The influence of short-term cold stress on the metabolism of non-structural carbohydrates in polar grasses

Elżbieta ŁOPIEŃSKA-BIERNAT[1]*, Marta PASTORCZYK[2], Irena GIEŁWANOWSKA[2], Krystyna ŻÓŁTOWSKA[1], Robert STRYIŃSKI[1] and Ewa ZAOBIDNA[1]

[1] *Department of Biochemistry, Faculty of Biology and Biotechnology, University Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-719 Olsztyn, Poland*

[2] *Department of Plant Physiology, Genetics and Biotechnology, Faculty of Biology and Biotechnology, University Warmia and Mazury in Olsztyn, Oczapowskiego 1A, 10-719 Olsztyn, Poland*

* *corresponding author <ela.lopienska@uwm.edu.pl>*

Abstract: Plants adapt to extremely low temperatures in polar regions by maximizing their photosynthetic efficiency and accumulating cryoprotective and osmoprotective compounds. Flowering plants of the family Poaceae growing in the Arctic and in the Antarctic were investigated. Their responses to cold stress were analyzed under laboratory conditions. Samples were collected after 24 h and 48 h of cold treatment. Quantitative and qualitative changes of sugars are found among different species, but they can differ within a genus of the family Poaceae. The values of the investigated parameters in *Poa annua* differed considerably depending to the biogeographic origin of plants. At the beginning of the experiment, Antarctic plants were acclimatized in greenhouse characterized by significantly higher content of sugars, including storage reserves, sucrose and starch, but lower total protein content. After 24 h of exposure to cold stress, much smaller changes in the examined parameters were noted in Antarctic plants than in locally grown specimens. Total sugar content and sucrose, starch and glucose levels were nearly constant in *P. annua*, but they varied significantly. Those changes are responsible for the high adaptability of *P. annua* to survive and develop in highly unsupportive environments and colonize new regions.

Key words: Arctic, Antarctic, cold stress, carbohydrates, enzymes, Poaceae, polar plants.

Introduction

The Arctic and the Antarctic are the least anthropogenically modified ecosystems on Earth due to their remote geographic location and extreme climate. Extensive research was conducted in those regions focused on global climate changes which have been observed in the last decades and which indirectly

influence the distribution of plant species in the tundra (Grobe *et al.* 1997; Bjorkman *et al.* 2017).

Main abiotic factors that significantly influence polar plants include short duration of growing season, low temperature, low moisture, high salinity and high radiation stress (Alberdi *et al.* 2002; Block *et al.* 2009; Taran *et al.* 2012). In the evolutionary process, polar plants have developed specific anatomic and physiological features that enable them to survive in unsupportive environments and colonize areas uncovered by melting glaciers (Giełwanowska 2005; Zůñiga-Feest *et al.* 2009; Giełwanowska *et al.* 2011a,b). Plants adapt to extremely low temperatures in polar regions by maximizing their photosynthetic efficiency (Xiong *et al.* 2000) and accumulating cryoprotective and osmoprotective compounds (Janská *et al.* 2010). They have developed two main mechanisms of tolerance to low, namely prevention of freeze damage to tissues and formation of ice crystals in the extracellular matrix to prevent damage of cellular structures. Supercooling, a process by which cells prevent liquids from solidifying below their freezing point, is a strategy that is widely deployed by plants in polar regions (Reyes-Diaz *et al.* 2006). Cryoprotective and osmoprotective compounds include amino acids (proline), proteins (HSP, LEA, PR, dehydrins) and sugars, mainly sucrose, raffinose family oligosaccharides and other polyols (Lineberge and Steponkus 1980; Newsted *et al.* 1991; Solhaug and Aares 1994; Hare and Cress 1997; Reyes *et al.* 2003; Bravo and Griffith 2005; Olave-Concha *et al.* 2005; Piotrowicz-Cieślak *et al.* 2005; Janská *et al.* 2010). The mechanism responsible for the protective effects of carbohydrates on proteins, biological membranes and cytoplasmic structures during dehydration has been extensively studied during freezing temperature. Sugars exert a protective influence by replacing water with hydroxyl groups to maintain hydrophilic interactions of phospholipid molecules of membranes and proteins. Carbohydrates have the ability to transform the cytoplasm into a glassy state *i.e.*, membrane rigidification (Hoekstra *et al.* 2001; Reyes-Diaz *et al.* 2006). The glassy state protects cell structures against damage, and it stabilizes macromolecules (Koster 1991; Buitink and Leprince 2004).

Studies of the adaptive strategies of plants inhabiting extreme polar regions focuse mainly on species such as *Colobantus quitensis* (Kunth) Bartl. and *Cerastium arcticum* Ange. of the family Caryophyllace and grasses such as *Deschampsia antarctica* Desv., *Poa arctica* R. Br. and *Poa annua* L. which have developed specific metabolic mechanisms adapted to very low temperature. Those plants have been the object of many studies, including experiments investigating the role of sugars during cold acclimation (Piotrowicz-Cieślak *et al.* 2005; Convey and Smith 2006; Parnikoza *et al.* 2009; Olech *et al.* 2011; Parnikoza *et al.* 2011; Pastorczyk *et al.* 2014; Kellmann-Sopyła *et al.* 2015).

Less is known about enzymes that participate in carbohydrate metabolism and their responses to chill stress. Present investigation focused mainly on sucrose metabolizing enzymes, synthases and invertases, which are responsible for sucrose

levels in plants and play an important role in protection against cold stress (Guy *et al*. 1992; Bascuňán-Godoy *et al*. 2006). Amylases, glycosidases that decompose starch, one of the main reserve materials in plants, supply low-molecular-weight oligosaccharides and glucose, which maintain the osmolarity of intracellular fluid. Recent research has expanded our understanding of the functions played by sucrose and monosaccharides in sugar sensing and signaling in plants (Roitsch and Gonzalez 2004; Rolland *et al*. 2006; Proels and Hückelhoven 2014). Those mechanisms enable sugardecomposing enzymes to control metabolic processes and defense responses (Dorion *et al*. 1996; Bonfig *et al*. 2010).

There are three types of invertase isoenzymes that differ in their localization in plants (EC 3.2.1.26): vacuolar invertases (Inv-v), cell wall invertases (Inv-cw) and cytoplasmic invertases (Inv-c). Inv-v and Inv-cw have similar properties, and their activity is optimized in an acidic environment (pH 5.0–5.5 and pH 3.5–5, respectively). Both enzymes are β-fructofuranosidases which break down sucrose and, less effectively, other fructofuranosides such as stachyose and raffinose. Inv-v is important for osmoregulation, whereas Inv-cw regulates the distribution of sugars, mainly sucrose, in plants. Inv-c is active under alkaline conditions (pH 6.8–8.0), and it is also referred to as neutral invertase. This sucrose-specific enzyme is strongly inhibited by high concentrations of its hydrolysis products, glucose and fructose (Roitsch and Gonzalez 2004). The multitude of mechanisms that regulate invertase expression and activity indicate that those enzymes play a very important role in the metabolism, development and defense responses of plants (Bournay *et al*. 1996; Cheng *et al*. 1999; Rojo *et al*. 2003; Rausch and Greiner 2004; Roitsch and Gonzalez 2004).

Plants contain three types of amylases: α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and γ-amylase, also known as glucoamylase or amyloglucosidase, (EC 3.2.1.3), which differ in their mechanism of action and the location of the hydrolyzed glycoside bond. All amylases hydrolyze α-1,4-glycoside bonds in starch (Beck and Ziegler 1989). Alpha-amylase plays the most important role. Depending on reaction time, α-amylase yields products characterized by different degree of glucose polymerization, including dextrins, oligosaccharides, maltose (which is also the main product of β-amylase) and glucose. Amylases degrade starch and increase the concentrations of soluble sugars in plants which act as osmolytes. Research has demonstrated that amylases are involved in low temperature tolerance, which is an important trait in polar plants (Vecchia *et al*. 1998; Kratsch and Wise 2000). Cold-induced changes in soluble carbohydrates have been identified in selected polar species of the families Poaceae (*Poa arctica vivipara* L., *Deschampsia antarctica*) and Caryophyllaceae (*Cerastium arvense*, L., *Colobanthus quitensis*) (Pastorczyk *et al*. 2014; Kellmann-Stopyła *et al*. 2015). Five other species (ecotypes) of the family Poaceae (*Festuca brachyphylla* Schultes, *Deschampsia alpina* (L.) Roem. *et* Schult., *Deschampsia cespitosa* (L.) Beauv, *Poa alpina* L. and *Poa annua*) were investigated in this experiment. The aim of this study was to analyze the content

and composition of carbohydrates and the activity of two main enzymes (amylase and invertase) which initiate the hydrolytic degradation of reserve sugars, starch and sucrose, in the shoots of plants of the family Poaceae harvested in polar regions, and to describe their responses to cold stress. The evaluated parameters were compared in *Poa annua* plants harvested in the Antarctic and in the Olsztyn area to analyze their adaptive strategies based on sugar metabolism.

Material and methods

Material. — The experiment was performed on flowering plants of the family Poaceae growing in the Arctic and the Antarctic. Live *Poa annua* plants (designated A) were harvested in 2010 in the area of the *Henryk Arctowski* Polish Antarctic Station on King George Island (South Shetland Islands) (62°09'S and 58°28'W) (Fig. 1A), whereas *Festuca brachyphylla*, *Deschampsia alpina*, *Deschampsia cespitosa* and *Poa alpina* plants were collected near the *Stanisław Siedlecki* Polish Polar Station in Hornsund, Spitsbergen (77°00'N and 15°33'E) (Fig. 1B–F). The sixth studied ecotype was *Poa annua* (designated O) growing in the area of Olsztyn, Poland (53°46'N and 20°29'E).

The harvested specimens were planted individually in pots filled with horticultural soil and grown at a temperature of 18–20°C, in the presence of daylight in the experimental greenhouse of the Faculty of Biology and Biotechnology of the University of Warmia and Mazury in Olsztyn (Fig. 1B–F).

Plant responses to cold stress were evaluated under controlled laboratory conditions. Twenty-four hours after transfer to the laboratory (19–20°C), plants were placed in an incubator (Heraeus, BK6160, Kendro Laboratory Products) at a constant temperature of 20°C and 20/4 h photoperiod (light/dark) which corresponds to the length of day during the growing season in polar regions. After 24 hours of acclimatization, plants were exposed to cold stress at 4°C for 2 days. Samples for analysis were collected from shoots before (control, 0 h), 24 h and 48 h after exposure to cold stress. Samples of *P. annua* (A) were collected only after 24 h due to a small number of specimens. The experiment was conducted in three replications. One sample was composed of shoot tissues from 5–10 plants. Three analytical samples of 3 g each were prepared from each sample and stored at -80°C.

Preparation of plant extracts. — Plant extracts were prepared by homogenizing 3 g analytical samples of plant tissues in 30 ml of PBS (Phosphate Buffered Saline) on ice at 35,000 rpm according to modified method of Sung *et al*. (1989). Half of the homogenate was centrifuged for 15 minutes at $12,000 \times g$ at a temperature of 4°C. The resulting supernatant (supernatant 1) was used to determine total protein content and enzyme activity. The second half of the
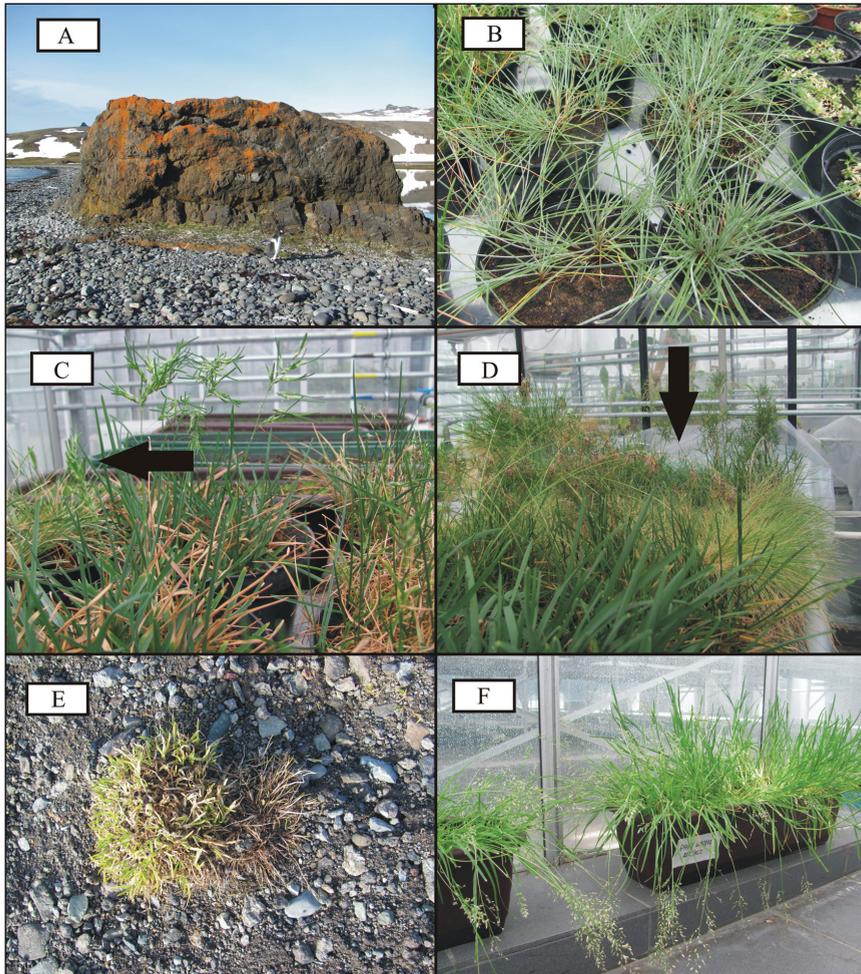
Fig. 1. The polar flowering plants of the family Poaceae under study. King George Island, neighborhood of the *Henryk Arctowski* Polish Antarctic Station (A), *Festuca brachyphylla* (B), *Deschampsia alpina* (arrow) (C), *Poa alpina var. vivipara* with viviparous culms, *Deschampsia cespitosa* (arrow) (D) and *Poa anua* in the Antarctic (E) and in the experimental greenhouse (F).

supernatant was heated at 90°C for 15 minutes to dissolve starch, precipitate proteins and deactivate enzymes. The homogenate was cooled and centrifuged as above. The resulting supernatant (supernatant 2) was used to determine the concentrations of starch and soluble carbohydrates.

Determination of protein content. — Protein content was determined by the method proposed by Bradford (1976) in 5-fold diluted supernatant. The protein content of the sample was read from the calibration curve for bovine serum albumin (Sigma-Aldrich) with the concentration of 500 µg/mL.

Determination of protein content. — Total sugar content was determined by the anthrone method (Morse 1947). Samples were diluted 100-fold. Sugar concentrations were read from the calibration curve for glucose solution (50 µg/mL) (Sigma-Aldrich) and were expressed in mg per g of fresh weight of tissue (ft).

Determination of starch content. — Starch content was determined by the modified method proposed by Sölling and Esmann (1975), which involves the measurement of the quantity of glucose released from endogenous starch by exogenous amyloglucosidase to the reaction environment. 10 µL of the supernatant was transferred to square pieces (1.5 × 1.5 cm) of Whatman 3 filter paper and left to dry at room temperature. Filter paper was placed in test tubes and soaked with 3 mL of cold 66% ethanol with the addition of 10% TCA for 20 minutes. The liquid was removed, filter paper was rinsed twice with cold 66% ethanol, and the samples were left to stand for 20 minutes each time. In the end, filter paper was rinsed for 10 minutes in cold acetone. Filter paper was dried, cut into pieces and quantitatively transferred to dry test tubes. Every test tube was filled with 0.5 mL of 0.2 M acetate buffer with pH 4.8 and 30 µl (containing 25.8 mU) of *Rhizopus* amyloglucosidase solution (Sigma-Aldrich), and incubated at 37°C for 30 minutes. Glucose released by the enzyme during starch hydrolysis was identified with the Oxy-Glucose glucose assay kit (Pointe Scientific, Poland). Starch content was read from the calibration curve for potato starch standard (1 mg/mL) (Sigma-Aldrich) which was handled in the same manner as the analyzed samples. Starch content was expressed in mg per g of ft.

Determination of glucose content. — Glucose content was determined with the Oxy-Glucose glucose assay kit (Pointe Scientific, Poland) according to the manufacturer's instructions.

Determination of soluble sugar content by HPLC. — Sample preparation: 0.5 mL of the supernatant was combined with 1 mL of cold (-18°C) 96% ethanol. The mixture was stirred and left to stand at 4°C for 30 minutes, after which it was centrifuged at 12,000 × g for 6 minutes. The resulting supernatant was poured into dry test tubes and incubated at 50°C until solvent evaporation. Dried samples were stored in a desiccator at room temperature until analysis.

Chromatography: The sediment was dissolved in 0.5 mL mixture of acetonitrile and deionized water (3:2), passed through the Alltech Micro-Spin® Filter Tubes nylon filter (0.2 µm) (Deerfield, Ireland) and centrifuged at 12,000 × g for 1 minute. 30 µL of the supernatant was applied onto a chromatography column.

Carbohydrates were separated in the Shimadzu HPLC SCL-10A (Kyoto, Japan) chromatography system, on a 4.6 × 250 mm High Performance Carbohydrate Column (Shimadzu) with a 3.9 × 20 mm Sentry™ High

Performance Carbohydrate Guard Column (Shimadzu). Carbohydrates were analyzed with the use of the Shimadzu RID-10A refractometer. The mobile phase was a solution of acetonitrile and deionized water (3:2). Separation was carried out at a flow rate of 1 mL/min and temperature of 35°C. Sugars were identified based on their retention times. They were determined quantitatively by comparing the peak area from the sample elution curve with the peak area of reference standards in the Chromax 2004 program for processing chromatographic data (POL-LAB Artur Dzieniszewski, Poland). The results were expressed in mg of sugar per g of ft.

Determination of enzyme activity. — Amylase activity was determined by the modified method proposed by Caraway (1959) based on the decrease in the color intensity of the starch-iodine solution. The experimental samples contained 50 µL of the tested supernatant, 0.85 µL of 0.2 M acetate buffer with pH 4.8 and 0.1 mL of 1% potato starch solution (Sigma-Aldrich). They were incubated at 37°C for 120 minutes. Control samples containing 0.85 mL of acetate buffer and 0.1 mL of 1% starch solution were incubated simultaneously with the experimental samples. They were combined with 50 µL of the supernatant after incubation. Immediately after incubation, all samples were combined with 4 ml of 10-fold diluted iodide in potassium iodide solution. Absorbance was read immediately at a wavelength of 650 nm. The results were expressed in µg of decomposed starch per mg of protein.

Invertase activity was determined by the method proposed by Dahlquist (1968) and Bonfig *et al*. (2010) by measuring the amount of glucose released by the enzyme from the sucrose substrate at pH 4.8 and pH 6.8. Glucose was determined with an enzymatic assay kit (Pointe Scientific, Poland). Enzyme activity was expressed in U per mg of protein.

Statistical analysis. — The results were analyzed statistically in the Statistica 10.0 program (StatSoft Inc.). Significant differences between means ($p \leq 0.05$) were determined by ANOVA, followed by Tukey's test.

Results

The protein content of vegetative tissues in control plants was determined in a broad range of 0.48 mg/g in *P. annua* (A) to 16.56 mg/g ft in *F. brachyphylla*. Mean values differed significantly between species. The only exception was protein content which was similar in both species of the genus *Deschampsia* (Fig. 2). Cold stress (4°C for 24 h) led to a significant increase in protein concentrations in *P. annua* plants harvested in both Antarctic (A) and Olsztyn (O). The protein content of *D. alpina*, *D. cespitosa* and *P. alpine* decreased relative
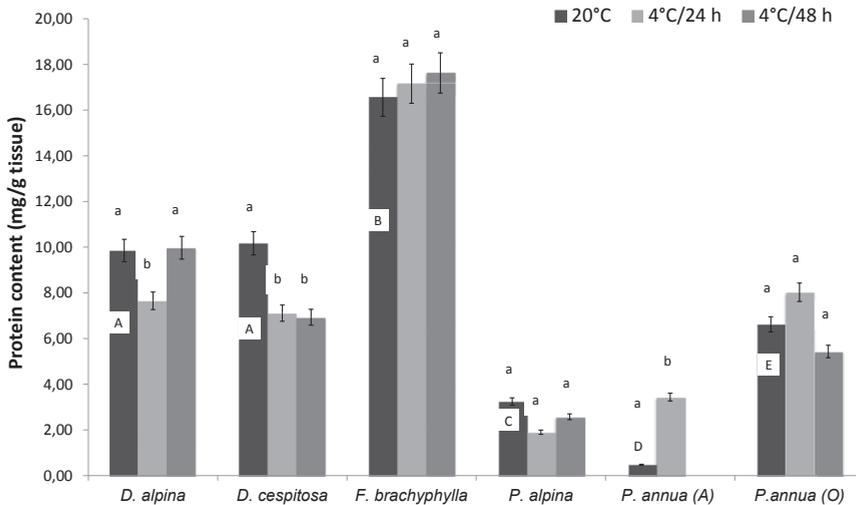
Fig. 2. Changes in the content of protein (mg/g fresh tissue) in shoots of polar Poaceae plants growing at 20ºC (control, 0 h) and at 4ºC (chilling stress).

Explanation: Mean ± SD, values are means of three replicates, each of them consists material from five plants. The same capital letters in the column indicate no significant differences ($P < 0.05$) between means of studied species controls. The same small letters in the column indicate no significant differences ($P < 0.05$) between means of protein for the same species depended of time treatment at 4ºC.

to control plants. After 48 h of exposure to cold stress, significant differences in protein content were not observed between control and experimental plants, excluding *D. cespitosa* and *P. annua* (A) (Fig. 2).

The total sugar content of control plants from each species also differed significantly and ranged from 17.9 mg/g to 54.0 mg/g ft (Fig. 3). The noted difference was not statistically significant ($p = 0.072$) only between *D. alpina* and *P. annua* (O). Significant changes induced by cold stress were not noted in *D. alpina* and *P. annua* (A). Cold stress lowered the total carbohydrate content of *D. cespitosa* and *P. alpina*. Similar changes were noted after 24 h in *P. annua* (O), whereas an increase in carbohydrate concentrations was observed in *F. brachyphylla*. Sugar concentrations in both *P. annua* (O) and *F. brachyphylla* returned to control group levels after 48 h of cold stress (Fig. 3).

The control samples of the evaluated species also differed in the levels of different soluble sugars (Table 1). After 24 h of storage at 4ºC, changes in sucrose concentrations were not observed, except in *P. alpina* and *P. annua* (O), whereas after 48 h, sucrose levels increased significantly in *D. alpina*, *D. cespitosa*, *F. brachyphylla* and *P. annua* (O) (Table 1). The noted differences in sucrose levels were not always accompanied by the corresponding changes in hexoses – free glucose and fructose (Table 1). The remaining sugars were not identified in
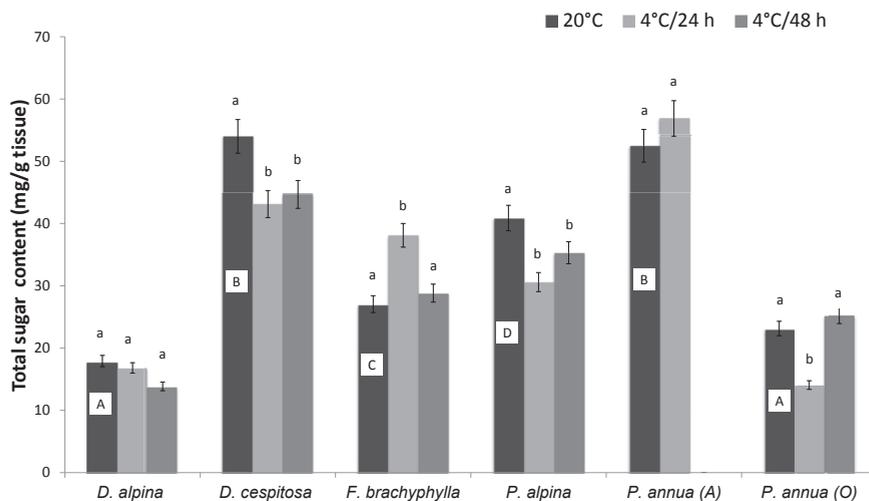
Fig. 3. Changes in the content of total sugars (mg/g fresh tissue) in shoots of polar Poaceae plants growing at 20ºC (control, 0 h) and at 4ºC (chilling stress). Explanation the same as in Fig. 2.

all species. Raffinose was detected only in *D. cespitosa*, and its concentrations increased significantly after 48 h of cold stress (Table 1). The presence of ribose was noted in control plants of three species, and its concentrations were highest after 24 h of cold storage (Table 1). The analyzed plants also contained other polyols – glycerol and myo-inositol. Glycerol was present in all species, and its concentrations were similar in control samples (Table 1). Glycerol levels increased after 48 h of exposure to cold stress in *D. cespitosa* and *F. brachyphylla*, and already after 24 h in *P. annua* (A) and *P. annua* (O). Myo-inositol was detected in *F. brachyphylla* and *P. annua* (A), and it was less abundant in experimental than in control plants (Table 1).

The evaluated plant species were divided into two groups based on their starch content. The first group comprised plants with low starch levels (3.1 mg/g wt to 4.0 mg/g ft): *D. alpina*, *F. brachyphylla* and *P. annua* (O) (Fig. 4). Plants from the second group, *D. cespitosa*, *P. alpina* and *P. annua* (A), were more abundant in starch (6.2 mg/g to 9.2 mg/g ft) (Fig. 4). Cold stress did not induce significant changes in starch concentrations, and the only exception was a transient (after 24 h) decrease in starch levels in *P. annua* (O) (Fig. 4).

Amylase activity was very low, except in *D. alpina*, *F. brachyphylla* and *P. alpina*, and it was further decreased in most species after 24 of cold stress. After 48 h of cold storage, amylase activity increased significantly relative to the level noted after 24 h except *D. alpina* (Fig. 5). Under control conditions, invertase activity was minimal at pH 4.8 (data not shown). At pH 6.8, invertase activity was high in *D. alpina*, *P. alpina* and *P. annua* (A), low in *F. brachyphylla* and very low in *D. cespitosa* and *P. annua* (O) (Fig. 6). After 24 h of cold stress,

Table 1

Changes in the concentration of soluble carbohydrates (mg/g wet tissue) in shoots of polar Poaceae plants growing at 20°C (control, 0 h) and at 4°C (chilling stress)

Species	Time at 4°C (h)	Saccharose	Glucose	Fructose	Ribose	Raffinose	Glycerol	Myo-inositol
Deschampsia alpina	0	6.50 ± 0.33*[a,A]	2.46 ± 0.12[a,A]	1.80 ± 0.09[a,A]	1.69 ± 0.58[A]	0	1.75 ± 0.09[ab,A]	0
	24	7.79 ± 0.39 [ab]	4.30 ± 0.22[b]	3.89 ± 0.19[b]	0	0	1.81 ± 0.09[a]	0
	48	10.08 ± 0.5[b]	2.98 ± 0.15[a]	4.37 ± 0.22[b]	0	0	1.25 ± 0.06[b]	0
Deschampsia cespitosa	0	17.91 ± 0.9[a,B]	1.72 ± 0.09[a,B]	3.13 ± 0.16[a,B]	0	1.37 ± 0.07[a]	0.88 ± 0.04[a,,A]	0
	24	16.14 ± 0.81[a]	1.68 ± 0.08[a]	3.09 ± 0.15[a]	7.17 ± 0.36	1.28 ± 0.06[a]	1.26 ± 0.06[a]	0
	48	31.49 ± 1.57[b]	7.80 ± 0.39[b]	8.55 ± 0.43[a]	0	14.69 ± 0.73[b]	4.18 ± 0.21[b]	0
Festuca brachyphylla	0	14.82 ± 0.74[aC]	3.57 ± 0.18[a,C]	4.89 ± 0.24[a,,C]	22.35 ± 1.12[a,B]	0	2.28 ± 0.11[a,A]	3.73 ± 0.19[a,A]
	24	16.62 ± 0.83[a]	3.24 ± 0.16[a]	5.72 ± 0.29[a,]	39.11 ± 1.96[b]	0	1.96 ± 0.1[a]	3.08 ± 0.15[a]
	48	24.68 ± 1.23[b]	3.18 ± 0.15[a]	5.94 ± 0.3[a]	18.72 ± 0.94[a]	0	3.96 ± 0.2[b]	1.55 ± 0.08[b]
Poa alpina	0	1.95 ± 0.1[a,A]	3.96 ± 0.2[a,C]	5.84 ± 0.29[a,C]	0	0	1.31 ± 0.07[a,A]	0
	24	16.67 ± 0.83[b]	2.38 ± 0.12[b]	2.16 ± 0.11[b]	0	0	1.09 ± 0.05[a]	0
	48	2.60 ± 0.13[a]	11.87 ± 0.59[c]	10.82± 0.54[c]	0	0	1.67± 0.08[a]	0
Poa annua (A)	0	13.22 ± 0.66[a,C]	2.52 ± 0.13[a,A]	18.35 ± 0.92[a,D]	26.37 ± 1.32[a,B]	0	2.68 ± 0.13[a,A]	1.00 ± 0.05[a,,B]
	24	12.86 ± 0.64[a]	1.92 ± 0.1[a]	25.44 ± 1.27[b]	41.97 ± 2.1[b]	0	4.28 ± 0.21[b]	0.59 ± 0.03[b]
Poa annua (O)	0	9.24 ± 0.46[a,D]	0.37 ± 0.15[a,,D]	2.68 ± 0.13[a,,B]	1.21± 0.18[a,A]	0	1.12 ± 0.06[a,A]	0
	24	17.42 ± 0.87[b]	4.27 ± 0.21[b]	8.25 ± 0.41[b]	31.63 ± 1.58[b]	0	3.90 ± 0.2[b]	0
	48	14.92 ± 0.75[c]	0.97 ± 0.05[c]	5.91 ± 0.3[c]	23.41 ± 1.17[c]	0	3.05 ± 0.15[b]	0

Explanation: * mean ± SD, values are means of three replicates, each of them consists material from five plants. The same capital letters in the column indicate no significant differences (P<0.05) between means of studied species controls. The same small letters in the column indicate no significant differences (P≤0.05) between means of sugar for the same species depended of time treatment at 4°C.
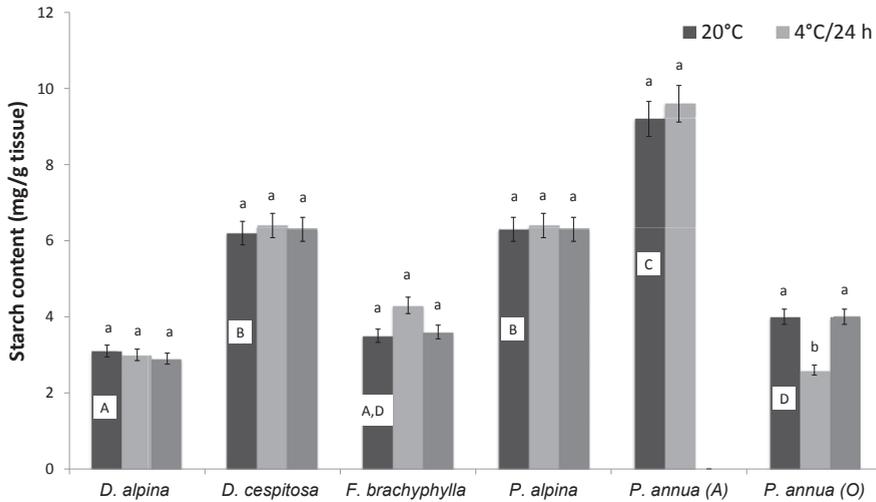
Fig. 4. Changes in the content of starch (mg/g fresh tissue) in shoots of polar Poaceae plants growing at 20ºC (control, 0 h) and at 4ºC (chilling stress). Explanation the same as in Fig. 2.

invertase activity decreased several fold in *D. alpina* and *P. annua* (A). After 48 h, invertase activity increased multi-fold in *P. annua* (O) and *F. brachyphylla* plants (Fig. 6).
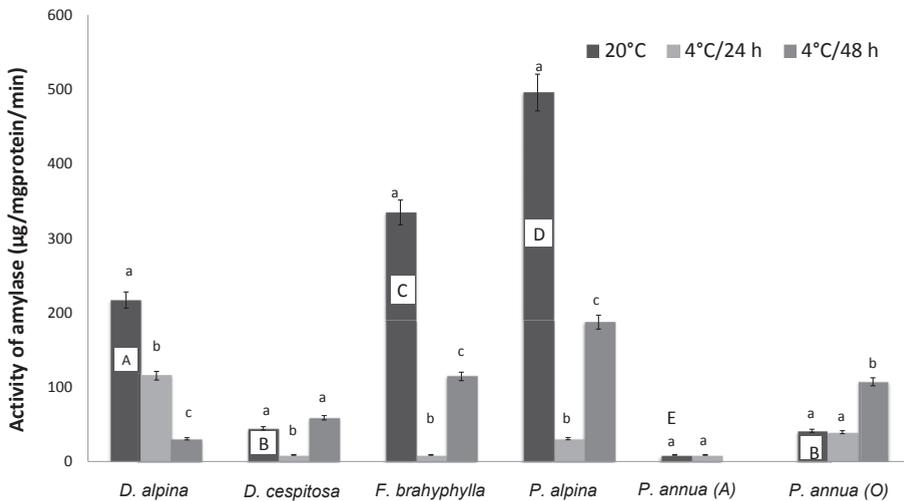

Fig. 5. Activity of amylase (µg/mg protein/min) in shoots of polar Poaceae plants growing at 20ºC (control, 0 h) and at 4ºC (chilling stress). Explanation the same as in Fig. 2.
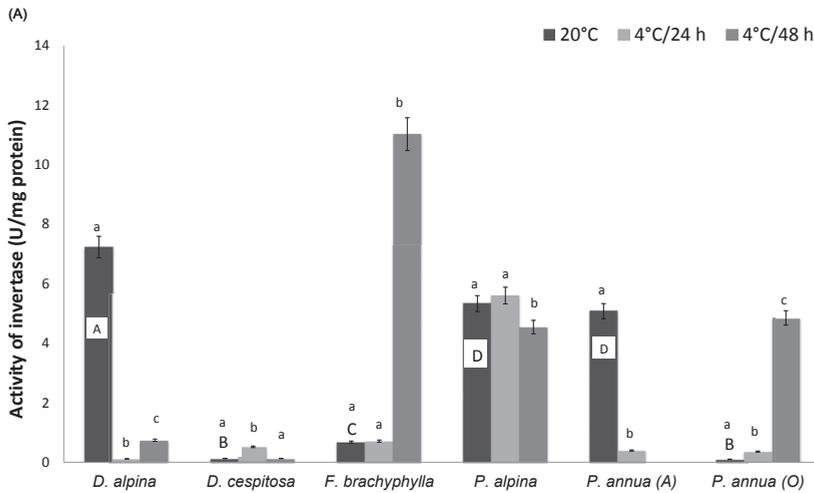
Fig. 6. Activity of invertase (U/mg protein) in shoots of polar Poaceae plants growing at 20ºC (control, 0 h) and at 4ºC (chilling stress). Explanation the same as in Fig. 2.

Discussion

Harsh climatic conditions have caused the formation of different morphological features in vascular plants of the polar regions, which are helping them to maintain in the cold and harsh climate. These features can include first of all reduced, vertical and compact plant growth, protect the shoots by reducing the structure *e.g.* the loss of water from the tissues or the influence of light intensity, phenotypic plasticity of individuals of the same species living in different microhabitats and communal growth of plants. In such difficult conditions it occurs mostly to reduce to a minimum the metabolic activity of the body, and thus the processes of plant growth and development (Block *et al.* 2009). The presence, development, reproduction and expansion of vascular plants, including *P. annua*, in the Arctic and in the Antarctic, indicate that they tolerate adverse environmental conditions, including cold stress.

Carbohydrates play the same roles in plants inhabiting polar and temperate climates. Sugars act as energy reserves, signaling molecules, free radical scavengers and compounds that protect plants against abiotic and biotic stresses. In plant species native to the Antarctic geobotanical zone, the accumulation of carbohydrates is one of the key mechanisms conditioning resistance to cold stress (Zúñiga *et al.* 1996; Bravo *et al.* 2001; Piotrowicz-Cieślak *et al.* 2005). In the present study, control plants of grass species belonging to the same genus differed in their total sugar content (Fig. 3). The greatest, more than 2-fold differences were noted between *P. annua* plants harvested in the Antarctic and in the Olsztyn area, and those variations could be attributed to adaptive mechanisms.

The above plants also differed in their responses after 24 h of exposure to cold stress (Fig. 3). Carbohydrate levels decreased in *D. cespitosa*, *P. alpina* and *P. annua* (O), probably due to higher demand for energy required for adaptation to cold stress. In *P. annua* (O), the observed decrease could be associated with starch decomposition (Table 1). In a similar experiment, Pastorczyk *et al*. (2015) reported an increase in sugar levels after 24 h of cold stress in two species of the family Caryophyllaceae (*C. quitensis* and *C. alpinum*) and two species of the family Poaceae (*D. antarctica* and *P. arctica*). The results noted by the cited authors differed from our findings, probably because Pastorczyk *et al*. (2015) analyzed only soluble sugars.

Non-reducing sugars, such as sucrose, raffinose and stachyose, protect plant cells by providing them with specific properties. They can be accumulated in higher concentrations because unlike monosaccharides, they do not enter into reactions with amino acids that lead to non-enzymatic glycation of proteins and peptides (Lapolla *et al*. 2005). Increased sucrose accumulation in the vegetative tissues of plants subjected to various environmental stressors is a well-known phenomenon. It has been observed in many cold-exposed plant species in different geographic latitudes and different stages of development, including *Triticum aestivum* L. seedlings (Crespi *et al*. 1991), *Spinacia oleracea* L. leaves (Guy *et al*. 1992) and *Lolium perenne* L. shoots (Bhowmik *et al*. 2006). Our findings (Table 1) and the results of studies analyzing the vegetative tissues and diaspores of several species of polar plants (Pastorczyk *et al*. 2014; Kellmann-Sopyła *et al*. 2015) indicate that sucrose, an osmotically active compound, is accumulated in response to cold stress. As expected, sucrose was the predominant sugar in the evaluated species (Table 1). Raffinose, one of the most important oligosaccharides with protective properties (Piotrowicz-Cieślak *et al*. 2005), was detected only in *D. cespitosa*. This is an interesting observation because the results reported by Pastorczyk *et al*. (2014) suggested that raffinose does not occur in polar plants of the family Poaceae. In the present study, raffinose concentrations in *D. cespitosa* increased in response to cold stress (Table 1). Similar results were noted by Pastorczyk *et al*. (2014) in Caryophyllaceae species.

Polyols, including cyclitols, are osmotically active compounds that increase plant resistance to salt stress, low temperature and soil drought (Chiera *et al*. 2006). Polyols stabilize cell membranes under exposure to stress (Farrar 1988; Leprince *et al*. 1993; Jennings and Lysek 1996). Glycerol is a major polyol found in the tissues of the analyzed plants (Table 1). In this study, a significant increase in glycerol concentrations was observed in most of the examined species (Table 1). An increase in the biosynthesis and content of glycerol was also reported in *Arabidopsis thaliana* (L.) exposed to salt stress (Bahieldin *et al*. 2014). The above observations indicate that glycerol has cryoprotective and osmoprotective properties. Myo-inositol is the most widely occurring cyclitol which is found in the cytoplasm of both plant and animal cells and in organelles

bounded by one or two membranes (Lackey *et al*. 2003). In this study, myo-inositol was found in only two plant species, but it could be present in the remaining plants in quantities below the method detection limit. Control plants of *F. brachyphylla* were much more abundant in myo-inositol (3.73 ± 0.19 mg/g wt) than *P. annua* (A) (1.0 ± 0.05 mg/g ft). Myo-inositol levels in vegetative tissues were nearly halved after cold storage at 4°C. The compound was not found in the vegetative tissues of *P. annua* (O) (Table 1). The concentrations of myo-inositol were very low (0.07 ± 0.00 mg/g DW) in mature seeds of *P. annua* (O), and were higher in *P. annua* (A) (0.50 ± 0.02 mg/g DW) (Kellmann-Sopyła *et al*. 2015). Myo-inositol seems to play a limited role in this species under exposure to cold stress.

Our study analyzed changes in the content of carbohydrates and their derivatives as well as changes in the activity of invertases and amylases hydrolyzing two major plant sugars in response to short-term cold stress. Sucrose is decomposed by invertase to glucose and fructose, hexoses which are a source of energy and play important anabolic and regulatory (sugar sensing) functions in plants (Van den Ende and Valluru 2009). Invertase activity was examined at pH 4.8 and pH 6.8. The results noted at pH 4.8 were surprising. High levels of activity were expected because pH 4.8 is close to the optimal values for two of the three main invertases: Inv-cw and Inv-v (Roitsch and Gonzalez 2004), whereas only minimal levels of activity were observed. This result is difficult to interpret, and it could be attributed to the release of invertase inhibitory proteins, whose presence has been noted in many plant species (Bonfig *et al*. 2010), during sample preparation. This observation indicates that our knowledge of invertases and their inhibitors in the analyzed group of plants is limited and should be expanded. Cytoplasmic invertase has different properties and is active at neutral pH (Roitsch and Gonzalez 2004). In this study, Inv-c activity was also low in the control plants of three species, but it increased after 24 h of chilling. The observed increase was significant in *D. cespitosa* and *P. annua* (O), whereas in *F. brachyphylla*, a significant rise in Inv-c activity was noted only after 48 h (Fig. 6). In the control plants of *D. alpina* and *P. annua* (A) characterized by high invertase levels, the activity of Inv-c clearly decreased after 24 h of exposure to cold stress. In the above plants, a simultaneous increase was observed in the concentrations of glucose and fructose, the products of sucrose hydrolysis catalyzed by invertase (Table 1). Vargas *et al*. (2003) demonstrated that Inv-c is strongly inhibited by those compounds, which could at least partially explain the accompanying decrease in enzyme activity levels.

Similarly to invertases, the activity of amylases also differed significantly in control plants. After 24 h of cold stress, a transitional decrease in amylase activity was observed in most species, except *P. annua* (Fig. 5). As a result, starch levels remained fairly constant.

The results of our study suggest that cold-induced changes in the content and composition of sugars are varied and species-specific, but they can differ within a genus of the family Poaceae. The values of the investigated parameters in *P. annua* differed considerably depending on the biogeographic origin of plants. At the beginning of the experiment, greenhouse acclimatized Antarctic plants were characterized by significantly higher content of sugars, including storage reserves, sucrose and starch, but lower total protein content. After 24 h of exposure to cold stress, much smaller changes in the examined parameters were noted in Antarctic plants than in locally grown specimens. Total sugar content and sucrose, starch and glucose levels were nearly constant in *P. annua* (A), but they varied significantly in *P. annua* (O). These observations point to epigenetically programmed changes in the species' genome which are influenced by habitat conditions. Those changes are responsible for the high adaptability of *P. annua* which enables this cosmopolitan species to survive and develop in highly unsupportive environments and colonize new regions.

References

ALBERDI M., BRAVO L.A., GUTIÉRREZ A., GIDEKEL M. and CORCUERA L. 2002. Ecophysiology of Antarctic vascular plants. *Plant Physiology* 115: 479–486.

BAHIELDIN A., SABIR J.S.M., RAMADAN A., ALZOHAIRY A.M., YOUNIS R.A., SHOKRY A.M., GADALLA N.O., EDRIS S., HASSAN S.M., AL-KORDY M.A., KAMAL K.B.H., RABAH S., ABUZINADAH O.A. and EL-DOMYATI F.M. 2014. Control of glycogen biosynthesis under high salt stress in *Arabidopsis*. *Functional Plant Biology* 41: 87–95.

BASCUÑÁN-GODOY L., URIBE E., ZÙÑIGA-FEEST A., CORCUERA L. and BRAVO L. 2006. Low temperature regulates sucrose-phosphate synthase activity in *Colobantus quitensis* (Kunth) Bartl. by decreasing activitation by glucose-6-phosphate. *Polar Biology* 29: 1011–1017.

BECK E. and ZIEGLER P. 1989. Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Biology* 40: 95–117.

BHOWMIK P.K., TAMURA K., SANADA Y., TASE K. and YAMADA T. 2006. Sucrose metabolism of perennial ryegrass in relation to cold acclimation. *Zeitschrift für Naturforschung* 61c: 99–104.

BJORKMAN A.D., VELLEND M., FREI E.R. and HENRY G.H.R. 2017. Climate adaptation is not enough: warming does not facilitate success of southern tundra plant populations in the high Arctic. *Global Change Biology* 23: 1540–1551.

BLOCK W., SMITH L.R.I. and KENNEDY A.D. 2009. Strategies of survival and resource exploitation in the Antarctic fell field ecosystem. *Biology Review* 84: 449–484.

BONFIG K.B., GABLER A., SIMON U.K., LUSCHIN-EBENGREUTH N., HATZ M., BERGER S., MUHAMMAD N., ZEIER J., SINHA A.K. and ROITSCH T. 2010. Post-translational depression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. *Molecular Plant* 3: 1037–1048.

BOURNAY A.S., HEDLEY P.E., MADDISON A., WAUGH R. and MACHRAY G.C. 1996. Exon skipping induced by cold stress in a potato invertase gene transcript. *Nucleic Acids Research* 24: 2347–2351.

BRADFORD M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytical Biochemistry* 72: 248–54.

BRAVO L.A. and GRIFFITH M. 2005. Characterization of antifreeze activity in Antarctic plants. *Journal of Experimental Botany* 56: 1189–1196.

BRAVO L.A., ULLOA N., ZÚÑIGA G.E., CASANOVA A., CORCUERA L.J. and ALBERDI M. 2001. Cold resistance in Antarctic angiosperms. *Plant Physiology* 111: 55–65.

BUITINK J. and LEPRINCE O. 2004. Glass formation in plant anhydrobiotes: survival in the dry state. *Cryobiology* 48: 215–228.

CARAWAY W.T. 1959. A stable starch substrate for the determination of amylase in serum and other body fluids. *American Journal of Clinical Pathology* 32: 97–99.

CHENG W.H., TALIERCIO E.W. and CHOUREY P.S. 1999. Sugars modulate an unusual mode of control of the cell-wall invertase gene (*Incw1*) through its 3' untranslated region in a cell suspension culture of maize. *Proceedings of the National Academy of Sciences of the United States of America* 96: 10512–10517.

CHIERA J.M., STREETER J.G. and FINER J.J. 2006. Ononitol and pinitol production in transgenic soybean containing the inositol methyl transferase gene from *Mesembryantheum crystalinum*. *Plant Science* 171: 647–654.

CONVEY P. and SMITH R.I.L. 2006. Responses of terrestrial Antarctic ecosystems to climate change. *Plant Ecology* 182: 1–10.

CRESPI M.D., ZABALETA E.J., PONTIS H.G. and SALERNO G.L. 1991. Sucrose synthase expression during cold acclimation in wheat. *Plant Physiology* 96: 887–891.

DAHLQVIST A. 1969. Assay of intestinal disaccharidases. *Analytical Biochemistry* 22: 99–107.

DORION S., LALONDE S. and SAINI H.S. 1996. Induction of male sterility in wheat by meiotic-stage water decline is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. *Plant Physiology* 111: 137–145.

FARRAR J.F. 1988. Physiological buffering. *In*: M. Galun (ed.) *CRC Handbook of Lichenology*, vol. 2. CRC Press., Boca Raton: 101–105.

GIEŁWANOWSKA I. 2005. Specyfika rozwoju antarktycznych roślin naczyniowych *Colobanthus quitensis* (Kunth) Bartl. i *Deschampsia antarctica* Desv. 112. Wydawnictwo Uniwersytetu Warmińsko-Mazurskiego w Olsztynie, Olsztyn: 150 pp.

GIEŁWANOWSKA I., BOCHENEK A., GOJŁO E., GÓRECKI R., KELLMANN W., PASTORCZYK M. and SZCZUKA E. 2011. Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Island. *Polish Polar Research* 32: 139–155.

GIEŁWANOWSKA I., PASTORCZYK M. and KELLMANN-SOPYŁA W. 2011a. Influence of environmental changes on physiology and development of polar vascular plants. *Papers on Global Change* 18: 53–62.

GROBE C.W., RUHLAND C.T. and DAY T.A. 1997. A new population of *Colobanthus quitensis* near Arthur Harbor, Antarctica: correlating recruitment with warmer summer temperatures. *Arctic, Antarctic and Alpine Research* 29: 217–221.

GUY C.H.L., HUBER J.L.A. and HUBER S.C. 1992. Sucrose phosphate synthase and sucrose accumulation at low temperature. *Plant Physiology* 100: 502–508.

HARE P.D. and CRESS W.A. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* 21: 79–102.

HOEKSTRA F.A., GOLOVINA E.A. and BUITINK J. 2001. Mechanisms of plant desiccation tolerance. *Trends in Plant Science* 6: 431–438.

JANSKÁ A., MARŠÍK P., ZELENKOVÁ S. and OVESNÁ J. 2010. Cold stress and acclimation-what is important for metabolic adjustment? *Plant Biology* 12: 295–405.

JENNINGS D.H. and LYSEK G. 1996. *Fungal biology: understanding the fungal lifestyle*. BIOS Scientifc Publishers, Guildford, UK: 156 pp.

KELLMANN-SOPYŁA W., LAHUTA L.B., GIEŁWANOWSKA I. and GÓRECKI R.J. 2015. Soluble carbohydrates in developing and mature diaspores of polar Caryophyllaceae and Poaceae. *Acta Physiologiae Plantarum* 37: 118.

KOSTER K.L. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiology* 96: 302–304.

KRATSCH H.A. and WISE R.R. 2000. The ultrastructure of chilling stress. *Plant Cell and Environment* 23: 337–350.

LACKEY K.H., POPE P.M. and JOHNSON M.D. 2003. Expression of 1L-moy-inositol-1-phosphate synthase in organelles. *Plant Physiology* 132: 2240–2247.

LAPOLLA A., TRALDI P. and FEDELE D. 2005. Importance of measuring products of non-enzymatic glycation of proteins. *Clinical Biochemistry* 38: 103–115.

LEPRINCE O., HENDRY G.A.F. and MCKERSIE B.D. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research* 3: 231–246.

LINEBERGER D.R. and STEPONKUS P.L. 1980. Cryoprotection by glucose, sucrose, and raffinose to chloroplast thylakoids. *Plant Physiology* 65: 298–304.

MORSE E.E. 1947. Anthrone in estimating low concentration of sugar. *Analytical Chemistry* 19: 10–12.

NEWSTED W.J., CHIBBAR R.N. and GEORGES F. 1991. Effect of low temperature stress on the expression on sucrose synthase in spring and winter wheat plants. Development of monoclonal antibody against wheat germ sucrose synthase. *Biochemistry and Cell Biology* 69: 36–41.

OLAVE-CONCHA N., BRAVO L.A., RUIZ-LARA S. and CORCUERA L.J. 2005. Differential accumulation of dehydrin-like proteins by abiotic stresses in *Deschampsia antarctica* Desv. *Polar Biology* 28: 506–513.

OLECH M., WĘGRZYN M., LISOWSKA M., SŁABY A. and NAGIEL P. 2011. Contemporary changes in vegetation of Polar Regions. *Papers on Global Change* 18: 35–51.

PARNIKOZA I., CONVEY P., DYKYY I., TROKHYMETS V., MILINEVSKY G., TYSHCHENKO O., INOZEMTSEVA D. and KOZERETSKA I. 2009. Current status of the Antarctic herb tundra formation in the Central Argentine Islands. *Global Change Biology* 15: 1685–1693.

PARNIKOZA I., KOZERETSKA I. and KUNAKH V. 2011. Vascular Plants of the Maritime Antarctic: Orgin and Adaptation. *American Journal of Plant Sciences* 2: 381–395.

PASTORCZYK M., GIEŁWANOWSKA I. and LAHUTA L.B. 2014. Changes in soluble carbohydrates in polar Caryophyllaceae and Poaceae plants in response to chilling. *Acta Physiologiae Plantarum* 36: 1771.

PIOTROWICZ-CIEŚLAK A.I., GIEŁWANOWSKA I., BOCHENEK A., LORO P. and GÓRECKI R.J. 2005. Carbohydrates in *Colobanthus quitensis* and *Deschampsia antarctica*. *Acta Societatis Botanicorum Poloniae* 74: 209–217.

PROELS R.K. and HÜCKELHOVEN R. 2014. Cell-wall invertases, key enzymes in the modulation of plant metabolism during defence responses. *Molecular Plant Pathology* 15: 858–864.

RAUSCH T. and GREINER S. 2004. Plant protein inhibitors of invertases. *Biochimica et Biophysica Acta* 1696: 253–261.

REYES M.A., CORCEUERA L.J. and CARDEMIL L. 2003. Accumulation of HSP70 in *Deschampsia antarctica* Desv. leaves under thermal stress. *Antarctic Science* 15: 345–352.

REYES-DIAZ M., ULLOA N., ZÙÑIGA-FEEST A., GUTIERREZ A., GIDEKEL M., ALBERDI M., CORCUERA L.J. and BRAVO L.A. 2006. *Arabidopsis thaliana* avoids freezing by supercooling. *Journal of Experimental Botany* 57: 3687–3696.

ROITSCH T. and GONZALEZ M.C. 2004. Function and regulation of plant invertases: sweet sensation. *Trends in Plant Science* 9: 606–613.

ROJO E., ZOUHAR J., CARTER C., KORALEVA V. and RAIKHEL N.V. 2003. A unique mechanism for protein processing and degradation in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 100: 7389–7394.

ROLLAND F., BAENA-GONZALEZ E. and SHEEN J. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review Plant Biology* 57: 675–709.

SOLHAUG K.A. and AARES E. 1994. Remobilization of fructans in *Phippsia algida* during rapid inflorescence development. *Plant Physiology* 91: 219–225.

SÖLLING H. and ESSMAN V. 1975. A sensitive method of glycogen determination in the presence of interfering substances utilizing the filter-paper technique. *Analytical Biochemistry* 68: 664–668.

SUNG S.J.S., XU D.P., and BLACK C.C. 1989. Identification of actively filling sucrose sinks. 1. *Plant Physiology* 89: 1117–1121.

TARAN N.Y.U., STOROZHENKO V.O., SVIETLOVA N.B. and TOPCHIY N.M. 2012. Lipids and pigment-protein complexes of photosynthetic apparatus of *Deschampsia antarctica* Desv. Plants under UV-B radiation. *Biopolymers Cell* 28: 39–43.

VAN DEN ENDE W. and VALLURU R. 2009. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? *Journal of Experimental Botany* 60: 9–18.

VARGAS W., CUMINO A. and SALERNO G.L. 2003. Cyanobacterial alkaline/neutral invertases. Origin of sucrose hydrolysis in the plant cytosol? *Planta* 216: 951–960.

VECCHIA F.D., ASMAR T., CALAMASSI R. and RASCIO C.V. 1998. Morphological and ultrastructural aspects of dehydratation and rehydratation in leaves of *Sporobolus stapfianus*. *Plant Growth Regulation* 24: 219–228.

XIONG F.S., MUELLER E.C. and DAY T.A. 2000. Photosynthesis and respiratory acclimation and growth response of Antarctic vascular plants to contrasting temperature regimes. *American Journal of Botany* 87: 700–710.

ZÙÑIGA G.E., ALBERDI M. and CORCUERA L.J. 1996. Non-structural carbohydrates in *Deschampsia antarctica* Desv. from South Shetland Islands, Maritime Antarctic. *Environmental Experimental Botany* 36: 393–398.

ZÙÑIGA-FEEST A., BASCUÑÁN-GODOY L., REYES-DIAZ M., BRAVO L.A. and CORCUERA L.J. 2009. Is survival after ice encasement related with sugar distribution in organs of the Antarctic plants *Deschampsia antarctica* Desv. (Poaceae) and *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae)? *Polar Biology* 32: 583–591.