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Accumulation of dehydrin-like proteins in pear (*Pyrus communis* L.) microshoots during cold acclimation

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Abstract

Climatic conditions and plant diseases constitute a serious hazard to genetic resources stored in field collections. Therefore, *in vitro* techniques are being developed and used to store vegetatively propagated plants under low temperature conditions. Plants are capable of acclimating to low temperatures, and this process plays a crucial role on plant tolerance of low temperature stress and survival during tissue storage and regeneration under *in vitro* conditions. It has been demonstrated, that dehydrin proteins ensure plant resistance to stress factors, including cold treatment. The dehydrin proteins accumulate during plant acclimation and could be used as biochemical markers that indicate parameters of the acclimation process. Yet, so far little is known about dehydrin-like proteins of *Pyrus* sp. plants. In this study, we investigated dehydrin-like protein accumulation in microshoots of five representative European pear (*Pyrus communis* L.) genotypes cold acclimated under *in vitro* conditions. Immunoblot analysis using plant dehydrin specific antibody revealed the presence of eight dehydrin-like protein bands. The effect of medium and acclimation conditions on the accumulation of dehydrin-like proteins in pear microshoots was assessed during cold acclimation. The results of the analysis demonstrated that differential expression of eight protein bands of molecular weight estimated at 31, 42, 50, 54, 57, 64, 69 and 82 kDa corresponding to dehydrin-like proteins was characteristic of all selected genotypes. The small difference in the effect of acclimation conditions demonstrated that low temperature treatment was sufficient for the induction of cold acclimation process under *in vitro* conditions, and it led to a similar outcome as combination of short day photoperiod with varying temperature in most cases. Similarly, the results of the effect of medium composition indicated that plant growth regulator cytokinin and sugar alcohol mannitol had limited effect on the low temperature-induced plant response under *in vitro* conditions. Acclimation parameters identified for the specific genotypes provided guidelines for the development of methods of *in vitro* storage required for conservation of genetic resources of *Pyrus* sp. and other plants of the *Rosaceae* family.

Key words: cold hardiness, germplasm conservation, *Pyrus communis*, gene expression.

Introduction

The European pear (*Pyrus communis* L.) is a temperate-zone species of pomme fruit tree common in Lithuania and surrounding regions. The Institute of Horticulture of the Lithuanian Research Centre for Agriculture and Forestry maintains collection of over 200 cultivars and genetic forms of *Pyrus* sp. and a unique collection of 127 accessions of wild pear indigenous to the region (Petryla, 1973; Petrokas et al., 2007). Many of the pear genotypes distributed in Lithuania are fairly susceptible to cold damage and plant diseases, such as fire blight, that constitute a serious hazard to genetic resources stored in field collections. Therefore *in vitro* techniques are being employed to ensure efficient preservation of genetic resources of the vegetatively propagated plants. Most common methods used for long-term *in vitro* preservation of microshoots are cryo-preservation and storage under growth retarding, low temperature conditions (Chang, Reed, 2000; Shibli et al., 2006; Shatnawi et al., 2007; Kushnarenko et al., 2009).

Knowledge about plant acclimation capacity and tolerance of low temperature stress is required for suc-

cessful development of *in vitro* preservation methods. Cold acclimation is a process characteristic of woody plants of temperate zone, including pear and other plants of the *Rosaceae* family. A short day photoperiod and low temperatures control expression of genes involved in physiological changes leading to cold hardiness (reviewed by Wisniewski et al., 2003). It has been demonstrated that physiological changes induced during cold acclimation ensure higher plant tolerance of long term storage conditions, and higher survival rates after treatment with low temperatures are being achieved for cold acclimated plant shoots (Chang, Reed, 2000; Kushnarenko et al., 2009).

It has been established that dehydrin accumulation fluctuations are characteristic of woody plants of temperate regions and the dehydrin accumulation directly correlates to plant cold acclimation (Wisniewski, Arora, 2000). Dehydrins are proteins that accumulate in tissues during dehydration stress induced by low temperature, drought or salinity (Close, 1996). Investigation of accumulation of dehydrin proteins in plant tissue has been

used to assess acclimation process of *Prunus* sp. plants (Lukoševičiūtė et al., 2009). However, studies on the composition of dehydrins and their role in cold acclimation of different plants of the *Rosaceae* family are scarce, and information on the expression of dehydrin proteins in pear is vague.

The aim of our study was to identify dehydrin-like proteins characteristic of the selected genotypes of *Pyrus* sp., and to assess accumulation of the proteins during the cold acclimation process of microshoots maintained under *in vitro* conditions. The study identified media and acclimation conditions optimal for cold acclimation of microshoots of the selected pear genotypes.

Materials and methods

The experiments were performed in 2011 at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry.

Materials. Polyvinylpyrrolidone was purchased from "Sigma-Aldrich Ltd." (USA); defatted milk protein was from "Bio-Rad Laboratories Inc." (USA). Rabbit polyclonal anti-dehydrin antibody and secondary mouse anti-rabbit antibody labelled with alkaline phosphatase were obtained from "Agriser AB" (Sweden); alkaline phosphatase substrate was from "Novagen Ltd." (USA). Unless otherwise specified, all other reagents were purchased from "Carl-Roth Ltd." (Germany).

Plant material. Shoots of four European pear (*Pyrus communis* L.) cultivars including 'Karaliene Jadvyga', 'Koncentrat', 'Oranzhevaya', 'Princess Dagmar' and one breeding line No. 0408 were maintained on MS (Murashige, Skoog, 1962) medium supplemented with 3.2 μM benzylaminopurine, 3% sucrose and 0.8% plant agar. For cold acclimation experiments, the pear shoots were propagated and grown for 7 days at $22 \pm 3^\circ\text{C}$ under fluorescent lamp illumination $50\text{--}150 \mu\text{mol m}^{-2} \text{s}^{-1}$ intensity and 16/8 h photoperiod.

Cold acclimation. For cold acclimation experiments, three different modifications of MS medium were used: 1) medium without plant growth regulators, 2) medium supplemented with 3.2 μM benzylaminopurine, 3) medium without plant growth regulators, supplemented with 2% mannitol. Two different cold acclimation conditions were used: 1) short day 8/16 h photoperiod of $25\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and variation of temperature (22°C for light and 4°C for dark period) (Kovalchuk et al., 2009), 2) constant temperature of 4°C in the dark. Cold acclimation was carried out for 7 days. Microshoots of non-acclimated control were maintained under conditions used for shoot growth. Plant material was frozen in liquid nitrogen and used for protein extraction.

Dehydrin-like protein expression analysis. Thermostable proteins of the control and cold acclimated samples were isolated using the method described by Dhanaraj et al. (2005) with several modifications. Plant tissue was homogenized in extraction buffer (30 mM Tris-HCl, 25 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride, 5 mM benzamidine, 5 mM ϵ -aminocaproic acid, 5% polyvinylpyrrolidone, pH 8.5) at 2 ml g^{-1} of fresh weight using a homogenizer MM400 ("Retsch Ltd.", Germany). The extract was centrifuged at $16000 \times \text{g}$ for 20 min at 4°C . Supernatant was transferred into a new tube, boiled in a water bath for 10 min and centrifuged under the same conditions as before. Supernatant was transferred into a new tube. Protein concentration was determined

by Bradford (1976) method using "Roti-Quant" reagent. The protein solution was combined with three volumes of cold acetone and incubated at -20°C overnight. Protein precipitate was isolated by centrifugation at $16000 \times \text{g}$ for 30 min at 4°C and air dried. Protein pellets were resuspended in Laemmli electrophoresis buffer and 10 μg of protein was separated on 12% polyacrylamide gel as described by Laemmli (1970) using "Protean Mini 3" system ("Bio-Rad Laboratories Inc.", USA). "Roti-Mark Prestained" protein marker was used as a molecular weight standard. Gel was stained with colloid "Coomassie Brilliant Blue G-250" stain (Kang et al., 2002).

Protein was transferred from the polyacrylamide gel onto a nitrocellulose membrane using "Mini Trans-Blot" system ("Bio-Rad Laboratories Inc.", USA). The membrane was blocked in TBS solution (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% of defatted milk protein. Protein bands containing conservative amino acid sequence characteristic of dehydrin family proteins were identified by immunoblotting using the specific polyclonal anti-dehydrin antibody at 1:500 dilution for 2 h. After rinsing with TBS containing 0.01% Tween-20, the membrane was incubated with secondary antibody diluted at 1:1000, for 1 h. After wash, the membrane was developed using alkaline phosphatase substrate.

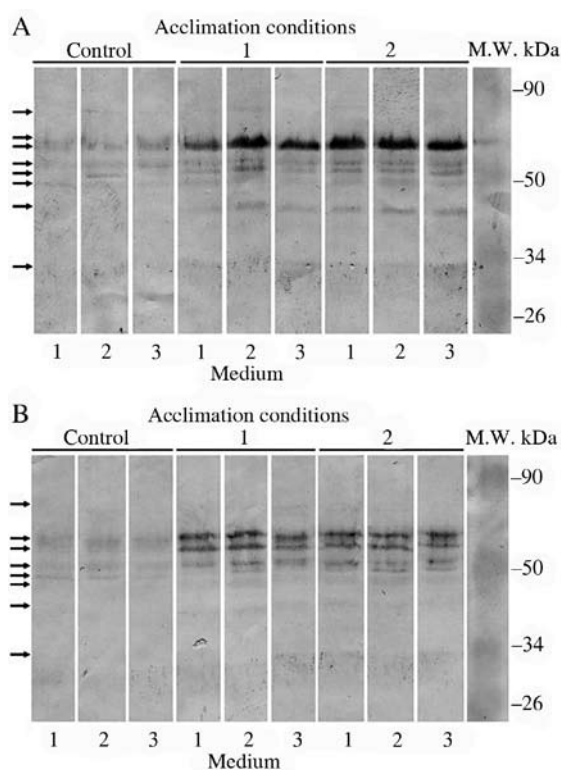
Protein gel densitometry analysis was performed using the program *ImageJA v.1.42* (Schindelin, 2008). Relative intensity of staining of anti-dehydrin antibody immunoblots was measured; mean and standard error of the mean were calculated using at least three repeats from independent protein extraction and immunoblotting analysis experiments.

Results and discussion

Previous studies on cold acclimation in herbaceous plants of genus *Arabidopsis*, *Brassica*, *Fragaria*, *Miscanthus* and woody plant species of genus *Prunus*, *Cydonia* revealed that accumulation of cold stress related metabolites and proteins, including dehydrin-like proteins, was essential for cold acclimation of the plants under field or *in vitro* conditions (Welling et al., 2004; Wisniewski et al., 2006; Patton et al., 2007; Nishizawa et al., 2008; Lukoseviciute et al., 2009; Lukoševičiūtė et al., 2009; Rugienius et al., 2009). It has been also demonstrated that cold acclimation is important for plant germplasm survival under low-temperature storage conditions (Chang, Reed, 2000; Kushnarenko et al., 2009). As cold acclimation trait varies for different genotypes of the same plant species (Lukoseviciute et al., 2009; Lukoševičiūtė et al., 2009; Rugienius et al., 2009), assessment of conditions for optimal cold acclimation is an important step in establishment of low temperature preservation procedure for individual clones of vegetatively propagated plants. Therefore, in this study, an accumulation of biochemical markers of plant cold stress response was used to assess properties of cold acclimation of individual plant genotypes. Pear genotypes of diverse genetic background were selected based on previous study on genetic polymorphism of pear tree genetic resources maintained at *in vitro* collection of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry (unpublished data). Dehydrin-like protein accumulation during cold acclimation under different acclimation conditions *in vitro* was assessed for five representative pear genotypes including cultivar 'Karaliene

Jadvyga', 'Koncentrat', 'Oranzhevaya', 'Princess Dagmar' and breeding line No. 0408.

Immunochemical analysis of acclimated microshoots of the selected pear genotypes using plant dehydrin-like protein specific antibody identified eight protein bands of molecular weight estimated at 31, 42, 50, 54, 57, 64, 69 and 82 kDa (Fig. 1). Previous studies demonstrated that the number of dehydrin genes characteristic of different plants varied. Six dehydrin genes were identified in *Arabidopsis thaliana* (Puhakainen et al., 2004), thirteen in *Hordeum vulgare* (Choi et al., 1999; Rodriguez et al., 2005). At least two genes were described in woody plant species of *Prunus* sp. (Wisniewski et al., 2006). The role of the cold stress induced proteins identified in our study in cold hardiness process of pear was elusive and would require further genetic study involving identification of genes encoding the proteins and mutation analysis of their function. However, the obtained results clearly indicated upregulation of cold induced stress response pathways and could provide quantitative information on the development of response in the selected genotypes.



Notes. Acclimation conditions and medium indicated by numbers are described in the Materials and methods section. Arrows on the left indicate the eight dehydrin-like protein bands identified in the analysis. M.W. – protein molecular weight standard.

Figure 1. Representative results of the immunoblot analysis of dehydrin-like proteins in pear microshoots of 'Oranzhevaya' (panel A) and breeding line No. 0408 (panel B) during cold acclimation *in vitro*

It has been demonstrated that acclimation process in woody plants is induced by complex interaction of two factors, low temperature and short day (Welling et al., 2004; Wisniewski et al., 2006). However, in some cases only low temperature treatment could be sufficient for induction of the genes involved in cold acclimation. In our study, the effect of the two factors was assessed using the selected pear genotypes. The accumulation

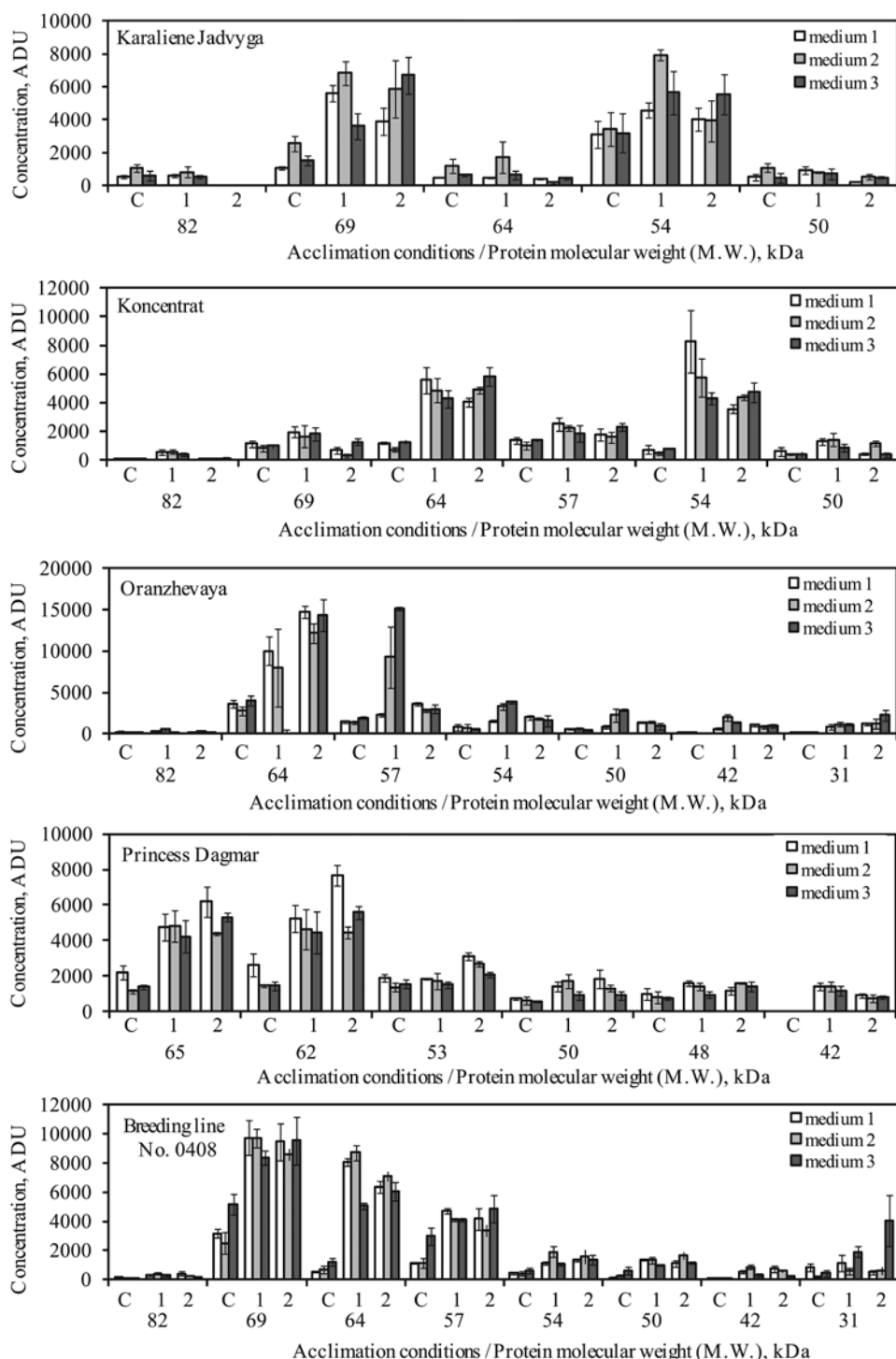
of dehydrin-like proteins in microshoots incubated under constant low temperature in the dark was compared to the microshoots treated under short day photoperiod combined with variation of temperature (high temperature during light period and low temperature during dark period) (Kovalchuk et al., 2009). Medium composition was another important factor found to be involved in plant microshoot acclimation and cold tolerance under *in vitro* conditions (Kovalchuk et al., 2009). It was shown that these physiological processes were influenced by the presence of plant growth regulators and carbohydrate content (Hassan, Bekheet, 2008). Therefore, the effect of presence of plant growth regulator benzamidine on the accumulation of the markers of acclimation process was assessed. Additionally, sugar alcohols mannitol or sorbitol are often included in plant storage medium (Montalvo-Peniche et al., 2007; Hassan, Bekheet, 2008). Therefore we assessed the effect of mannitol sugar on the accumulation of dehydrin-like proteins during cold acclimation.

A differential expression of the identified eight dehydrin-like protein bands was observed for the five pear genotypes under the acclimation conditions used in our study (Fig. 2). The 31 kDa protein was of low abundance and was observed only for the cultivar 'Oranzhevaya' and breeding line No. 0408. The largest upregulation in the expression of the protein (approximately 10 times as compared to control microshoots) was observed in microshoots acclimated on medium with mannitol under constant low temperature conditions. Accumulation of another low abundance protein of 42 kDa molecular weight was identified for cultivars 'Oranzhevaya', 'Princess Dagmar' and breeding line No. 0408. Accumulation of the protein was only slightly affected by different acclimation conditions.

Cold acclimation had no significant effect on 50 kDa protein accumulation for cultivars 'Karaliene Jadvyga' and 'Princess Dagmar'. Meanwhile, microshoots of the other three genotypes had the protein expression upregulated approximately 3 to 7 times. Accumulation of the protein was only slightly affected by the acclimation conditions. Only for 'Oranzhevaya', significantly higher expression was characteristic of microshoots acclimated on medium with cytokinin or mannitol under varying temperature and photoperiod conditions.

Accumulation of 54 kDa protein was observed for microshoots of all genotypes. The highest upregulation was identified for the cultivar 'Koncentrat' (approximately 12 times increase as compared to the control) on medium without growth regulators under varying temperature and photoperiod conditions. This protein was also the most abundant in microshoots of 'Karaliene Jadvyga' acclimated on the medium containing cytokinin under the varying temperature and photoperiod conditions. In other cases, acclimation conditions had little influence on the accumulation of the protein.

The highest upregulation of expression of 57 kDa protein was observed in microshoots of 'Oranzhevaya' that was acclimated on the medium containing cytokinin or mannitol (approximately 6 and 8 times, respectively). Meanwhile, no significant amount of the protein was detected for the cultivar 'Karaliene Jadvyga'. Lower expression levels and only slight variation among different acclimation conditions were observed for microshoots of 'Koncentrat', 'Princess Dagmar' and breeding line No. 0408. Protein of molecular weight of 64 kDa was



Notes. Concentrations were determined by immunoblot analysis using specific anti-dehydrin antibody and expressed in arbitrary density units (ADU). The results are presented as mean and SEM of results from at least three independent analyses. Acclimation conditions and medium indicated by numbers are described in the Materials and methods section.

Figure 2. Expression of dehydrin-like proteins for the selected pear genotypes under different acclimation conditions

one of the most abundant and highly upregulated (up to 13 times for breeding line No. 0408) in the microshoots of most of the genotypes. A predisposition for particular acclimation conditions was observed only for cultivar 'Princess Dagmar' where the largest increase in the protein expression was found in shoots incubated on the medium without growth regulators under varying temperature and photoperiod conditions. Surprisingly, no significant difference in the expression of the protein was detected for the cultivar 'Karaliene Jadvyga'.

Protein of 69 kDa molecular weight was another highly upregulated (up to approximately 13 times) and abundant protein in acclimated shoots of cultivars 'Karaliene Jadvyga', 'Princess Dagmar' and breeding line No. 0408. However, only slight upregulation of expression of this protein was detected for 'Koncentrat', and the protein was absent in microshoots of 'Oranzhevaya'. Protein of 82 kDa molecular weight was detected at low abundance in the microshoots of most cultivars with the exception of 'Princess Dagmar'. Significant upregulation

of the protein expression was observed for microshoots of 'Koncentrat' and breeding line No. 0408 (up to 9 and 5 times, respectively) under varying temperature and photoperiod treatment.

The results of the analysis of dehydrin-like protein expression in response to treatment under cold adaptation inducing conditions demonstrated that accumulation of dehydrin-like proteins was characteristic of microshoots of all the selected pear genotypes. The expression pattern of the eight dehydrin-like protein bands was variable and depended on the pear genotype and acclimation conditions used. The overall trends demonstrated that the combination of short day photoperiod and varying temperature treatment resulted in higher accumulation of the dehydrin-like proteins only in discrete cases, while constant low temperature treatment often was equally effective in induction of expression of the marker protein bands. This observation is in agreement with the results of previous studies that demonstrated that low temperature treatment may suffice to induce cold acclimation in woody plant species (Welling et al., 2004; Wisniewski et al., 2006). Such observation is of important practical significance in the development of germplasm conservation methods. The treatment of plants under constant low temperature conditions in the dark would require less elaborate experimental setup used for plant acclimation.

Similarly, a difference in the effect of medium composition on the expression of dehydrin-like proteins was weakly expressed in most cases. In several cases where such difference was observed, there was no consistent preference for particular medium conditions among the different genotypes or proteins of different molecular weight. Such results suggested that the presence of cytokinin or mannitol had only limited effect on cold-induced stress response under conditions used in the experiment. Therefore it could be concluded that the protective effect of alcohol sugars, such as sorbitol or mannitol, during low temperature treatment might be directly involved in the stabilization of plant tissue integrity or physiological homeostasis and their role in upregulation of cold stress response signalling pathways would be less significant.

The obtained results revealed trends in the protein accumulation that provided suggestions about acclimation requirements of the pear genotypes that would be essential for the development of genetic resources conservation methods for *Pyrus* sp. and other plants of the *Rosaceae* family. Based on the observed trends, optimal conditions for the acclimation of the genotypes were identified (Table).

Table. Optimal conditions for cold acclimation of the selected pear genotypes

Genotype	Medium *	Acclimation conditions*
'Karaliene Jadvyga'	2-3	1
'Koncentrat'	1-3	1-2
'Oranzhevaya'	2-3	1-2
'Princess Dagmar'	1-3	2
Breeding line No. 0408	1-2	1-2

Note. * – numbers corresponding to medium and acclimation conditions are described in the Materials and methods section.

The optimal conditions were estimated based on the highest expression values observed for the most abundant genotype specific dehydrin-like proteins.

Conclusions

1. Eight protein bands of molecular weight estimated at 31, 42, 50, 54, 57, 64, 69 and 82 kDa and corresponding to dehydrin-like proteins were identified using an immunoblot analysis in the microshoots of five representative genotypes of *Pyrus* sp. grown and cold acclimated under *in vitro* conditions.

2. During cold acclimation, the dehydrin-like protein bands were differentially expressed in microshoots of all selected genotypes. The small difference in the effect of acclimation conditions demonstrated that low temperature treatment was sufficient for the induction of cold acclimation process under *in vitro* conditions and in most cases it led to similar outcome as a combination of short day photoperiod with varying temperature. Similarly, the investigation of the effect of medium composition indicated that plant growth regulator cytokinin or sugar alcohol mannitol had limited effect on low temperature-induced response during acclimation under *in vitro* conditions.

3. Based on the observed trends of the expression of the most abundant dehydrin-like protein bands, optimal conditions for the acclimation of the selected pear genotypes were identified.

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Kultūrinės kriaušės (*Pyrus communis* L.) mikroūglių dehidrinų tipo baltymų sudėtis grūdinimo *in vitro* sąlygomis

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Santrauka

Klimato sąlygos ir augalų ligos kelia pavojų augnuose saugomiems augalų genetiniams ištekliams, todėl vegetatyviniu būdu dauginamiems augalams saugoti žemoje temperatūroje yra kuriamos *in vitro* technologijos. Žemoje temperatūroje augalai užsigrūdina, ir šis procesas lemia augalų atsparumą šalčio stresui bei padidina jų išgyvenimo galimybę audinius saugant žemoje temperatūroje ir regeneraciją *in vitro* sąlygomis. Tyrimai atskleidė, kad baltymai dehidrinai užtikrina augalų atsparumą stresui, taip pat ir šalčiui. Dehidrinai kaupiasi augalų grūdinimo metu ir gali būti naudingi kaip biocheminiai žymekliai, apibūdinantys grūdinimosi proceso parametrus. Duomenų apie dehidrinų tipo baltymų sudėtį *Pyrus* sp. augaluose yra nepakankamai.

Tyrimu siekta įvertinti penkių būdingų kultūrinės kriaušės genotipų dehidrinų tipo baltymų sudėtį grūdinimo *in vitro* sąlygomis. Imunodetekcijos metodu, panaudojant dehidrinams specifinį antikūną, nustatyti aštuoni dehidrinų tipo baltymai. Šie baltymų dehidrinų žymekliai buvo panaudoti skirtingoms grūdinimo sąlygoms ir terpės sudėties įtakai grūdinant mikroūglius įvertinti. Tyrimų rezultatai atskleidė, kad visiems tirtiems genotipams būdingi dehidrinų tipo baltymų raiškos pakitimai. Nedideli skirtumai tarp rezultatų, gautų esant skirtingoms grūdinimo sąlygoms, parodė, kad žemos temperatūros poveikis buvo pakankamas veiksnys, užtikrinantis užgrūdinimą *in vitro* sąlygomis, ir daugeliu atvejų rezultatai buvo artimi grūdinimo trumpos dienos fotoperiodo ir kintamos temperatūros sąlygomis tyrimų rezultatams. Taip pat ir terpės sudėties poveikio tyrimų rezultatai siūlo prielaidą, kad augimo reguliatorius citokininas ir angliavandenis alkoholis manitolis turi nedidelę įtaką šalčio sukkelto streso atsakai *in vitro* sąlygomis. Tyrimo metu nustatytų grūdinimo sąlygų, būdingų tirtiems genotipams, duomenys yra reikšmingi kuriant *Pyrus* sp. ir kitų *Rosaceae* šeimos augalų genetinių išteklių saugojimo *in vitro* sąlygomis metodus.

Reikšminiai žodžiai: atsparumas šalčiui, genetinių išteklių saugojimas, *Pyrus communis*, genų raiška.