rye plants under chilling was suppressed, and an increase in the zeatin-type cytokinin level in the shoots occured due to zeatin-*O*-glucoside degradation. Cytokinin-*O*-glucosides are usually considered as storage forms that can be easily cleaved by β-glucosidase with the release of free active hormones (Frébort et al., 2011).

Prolonged treatment with low temperature resulted in changed growth of young rye plants. This period of plant stress response (acclimation phase) is accompanied by the synthesis of new proteins, changes in plasma membrane density and plant energy metabolism (Kosová et al., 2015). The alteration in proportion between the length of the shoots and roots in favor of the latter, which we observed at this stage, was probably a result of the hormonal balance established earlier. Cytokinins act as negative regulators of root growth and development (Dello Ioio et al., 2012), therefore, disappearance of active cytokinins (zeatin-type isoforms) under cold shock (2 h) could be the reason for further root elongation in rye plants. We observed a decrease in the cytokinin level, except for *trans*-zeatin riboside, in shoots and roots after 2 d of low temperature treatment. Previously, ambiguous results about cytokinin levels at the acclimation phase of cold stress have been obtained. In tomato leaves, exposed for 3 d at 10 °C, zeatin-type cytokinin content decreases by 3.11 times in the susceptible species, while it increases by 2.39 times in the tolerant species (Heidari et al., 2021). A decline in the *trans*-zeatin and *trans*-zeatin riboside level in *Arabidopsis* apices and leaves after 7 d at 5 °C has been associated with a strong up-regulation of cytokinin oxidase activity, indicating a high rate of hormone degradation; wherein in the roots, cytokinin content increases slightly (Prerostova et al., 2021). Prolonged acclimation (21 d) of *Carpobrotus edulis* plants at 5 °C results in the increase of endogenous contents of *trans*-zeatin and *trans*-zeatin riboside (Fenollosa et al., 2018). Acclimation of barley plants at 5 °C during 7 d is accompanied by a decrease in the *trans*-zeatin content and an increase in *cis*-zeatin (Ahres et al., 2021). In winter wheat, elevation in active cytokinins content has been found during 7–31 d of acclimation at 4 °C after its transient drop at the alarm phase of stress response (Vanková et al., 2014). It should be noted that cytokinin response to low temperature shows a strong variety dependent effect in cereals (Kosová et al., 2012, Kalapos et al., 2017). In the present study, the observed decrease in the content of active cytokinins (*trans*-zeatin and isopentenyladenine) in the shoots and roots of rye plants at the acclimation phase was probably directed to the inhibition of growth processes in order to use the energy resources of an organism to overcome the negative effect of stress (protective proteins synthesis, antioxidative system activation etc.) (Khan et al., 2017). At the same time, the increase in the *trans*-zeatin riboside level was found in both shoots and roots of rye at the acclimation phase. Zeatin riboside is considered as a transport form of the hormones without their own biological activity (Romanov, Schmülling, 2021). Nevertheless, our data support the point of view that it can play an important role in the plant development and responses to environmental stress (Nguyen et al., 2021).

Cytokinin metabolism and endogenous hormone levels are directly related to cold-tolerance of plants. Treatment with exogenous cytokinins or enhancement of their endogenous content by gene modification improve resistance to low temperature. Thus, benzyladenine application on wheat leaves improves cold tolerance through the increase of endogenous cytokinin concentrations (Veselova et al., 2005). Seed priming and foliar treatment of *Zea mays* L. with kinetin improve the germination, increases the leaf area index, hydration, photosynthesis efficiency, phenol content and yield under chilling conditions (Bakhtavar et al., 2015). *Arabidopsis* mutants with higher cytokinin level or wild-type plants treated with zeatin demonstrate better growth rates due to increase in the cell number when exposed at 4 °C (Xia et al., 2009). Induced overexpression of isopentenyl transferase gene (*IPT*) also enhances cold tolerance of non-acclimated sugarcane (*Saccharum officinarum* L.) plants (Belintani et al., 2011). All these results together with our current findings point out to the significant role of cytokinin pool in the regulation of plants acclimation to low temperatures.

It should be noted that in our experiments, the exposure of rye plants to low temperature was carried out during the dark phase of photoperiod since the cold snap usually occurs at night in the field conditions. Light is very important environmental signal for plants, which is known to affect the cold stress response (Janda et al., 2014). Recently, it has been shown that the combination of dark and cold arrests the growth of *Arabidopsis* plants and leads to a strong decrease of cytokinin levels (Prerostova et al., 2021). Treatment of young barley plants with far-red light stimulates better cold acclimation and is associated with significant changes in phytohormone balance, in particular, an increased *cis*-zeatin concentration (Ahres et al., 2021). Unfortunately, in most studies of plant adaptation to cold stress, the role of light is often not taken into account. Therefore, to obtain more unified data, in future investigations all details of the experimental setup should be considered, including the extent of plant tolerance, stress duration and temperature range, light, etc.

Conclusion

Despite the fact that *Secale cereale* plants are quite resistant to extreme environmental conditions, unhardened young plants are sensitive to low temperature exhibiting changes in the ratio between the shoot and root length. Response to cold is associated with significant alterations in cytokinin pool depending on the part of plant and duration of stress. During the short-term effect of stress (alarm phase of response), a decrease in the content of active cytokinins (*trans*-zeatin and *trans*-zeatin riboside) in the roots and an increase in the shoots were shown. After prolonged influence of chilling (acclimation phase of response) decline in the amount of cytokinins except for *trans*-zeatin riboside was detected in both roots and shoots. Significant rise in *trans*-zeatin riboside content in roots and shoots in this period evidenced an important role of this cytokinin during the acclimation of rye plants to cold. It appears that cytokinins are a key regulator of rye plant response and adaptation to low temperature. Endogenous levels of cytokinins must be taken into account when treating plants with growth regulators in order to increase their cold tolerance.

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Recommended for publication by O.K. Zolotareva

Веденичова Н.П., Щербатюк М.М., Косаківська І.В. 2022. **Вплив низької температури на ріст рослин *Secale cereale* (*Poaceae*) та вміст ендогенних цитокінінів у коренях і пагонах.** *Український ботанічний журнал*, 79(3): 184–192 [In English].

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Реферат. Фітогормони відіграють ключову роль у регуляції пристосування рослин до низьких температур. Для з'ясування ролі цитокінінів у реакції молодих рослин жита на гіпотермію досліджували динаміку цих гормонів у пагонах і коренях при короткочасному та тривалому впливі охолодження. 7-денні рослини піддавалися холодовому стресу (2 °C) упродовж 2 год (фаза тривоги) або 6 год упродовж двох днів (фаза аклімації). Вміст ендогенних цитокінінів аналізували методом ВЕРХ-МС. Холодовий стрес по-різному впливав на вміст окремих цитокінінів та їхню локалізацію в рослинах жита. Під час короткочасного впливу стресу виявлено зниження вмісту активних цитокінінів (*транс*-зеатину і *транс*-зеатин рибозиду) у коренях та їхнє збільшення у пагонах. Після тривалого впливу холоду як у коренях, так і в пагонах виявлено зниження кількості цитокінінів за винятком *транс*-зеатин рибозиду. Значне підвищення вмісту *транс*-зеатину рибозиду в коренях і пагонах у цей період свідчить про важливу роль цього цитокініну під час пристосування рослин жита до низьких температур.

Ключові слова: адаптація, цитокініни, ріст, *Secale cereale*, холодовий стрес