Plant Physiology and Biochemistry 139 (2019) 215–228

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Changes in content of steroid regulators during cold hardening of winter wheat - Steroid physiological/biochemical activity and impact on frost tolerance☆

Anna Janeczko[a,*], Ewa Pociecha[b], Michał Dziurka[a], Barbara Jurczyk[b], Marta Libik-Konieczny[a], Jana Oklestkova[c], Ondřej Novák[c], Maria Pilarska[a], Maria Filek[a,d], Elżbieta Rudolphi-Skórska[d], Iwona Sadura[a], Agata Siwek[e]

[a] Polish Academy of Sciences, The Franciszek Górski Institute of Plant Physiology, Niezapominajek 21, 30-239 Krakow, Poland
[b] Department of Plant Physiology, University of Agriculture in Krakow, Podłużna 3, 30-239 Krakow, Poland
[c] Laboratory of Growth Regulators, Institute of Experimental Botany, The Czech Academy Sciences & Palacký University, Šlechtitelů 27, 783 71 Olomouc, Czech Republic
[d] Department of Biochemistry, Biophysics and Biotechnology, Institute of Biology, Pedagogical University, Podchorążych 2, 30-084 Krakow, Poland
[e] Department of Pharmacobiology, Faculty of Pharmacy, Jagiellonian University Medical College, Medyczna 9, 30-688 Krakow, Poland

ARTICLE INFO

Keywords:
ABA
Brassinosteroids
Cold hardening
Frost
Progesterone
Rubisco
Sugars

ABSTRACT

The purpose of experiments was to describe the alterations of content of steroid regulators (brassinosteroids, progesterone) during cold hardening of winter wheat. Further we studied physiological and biochemical changes induced by these steroids in cold hardened winter wheat together with estimation of plant frost tolerance. The endogenous brassinosteroid content was elevated in winter wheat during cold hardening while level of progesterone was lowered. A higher content of brassinosteroids (but not progesterone) was connected to better frost tolerance of winter wheat cultivars. Plant supplementation with brassinosteroid (24-epibrassinolide) and progesterone before cold hardening reduced frost damage. Tests with the inhibitors of the biosynthesis of brassinosteroids and progesterone suggested that these steroids are one of players in regulating the antioxidant system in winter wheat during cold hardening. Their role in regulating the expression of Rubisco or the Rubisco activase gene was less clear. Steroid regulators did not affect the content of the stress hormone ABA. Model studies of the membranes, made on a Langmuir bath, showed an increase in the value of the parameter describing differences in membrane compressibility (resulting from stronger interactions among the molecules in the monolayers). This suggests that 24-epibrassinolide and progesterone enter into the lipid layer and - in a similar way to sterols – stabilise the interaction among lipids. It may be significant step for better frost tolerance. The use of steroid regulators (especially brassinosteroids) as agrochemicals improving frost tolerance of winter cereals will be discussed.

1. Introduction

Wheat (*Triticum* ssp.) is a main crop that is of strategic importance in Poland and one of the main cereals that are cultivated all over the world - annual world production of wheat is 700 000 000 tonnes. Wheat is characterised by a high yield potential and the multidirectional use of the grain. In Poland, it dominates the cultivation of the winter forms of wheat due to its greater yield and the efficiency of its flour compared to its spring forms. However, according to the Development of Polish Official Variety Testing (COBORU), the disadvantage of many cultivars is their insufficient winter survival rate (and particularly frost resistance). Winter wheat can tolerate frost quite well under snow cover but changes in the climate, which are often connected with the lack of snow during winter in regions such as Poland, contribute to the lower survival rate of winter wheat even at temperatures of about −10 °C. The relatively lower risk of losses of yield due to frost damages gives

Abbreviations: ABA, abscisic acid; BR, brassinosteroid(s); BRZ, brassinazole; CS, castasterone; D.W., dry weight; EBR, 24-epibrassinolide; F.W., fresh weight; TRIL, trilostane; P, progesterone

☆ Anna Janeczko web page: www.belanna.strefa.pl.

* Corresponding author.
E-mail addresses: ania@belanna.strefa.pl (A. Janeczko), m.dziurka@ifr-pan.krakow.pl (M. Dziurka), jana.oklestkova@upol.cz (J. Oklestkova), ondrej.novak@upol.cz (O. Novák).

https://doi.org/10.1016/j.plaphy.2019.03.020
Received 9 January 2019; Received in revised form 9 March 2019; Accepted 11 March 2019
Available online 15 March 2019
0981-9428/

farmers cultivation of cultivars with minimum average frost tolerance (score of 4.5–5 on a nine-point scale of COBORU). Meanwhile, of the 86 winter wheat cultivars that are registered in COBORU in Poland (January 2017) that have been tested for winter survival about half have a very low or low winter survival rate (1–3.5 on a nine-point scale) but only ten cultivars have 5.5–6 points, which are the highest values. One component of winter survival is frost tolerance - a complex trait that is affected by many factors, both morphological and metabolic. The most important metabolic changes that permit plants to withstand frost are formed during the process of cold hardening and include increasing cell membrane fluidity by increasing the proportion of unsaturated to saturated fatty acids, increasing the content of sucrose and other osmoprotectants and producing special protective proteins. Biochemical changes in cells are coordinated, among others, by the multidirectional effect of hormones on the metabolism of plants, which is also the basis for the adaptability of organisms to be able to function in changing environmental conditions. In the research on the tolerance of plants to low temperatures, the role of the stress hormone abscisic acid (ABA) is relatively well studied. The quantity of ABA may increase during the hardening of wheat plants in cold temperatures (Janeczko et al., 2018a). Similarly, the higher cold tolerance of maize is associated with its ability to accumulate a higher content of ABA (Janowiak et al., 2003). Other plant hormones such as polyamines, salicylic acid, gibberellins, cytokinins or auxins act in a complex network of metabolic relationships and may also participate in the plant response to low temperatures.

Part of the vast richness and diversity of the secondary metabolism of living organisms is the large group of compounds that are known as steroids. Steroid regulators are compounds that are based on a four-ring sterane structure. Numerous modifications in the number and type of functional groups that are attached to sterane determine their functions in living organisms. Plants also produce steroid regulators and two examples are the hormones brassinosteroids (BR) and progesterone (Fig. 1). BR are found in the grains of winter wheat and in the grains and in the aerial part of spring wheat (Yokota et al., 1994; Janeczko and Swaczynová, 2010; Janeczko et al., 2010). The amount of BR in spring wheat rarely reaches 1 ng g^{-1} F.W. and most often is significantly lower than 1 ng and on average is about 300 pg (Janeczko and Swaczynová, 2010). Similarly, the amount of progesterone that is found in the spring cultivars of wheat is significantly less than 1 ng g^{-1} F.W. (Janeczko et al., 2013).

The presence of progesterone and BR in cold hardened winter wheat as well as knowledge about their role in the process of cold hardening and the acquisition of frost tolerance in winter wheat is as yet not known. Moreover, the knowledge about the activity of these steroids in other plant species that had been exposed to low temperatures (especially temperatures below 0) is also rather limited. Winter rye and perennial ryegrass that had been sprayed with BR before a hardening process at 4 °C showed less frost damage and a higher plant survival rate (Pociecha et al., 2016, 2017). The importance of BR in the process of acquiring frost tolerance was confirmed in studies on model plants - *Arabidopsis thaliana* L. (Eremina et al., 2016). Compared to the wild type, plants of the BR-hypersignalling line demonstrated a higher frost tolerance at −10 °C while mutants with disturbances in the BR-signalling exhibited a drastically reduced frost tolerance. The method of action of BR in plants consists of, among others, accumulating larger amounts of sucrose and protective fructooligosaccharides (nystose and 1-kestose), regulating the expression of *COR* (cold-responsive genes) and reducing membrane damage (Pociecha et al., 2016; 2017; Eremina et al., 2016). Progesterone, on the other hand, increases cold tolerance in cold-sensitive species such as maize and chickpea, which is accompanied by the mobilisation of the antioxidant system and a reduction in membrane damage (Genisel et al., 2013; Erdal and Genisel, 2016). Generally, further studies are required to reveal more details about the mechanisms of action of steroid regulators in plants that are exposed to low temperature. Among abiotic stresses, temperature stress such as frost is a particularly serious problem in agriculture and horticulture because causes of losses of yield. Discovering and studying of regulators that stimulate processes counteracting the effects of temperature stress, is very important from the practical point of view. The results of these studies may contribute to obtain and incorporate new, more tolerant cultivars of crops or prepare formulas (agrochemicals) with protective/anti-stress properties for land farming applications.

The aims of this study were to investigate:

1) whether a higher accumulation of endogenous brassinosteroids in winter wheat cultivars is accompanied by a higher tolerance to frost in these cultivars,
2) whether a higher accumulation of endogenous progesterone as well as higher concentration of cellular progesterone binding sites is accompanied by a higher tolerance to frost of the wheat,
3) whether the application of exogenous brassinosteroids and progesterone before cold hardening improves the frost tolerance of six cultivars of winter wheat,
4) whether an induced deficiency of the content of brassinosteroids and progesterone (after the application of the inhibitors of their biosynthesis to plants) influences the transcript accumulation of the genes of Rubisco and Rubisco activase, the accumulation and activity of Rubisco, the accumulation of sugar, the efficiency of the selected elements of antioxidant system (SOD enzyme) and content of phytohormone ABA in cold-hardened winter wheat.
5) how brassinosteroids and progesterone interact with the cell membranes of wheat plants that were not hardened or cold hardened and how this modifies the membrane physicochemical properties (model studies).

2. Material and methods

2.1. Plant material

Six cultivars of winter wheat (Arkadia, Batuta, Bystra, Nutka, Smuga and Magic) were selected from the COBORU database (Development of Polish Official Variety Testing) in 2014. The cultivars differed in their winter survival rates according to COBORU field tests (nine-point scale). Cv. Bystra and cv. Magic had a low winter survival rate (1.5 point in a nine-point scale). Cv. Batuta and cv. Nutka had a moderate winter survival rate (3 points). Cv. Arkadia (6 points) and cv. Smuga (6.5 points) had the highest winter survival rate.

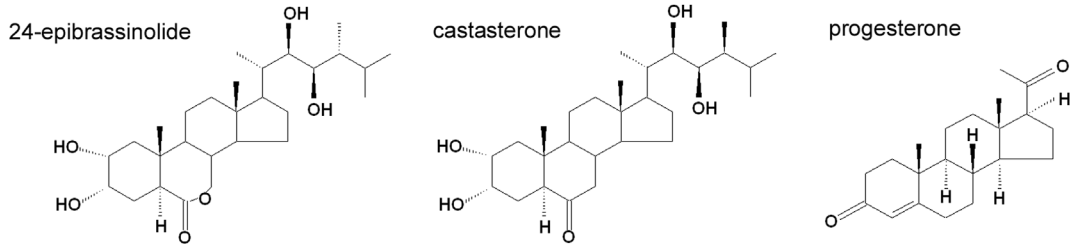

Fig. 1. Chemical structure of brassinosteroids and progesterone.

2.2. Experimental design

Four experiments were performed in the research. In experiment 1, changes in the content of endogenous BR and progesterone as well as changes in concentrations of progesterone binding sites in winter wheat that had not been hardened and after cold hardening were studied. In experiment 2, the frost tolerance of the six winter wheat cultivars was investigated after the application of the steroid regulators (brassinosteroids: 24-epibrassinolide (EBR), castasterone (CS)and progesterone (P)). The results of experiment 2 allowed two cultivars, Bystra and Smuga, to be selected for experiments 3 and 4. Cv. Bystra had a low frost tolerance and responded well to the steroid treatment and cv. Smuga had a high frost tolerance and responded weakly to the application of the steroids. In experiment 3 the biochemical changes (i.e. the expression of Rubisco, the accumulation of sugar, the activity of superoxide dismutase or ABA hormone content) were monitored during cold hardening in cv. Bystra and cv. Smuga after the induced deficiency of the steroid regulators (*via* the application of the inhibitors of steroid biosynthesis to plants) and after supplementing the steroid regulators (brassinosteroid or progesterone). The following inhibitors were used: brassinazole (BRZ) - brassinosteroid biosynthesis inhibitor (Asami et al., 2000) and trilostane - progesterone biosynthesis inhibitor (TRIL) (Janeczko et al., 2015). Finally, in experiment 4 (model studies), we attempted to answer the question of whether and how BR and progesterone change the physicochemical properties of wheat cell membranes because membranes are an important element in the mechanisms of the cell response to low temperatures.

2.2.1. Design of experiments 1 and 4

Seeds of six cultivars of winter wheat were germinated in pots (38 cm × 13 cm x 13 cm) at 25 °C (darkness) in a growth chamber. There were 12 pots - two pots for each cultivar, 50 seeds per pot. After 48 h, the temperature was set to 20 °C and the growth conditions were changed to a 12 h photoperiod [12/12 h (d/n)]. After ten days, the pots were divided into two groups. Six cultivars of the first group (six pots) continued growing at 20 °C for four more days (as plants that were not hardened). Six cultivars of the second group were exposed to three weeks of cold hardening at 5 °C, 8 h photoperiod [8/16 h (d/n)]. The plants that were not hardened and the cold-hardened plants were all at the same two-leaf stage of growth because low temperatures slow down the growth processes. Samples (the aerial part of plants) for the analysis of the content of BR and progesterone (experiment 1), the specific binding sites of progesterone (experiment 1) and Langmuir bath studies (experiment 4, only cv. Smuga and Bystra) were taken from the plants that were not hardened and from the hardened plants.

2.2.2. Design of experiment 2

Seeds of all six cultivars of winter wheat were sown in pots (50 cm x 38 cm x 16 cm) with soil and were germinated at 25 °C (darkness) in a growth chamber. In one pot was plants of three cultivars planted in six rows (two rows per cultivar, 20 plants per row). After 48 h, the pots were moved to a glasshouse (temperature 20 °C), natural day length and light (September/October; latitude: 50°03′ North, longitude: 19°55' East). Two-week-old plants were sprayed with the steroids. The following treatments were used: control - plants were not sprayed; CS 0.05 - plants were sprayed with brassinosteroid - castasterone (0.05 mg l^{-1}); CS 0.25 - plants were sprayed with castasterone (0.25 mg l^{-1}); EBR 0.05 - plants were sprayed with brassinosteroid - 24-epibrassinolide (0.05 mg l^{-1}); EBR 0.25 - plants were sprayed with 24-epibrassinolide (0.25 mg l^{-1}); P 0.05 - plants were sprayed with progesterone (0.05 mg l^{-1}); P 0.25 - plants were sprayed with progesterone (0.25 mg l^{-1}). There were two pots per one treatment because plants of the three cultivars were growing in each pot. BR were purchased from OlChemIm (Olomouc, Czech Republic) and the progesterone was purchased from Sigma-Aldrich (Poznań, Poland). Stock solutions (2 mg of the steroid per 0.5 ml 96% ethanol) were used to prepare water working solutions of the appropriate concentration for spraying the plants. When the plants were sprayed, they had two developed leaves. Twenty-four hours after they were sprayed, the plants were moved to a growth chamber with an 8 h photoperiod [8/16 h (d/n); lamps HPS Philips SON-T AGRO 400 W; light 200 μmol m^{-2} s^{-1}] and a temperature of 12 °C. After two days, the temperature was changed to 5 °C for the cold hardening of the plants (three weeks). After cold hardening, the pots with plants were taken to a dark chamber and exposed to frost at −12 °C. The temperature for the treatment was selected based on the experience of the authors and preliminary trials. The temperature was lowered three degrees per hour in order to reach −12 °C. The plants were kept at −12 °C for 6 h and then the temperature was increased slowly by three degrees each hour in order to reach 5 °C. The plants were then moved back to the growth chamber where they continued to grow at 12 °C, 8 h photoperiod [8/16 h (d/n)]. After two weeks, the plant regrowth rate was estimated based on the Larsen (1978) scale.

2.2.3. Design of experiment 3

The seeds of two cultivars (Bystra, Smuga) were germinated (72 h at 24 °C, darkness) in plastic vessels (12 cm × 10 cm x 3 cm) with H_2O moistened filter paper. The germinated seeds were transferred into Petri dishes (10 cm diameter, 30 seeds per dish) and treated as follows: (1) Control - the plants were watered with a solution of water with traces of DMSO (a solvent of brassinazole and trilostane), (2) the BRZ treated plants - plants watered with a brassinazole solution (BRZ, 0.5 mg l^{-1}) and (3) the TRIL treated plants - plants watered with a trilostane solution (TRIL, 0.5 mg l^{-1}). BRZ and TRIL are inhibitors of the biosynthesis of brassinosteroids and progesterone, respectively. The chemicals were purchased from Sigma-Aldrich (Poznań, Poland) and dissolved in DMSO (2 mg of the inhibitor per 0.5 ml DMSO) for the further preparation of the working solutions in water. On the first day, 50 ml of solution was applied to each Petri dish; on the second day 10 ml of solution was applied. On third and fourth days only water (30 ml) was applied to each Petri dish for all of the treatments each day. The Petri dish culture was placed in a greenhouse with natural day length and light (September/October; latitude: 50°03′ North, longitude: 19°55' East; temperature 20 °C). On the fifth day, the plants were transferred to pots (18 cm × 18 cm x 18 cm; 25–30 plants per pot). The potted plants were kept in the growth chamber for the next 5 day s at a temperature of 20 °C, 12 h photoperiod [(12/12 h (d/n)]. Then, the potted plants, which had previously been treated with BRZ, were divided into two groups and one of these groups was sprayed with 0.05 mg l^{-1} 24-epibrassinolide (EBR). The pots with plants, which had previously been treated with TRIL, were divided into two groups and one of these groups was sprayed with 0.05 mg l^{-1} progesterone. EBR was applied to counteract the BRZ-induced deficit of endogenous brassinosteroids in the plants. Exogenous P was applied to counteract the TRIL-induced deficit of endogenous progesterone in the plants. There was a total of five treatments for each of the two cultivars - the control (untreated plants), BRZ, BRZ + EBR, TRIL and TRIL + P treated plants. Twenty-four hours after the plants were sprayed with EBR and P, the temperature in growth chamber was set to 5 °C [8 h photoperiod, 8/16 h (d/n)] for the cold hardening of the plants (3 weeks). At the end of the cold period when the plants had two well-developed leaves, samples were taken for all of the biochemical measurements (Rubisco expression and activity, sugar content, antioxidative enzyme activity, phytohormone content). The first (older) leaf of a plant was always taken for the analysis.

3. Measurements and observations

3.1. Analysis of the brassinosteroid and progesterone content

Steroids were analysed as described in article of Oklestkova et al. (2017) (brassinosterois) and Simerský et al. (2009) (progesterone) with minor modifications. Plant material (1 g F.W.) was extracted (80%

methanol) and then purified on Discovery DPA-6S columns (Supelco, Bellefonte, PA USA) and immunoaffinity columns (Laboratory of Growth Regulation, Olomouc, Czech Republic; Oklestkova et al., 2017). Steroid content was measured using liquid chromatography – mass spectrometry UHPLC-MS/MS. Detail conditions of analysis are given in aforementioned articles. The analyses were made in three replicates where each replicate = material from five to seven plants.

3.2. Progesterone binding assay

Microsomal and cytosolic fractions that were isolated from wheat as described in Janeczko et al. (2008) were used for the progesterone binding assay. Aliquots of the microsomal or cytosolic fraction containing 150 µg of protein were incubated in triplicate with 10 nM [^{3}H] progesterone in a buffer (5 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 10 mM sodium molybdate, 10% glycerol) at 4 °C (16 h). Identical samples were incubated in triplicate with a 500-fold excess of unlabelled progesterone added in order to determine nonspecific binding. The final volume of each sample was 200 µl. After incubation, 200 µl of a charcoal (1%)/dextran (0.1%) suspension was added to each sample. The samples were then incubated for 10 min at 2 °C and then centrifuged at 800 × *g* for 15 min at 4 °C. Next, 200 µl of the supernatant was removed and the radioactivity of the bound [^{3}H]progesterone was measured in a MicroBeta TriLux 1450 scintillation counter (PerkinElmer, Waltham, MA, USA). Progesterone binding, which is considered to be specific, was determined by subtracting the nonspecific binding from the total binding and the results were expressed as femtomoles (fmol) of the bound progesterone per mg of protein. Principle of the method described in Fang et al. (1997) and Di Cosmo et al. (1998) was adapted to plant material. The data was expressed as the mean ± SE of three independent experiments that were carried out in triplicate.

3.3. Estimation of frost damage to the leaves (Larsen method)

Plant tolerance to frost was estimated using the modified method of Larsen (1978). The method is based on a visual score (0–9 point scale of plant regrowth after frost). 0 - dead plants, not regrowing; 1 - dying plants with a 0.5 cm leaf elongation; 2 - dying plants with a 1–2 cm leaf elongation; 3 - dying plants with more than 2 cm of leaf elongation; 4 - plants may continue to grow or die but the inner leaves will be brown; 5 - plants may survive but regrown leaves will be discoloured/curled; 6 - plants may survive but damage will be visible on about 50% of the leaves; 7 - plants are alive but symptoms of freezing injury are visible and some of the leaves are discoloured/deformed; 8 - only the tops of the inner leaves are discoloured/deformed and 9 - no symptoms of frost damage.

3.4. Transcript accumulation of Rubisco and Rubisco activase

For the transcript accumulation analysis, fragments of the first leaf (30–50 mg) from three individual plants (three samples/treatment) were collected. Additionally, each sample was measured four times (four technical repetitions). An RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) was used to extract total mRNA. A reverse transcription reaction was performed after the elimination of any traces of genomic DNA contamination using a QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). The primers and probes for the PCR reactions were designed using Primer Express Software v 3.0.1 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) based on the *Triticum aestivum* sequences that were derived from GenBank (ID: M37477.1, DQ984669.1, AB181991.1). The sequences of the primers and probes are listed in Table 1. Singleplex PCR amplifications for *Rbc*S, *Rca*A and *actin* as an endogenous control gene were performed in 96-well plates as described by Jurczyk et al. (2012). The specificity of the amplicons was checked and confirmed. The amplification results were analysed using 7500 real time PCR Sequence Detection Software v 1.3 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The Relative Standard Curve Method (Applied Biosystems) with *actin* as the endogenous control (An et al., 1996) was used for the relative quantification. The presented results are the fold differences in the expression of the *Rbc*S (Rubisco small subunit gene) and *Rca*A gene (Rubisco activase gene) in the treated samples relative to the *actin* gene. cDNA was prepared from three different samples and each PCR reaction was run in four technical replicates (the final number of replications was 12 - three biological samples each in four technical replicates). The normal distribution of data was confirmed.

3.5. Rubisco accumulation

For the analysis of Rubisco accumulation, seven first leaves were collected from individual plants within each treatment. The proteins were extracted according to Niewiadomska et al. (2009). The protein concentration was estimated using a Roti® Nanoquant reagent (Roth, Karlsruhe, Germany). Proteins were denatured in 62.5 mM Tris-HCl pH 6.8 containing 2.5% SDS, 0.02% Bromophenol Blue, 5% β-mercaptoethanol and 10% glicerol and incubated for 5 min at 95 °C. The protein extracts (2 µg per lane) were loaded on to 4–20% polyacrylamide gels (Bio-Rad) and SDS-PAGE electrophoresis was performed in a standard Laemmli buffer system. After separation, the proteins were stained with Coomassie Brilliant Blue. Gels from three independent experiments were scanned using the Epson Perfection V700 Photo system and densitometric analysis was performed using the ImageJ program (GPL license). Small and large (SSU and LSU) Rubisco subunits were recognised according to their molecular weight.

3.6. Rubisco activity

Rubisco activity was analysed according to the method of Sharkey et al. (1991). The first leaves with a known (weighted) fresh mass and area (Leaf Area Meter Cl-202, CID Bio-Science, Camas, WA, USA) were poured with liquid nitrogen and homogenised at 4 °C with an extraction buffer (5 mM DTT, 1 mM EDTA, 100 mM Bicine pH 7.8, 5 mM $MgCl_2$, 0.002% BSA (w/v)) to which insoluble PVPP had been added. After centrifugation (16.000*g*) for 1 min, the reaction was initiated by adding the leaf extract to the assay buffer. The assay buffer contained 50 mM Bicine pH 8.0, 1 mM EDTA, 15 mM $MgCl_2$, 18.5 mM NaCl, 9.2 mM DTT, 0.6 mM RuBP, 9.2 mM $NaHCO_3$, 0.4 mM NADH, 0.5 mM ATP, 4.6 mM phosphocreatine, 1.3 U of phosphocreatine kinase, 47 U of phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. Rubisco activity is coupled with NADH oxidation using 3-phosphoglycerate (PGA) kinase and glyceraldehyde 3-phosphate (GAP) dehydrogenase. The oxidation of NADH was continuously monitored on spectrophotometer (Ultrospec 2100; Biosciences Amersham, Sweden) at 340 nm (Sharkey et al., 1991). Five first leaves from independent plants within each treatment were collected for the analysis of the Rubisco activity.

3.7. Soluble sugar content

The plant material (250 mg of fresh mass of first leaves per sample) was ground in a mortar in liquid N_2. The powdered material was washed with 5 ml of H_2O and moved to a tube. The tube was boiled in a water bath (15 min). Next, the samples were centrifuged (10 min, 2000 g, 4 °C), the supernatant was removed and supplemented with 5 ml of H_2O. To estimate the soluble sugars, the extract was diluted with water 20-fold. Next, 1 ml was removed and mixed with 2 ml of 0.2% anthrone in concentrated H_2SO_4. The samples were heated in a water bath (90 °C) for 3 min. After the samples had cooled down, the absorbance was measured (620 nm) on a background blind sample (2 ml of anthrone solution in 1 ml of H_2O) using a Ultrospec 2100 spectrophotometer (Biosciences Amersham, Sweden). The analyses were performed in five

Table 1
Sequences of primers and probes that were designed and used in the study.

Gene name	Forward primer	Reverse primer	Probe
*Rbc*S	TGGCCAATTGAGGGCATCA	CAAGTAGTCGACCTGCTTCAAGAG	FAM-CAGGGTCTCGAACTTC
*Rca*A	CTGCCTCTTCATCAACGATCTTG	GGTTGTTGACGGTGTACTGTGT	FAM-CCGCCCATCCGACCCG
actin	AGATCCTGACAGAAAGAGGGTACTC	GCCACGTAAGCGAGCTTCT	FAM-TCTCTGACAATTTCCC

replicates.

3.8. Superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured according to Droillard et al. (1987). First leaf samples were homogenised at 4 °C with a 50 mM phosphate buffer (pH 7.8) and 1% PVPP and then centrifuged. The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 1 mm EDTA, 1 unit of catalase, 56 mM nitroblue tetrazolium (NBT), 0.1 mM xantine, 0.03 units of xantine oxidase and the extract from the tissue. The absorbance was monitored at 560 nm. The inhibition percentage of the NBT reduction is a measure of the SOD activity. One unit of SOD is the amount of extract that provides a half-maximum inhibition. Five samples were collected for the analyses of the SOD activity. Five to seven first leaves that were collected from individual plants were in each sample (300 mg fresh weight).

3.9. Visualisation and identification of SOD isoforms on polyacrylamide gels

Plant material (1 g fresh weight of the first leaves) was homogenised at 4 °C in a 1.5 ml of homogenization buffer pH 7.8 (50 mM phosphate buffer with 1 mM DTT), 1% PVPP and protease inhibitor cocktail (Roche) in a mortar. The homogenates were centrifuged for 5 min (2000 *g*). The supernatants were collected and stored at −80 °C. The total protein concentration was determined according to the Bradford dye-binding method (Bradford, 1976) using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with BSA as the standard. To each well was loaded 10 μg of proteins. To determine the activity of SOD, protein fractions that were isolated as described above were separated on 12% polyacrylamide gels using the Laemmli (1970) buffer system without sodium dodecyl sulfate (SDS) at 4 °C and 180 V in. The SOD bands were visualised on the gels using the activity staining procedure described by Beauchamp and Fridovich (1971) - the gels were incubated in a staining buffer (a potassium phosphate buffer, pH 7.8, containing 0.0068 g l^{-1} KH_2PO_4, 0.0175 g l^{-1} Na_2HPO_4, 0.372 g l^{-1} EDTA, 31% (v/v) TEMED, 7.5 mg l^{-1} riboflavin and 0.2 g l^{-1} NBT) for 30 min in the dark at room temperature and then exposed to white light until the SOD activity bands became visible. H_2O_2 in the concentration of 5 mM was added to the staining buffer in order to inhibit Cu/ZnSOD and FeSOD. The selective inhibition of Cu/ZnSOD was achieved by incubating the gels in a buffer containing 3 mM KCN. The gels were scanned using an Epson Perfection V700 Photo scanner.

Densitometric analysis of the gels: The intensity of bands that corresponded with the activities of the SOD isoforms were analysed using the ImageJ program. The results were presented in the arbitrary units that correspond to the area under the densitometric curve. Analyses were performed for cv. Smuga and repeated three times.

3.10. Abscisic acid (ABA) analysis

The analyses of hormone ABA were performed as described in Dziurka et al. (2016). Briefly, the plant material (1 g of first leaves) was lyophilised and powdered and samples weighing 30 mg of dried material were prepared. The analyses were performed in three replicates on a UHPLC apparatus (Agilent Infinity 1260, Agilent, Germany), which was coupled to a triple quadruple mass spectrometer (6410 Triple Quad LC/MS, Agilent, Santa Clara, CA, USA) equipped with electrospray ionisation (ESI). Separation was performed on an Ascentis Express RP-Amide analytical column (2.7 μm, 2.1 mm × 75 mm; Supelco, Bellefonte, PA, USA). Technical details are given in Dziurka et al. (2016).

3.11. Model studies of membranes

Model membranes were prepared from polar lipids from the plasmalemma of seedlings of the wheat cv. Bystra and cv. Smuga cultivated at 20 and 5 °C (experiment 4). The plasmalemma was obtained from the leaves using the method demonstrated earlier by Gzyl-Malcher et al. (2007) and using a mixture of 250 mM sucrose, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 M ethylenediaminetetraacetic acid and 10 mM tris(hydroxymethyl)aminomethane. Mixtures of isopropanol with 0.01% butylated hydroxytoluene and chloroform:methanol (2:1; v:v) were added to the lipid extractions as was described in detail by Filek et al. (2002). Lipids, which contained polar fractions (phospholipids - PL, mono- and digalactosyldiacylglycerols - MGDG, DGDG), were separated from the fraction of neutral lipids using adsorptive and distributive column chromatography (on silica acid and with a low N_2 pressure) and purified using thin-layer chromatography. To prepare the monolayers, the polar lipids of the plasmalemma were dissolved in chloroform (Merck, Germany) and used as the control (0). Brassinosteroid (24-epibrassinolide) and progesterone were selected to study the interaction with the model membranes. The 24-Epibrassinolide was purchased from Olchemin (Olomouc, Czech Republic) and the progesterone from Sigma-Aldrich (Poznan, Poland). Model studies at the interface water/air (model for hydrophilic/hydrophobic part of the membrane) require strictly defined solvents for investigations of the properties of adsorbent substances on lipid monolayers i.e.: chloroform - for studies the effects of more-hydrophobic molecules and water - for those which are more soluble in water. The introduction of other solvent (such as acetone, ethanol, etc.) would result in its independent absorption at the interface (affecting changes in surface tension, which is the basic parameter determined for model lipid monolayers) and thus the impossibility of precise interpretation of the physicochemical parameters of studied substances. Both chosen steroids are characterised by very poor dissolving in water (Product Inf. Cayman Chemical; Haskins, 1949). Our preliminary experiments indicated however that progesterone was anyway better water-soluble than EBR and stabile solutions of progesterone (several days, without precipitation) were prepared at concentration 1×10^{-5} mol l^{-1}. EBR was better dissolved in chloroform. For experiments EBR was prepared in chloroform at concentration 1 mg ml^{-1} as a starting solution. Pure lipid solutions or a mixture of lipids with a more hydrophobic hormone - 24-epibrassinolide (dissolved in chloroform at a ratio of 4:1; lipids: EBR; v:v) were applied on the water surface in a Langmuir-standard trough (Minitrough, KSV, Finland) equipped with a platinum Wilhelmy plate. To describe the effects of the progesterone, pure lipids were spread on a water phase that contained this hormone at a concentration of 1×10^{-5} mol l^{-1}. The use of significantly higher concentrations of steroids, in relation to the amounts occurring in cells, was aimed at intensification the effects of their action with lipids to obtain of the conclusive and significant effects allowed more precisely describe the physicochemical and structural changes occurring in monolayers. The justification for select of higher (than physiological) content of amphiphilic substance in the mixture with lipids can be found in the

literature to demonstrate the possibility of incorporation of these substances in membrane structures (to creating specific domains) (Atkinson et al., 2010; Lecompte et al., 2015). Such domains in which sterol substances (or others with amphiphilic structure) may be present in concentrations higher than physiological, play an important role in modifying membrane properties in developmental and stress processes.

The isotherms of the surface pressure vs the area per molecule (π vs A) were obtained by compressing the monolayers using two symmetrical barriers of the Langmuir trough with the rate of the monolayer compression fixed as 5 $Å^2$ $molecule^{-1}$ min^{-1} at an established temperature of 20 °C. All of the experiments were repeated four or five times to ensure the reproducibility of the obtained isotherms to ± 0.1–0.2 $Å^2$.

4. Results and discussion

4.1. Presence of brassinosteroids and changes of their content during the process of the cold hardening of winter wheat

According to the studies of Yokota et al. (1994), castasterone, 3-dehydroteasterone (intermediate in the conversion of teasterone to typhasterol), teasterone, typhasterol and 6-deoxocastasterone were present in the grains of winter wheat (cv. Chihoku). Our studies confirmed the presence of castasterone and teasterone in the leaves of wheat and additionally revealed presence of homocastasterone (Fig. 2A–C). Homocastastasterone was accumulated in the highest amounts.

The content of BR in plants is changeable and may depend on plant growth conditions such as light, water availability, temperature. Rice cultured in blue and white light had about 4 times increased content of castasterone compared to rice in the dark (Asahina et al., 2014). The BR content increased in barley during drought (Gruszka et al., 2016). Castasterone accumulation increased also during cold hardening of winter rye (Pociecha et al., 2016). In current studies on wheat the picture was not such clear for castasterone and presence/changes of this steroid was cultivar-dependent (Fig. 2 A). In cv. Bystra, only trace amounts of castasterone were found. In cv. Batuta, cv. Magic and cv. Nutka, the content of CS was similar before and after cold hardening. Only in cv. Arkadia and cv. Smuga CS increased after cold hardening. Contrary to CS, changes of two other BR after cold treatment of winter wheat were very clear. The content of homocastasterone and teasterone generally increased after cold hardening (Fig. 2 B and C). One exception was frost sensitive cv. Bystra, in which the content of these two BR remained unchanged. An increase in the BR accumulation under stress is connected to the upregulation of the BR biosynthetic genes (Zhang et al., 2013).

The highest content of BR after cold hardening was observed in the cultivars with the highest level of frost tolerance (cv. Batuta and cv. Smuga). This information can be useful in practice for breeding new winter wheat cultivars with a higher level of frost tolerance. The earlier studies of Janowiak et al. (2003) showed that ABA - a main stress hormone - was accumulated as a result of cold treatment and that a higher concentration of ABA in maize was highly correlated with a higher cold tolerance of maize cultivars.

4.2. Changes of the content of progesterone and the concentration of cellular progesterone binding sites during the process of the cold hardening of winter wheat

The progesterone content in plants not only depends on the species but also varies between different organs and stages of plant development (Iino et al., 2007). This steroid was found in our studies both in not hardened and cold hardened winter wheat (Fig. 3 A). We earlier reported presence of progesterone in spring wheat (Janeczko et al., 2013), but to the best of our knowledge, the endogenous changes of progesterone have not been studied in respect to cold hardening or cold stress. In current studies, the content of progesterone decreased during

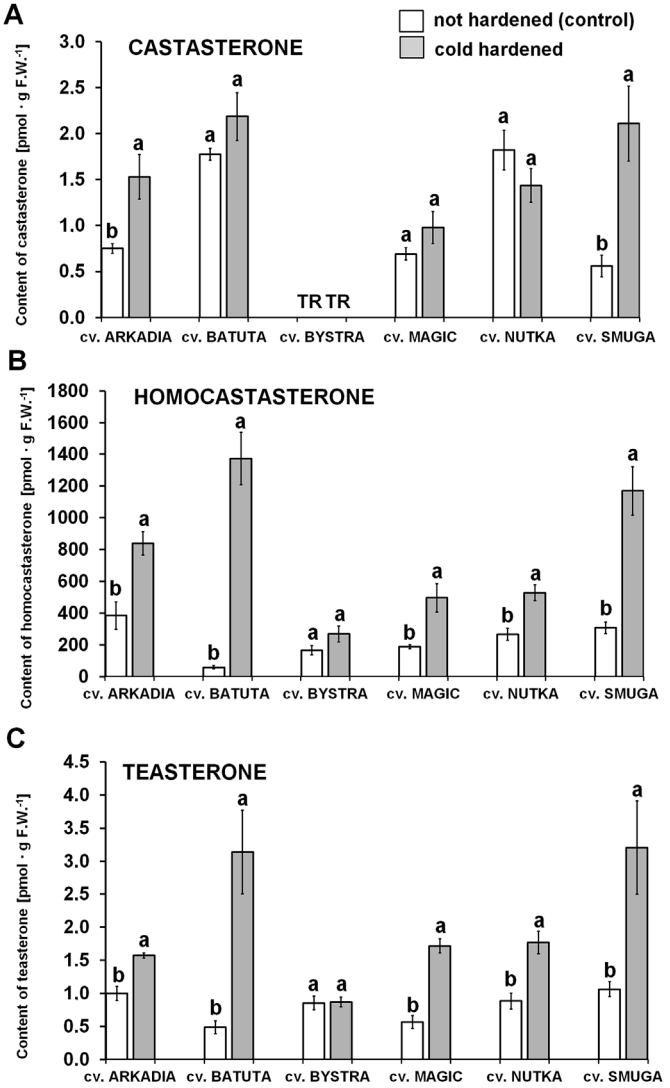

Fig. 2. Content of brassinosteroids in six cultivars of not hardened and cold-hardened winter wheat (A–C); TR – traces. *Values (± SE) marked with the same letters are not significantly different according to the Student test (P ≤ 0.05); comparison in pairs within one cultivar between not hardened and cold-hardened plants.*

cold hardening (in four of the cultivars, the effect was statistically significant) (Fig. 3 A). Interestingly, the progesterone content decreased gradually. The content of progesterone, which was measured in the middle of period of cold hardening (after 11 days, data not shown), was precisely between the values that were observed for the plants not hardened and the values for the plants after three weeks of cold hardening. Observed decrease of progesterone content in cold hardened winter wheat was counter to our expectations. We presumed that due to the antistress abilities of progesterone (Genisel et al., 2013; Erdal and Genisel, 2016), its accumulation during cold hardening would be beneficial. A similar phenomenon was observed earlier for another mammalian steroid hormone - androstenedione (Janeczko et al., 2018a). The endogenous content of androstenedione was lower in the winter wheat cv. Kobiera after a period of cold hardening although its exogenous application simultaneously increased the activity of the antioxidant system and increased the frost tolerance of winter wheat. Our earlier studies, which were devoted to drought stress, showed that the progesterone content decreased after a drought period in the spring wheat cultivar that was susceptible to drought but increased in the tolerant cultivar (Janeczko et al., 2013). In current experiment, we did not find

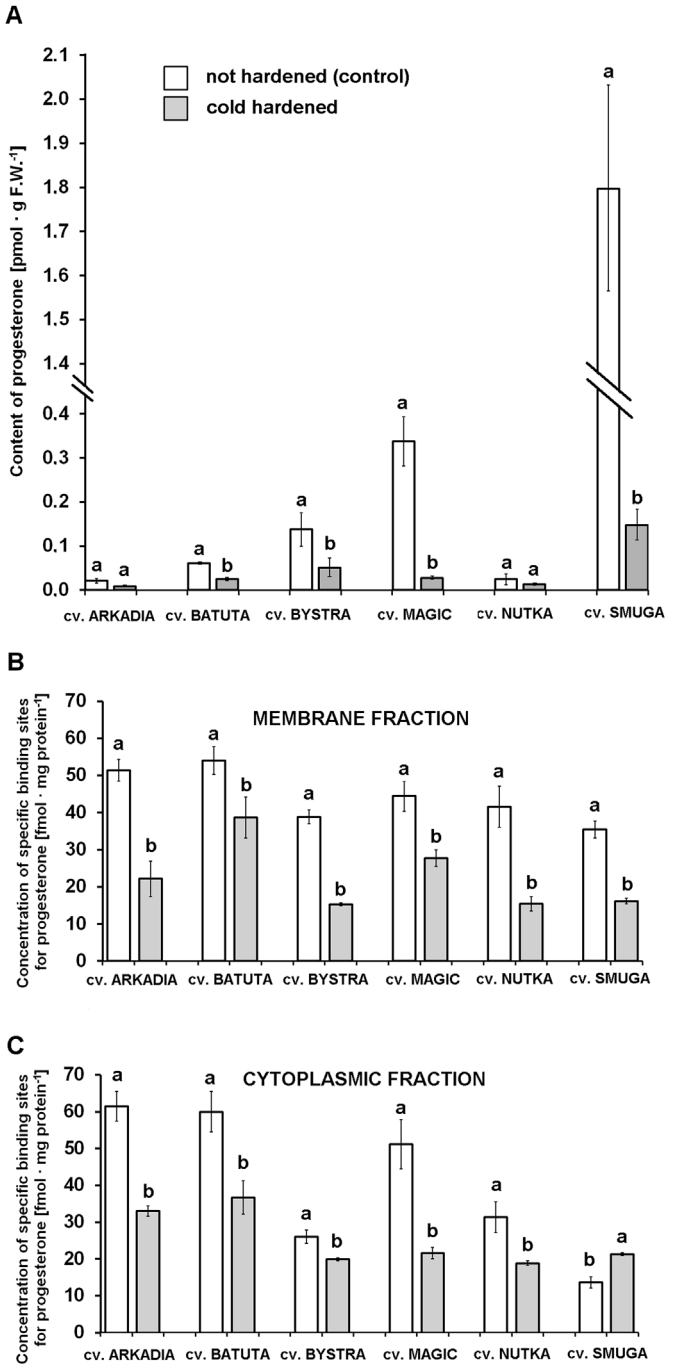

Fig. 3. Content of progesterone in six cultivars of not hardened and cold-hardened winter wheat (A). Binding of [^{3}H]progesterone in the membrane (B) and cytoplasmic (C) fractions that had been isolated from cells of not hardened and cold-hardened cultivars of winter wheat. *Values (± SE) marked with the same letters are not significantly different according to the Student's test ($P \leq 0.05$); comparison in pairs within one cultivar between not hardened and cold-hardened plants.*

any connection between the level of frost tolerance and the amount of progesterone in the cold hardened (nor in the not cold hardened) winter wheat cultivars.

As for explanation why accumulation of brassinosteroids is inversely proportional to the accumulation of progesterone we can propose only theory. In wheat brassinosteroids have activity delaying of generative development (Janeczko et al., 2015). On the other hand progesterone stimulates wheat flowering (Janeczko et al., 2015). In natural conditions, in early stages of winter it would be better for plant to maintain balance between BR and P with higher concentration of development inhibiting BR than development stimulating P (although P also has antistress activity). However it can be presumed that in late stages of winter or in early stages of spring progesterone content may increase. Currently we conduct experiments trying to prove it.

Binding sites for progesterone (a putative receptors) were present in the membrane fraction in all of the tested cultivars of wheat in amounts ranging from 35 to 60 fmol mg^{-1} protein (Fig. 3 B). The concentration of progesterone binding sites decreased during cold hardening by about 50%. Progesterone binding sites were also found in the cytoplasmic fraction. Their concentration in the cytoplasm decreased during cold hardening in five of the six tested cultivars from a few percent to 50% (Fig. 3 C). The concentration of progesterone binding sites increased only in the cytoplasm of cv. Smuga after cold hardening (Fig. 3 C).

In a work published in 2005, Yang et al. characterised the progesterone putative receptor in the cell membrane of *Arabidopsis thaliana* L. It was a membrane steroid binding protein (MSBP1). The *MSBP1* gene encoded a 220-amino acid protein that could bind to progesterone with a high affinity and to some other steroids, including brassinosteroids, with a low affinity. This phenomenon is known for steroid hormones in animal world, i.e. glucocorticosteroid receptors can not only bind glucocorticosteroids but also progesterone, testosterone or estrogen. It can be assumed that the binding of progesterone, which was found in the wheat membranes in our experiment, means the presence of MSBP1 at least in part. However, the existence of other binding proteins in addition to MSPB1 cannot excluded. According to Yang et al. (2005), a reduced expression of MSBP1 caused a hypersensitivity to progesterone but also to brassinosteroids in *Arabidopsis* grown in light conditions. On the other hand, an enhanced expression of MSBP1 resulted in a reduced response to these steroids. Furthermore, according to Song et al. (2009), MSBP1 suppressed the BR-signalling in *Arabidopsis* by interacting with BAK1. Typically, BAK1 is a co-receptor for the transmembrane brassinosteroid receptor BRI1. Its task is to enhance the BRI1-mediated BR-signalling. Although BAK1 does not directly participate in BR binding, the binding of ligand (brassinosteroid) to BRI1 affects the heterodimerisation of BRI1 and BAK1 and initiates the BR signal transduction. In the opinion of Song et al. (2009), MSBP1 accelerates BAK1 endocytosis, which results in the weakening of BR-signalling. Moreover, authors proved that the enhanced MSBP1 expression reduced the interaction between BRI1 and BAK1 thus demonstrating that MSBP1 acts as a negative factor at an early stage of the BR-signalling pathway. If we implement this model in our studies for cold-hardened winter wheat, a decrease in the concentration of progesterone binding sites (supposedly MSBP1) may be required for the more efficient BR-signalling in cold hardening, which may support our opinion that BR are important players in the cold acclimation processes in winter wheat although a detailed physiological/biochemical directions of their activity is yet to be explained. As was mentioned above, according to Yang et al. (2005), a decrease in the expression of MSBP1 increases the sensitivity of plants to progesterone (and BR). In our studies, decreasing the progesterone content in cold may then be compensated by an increased plant sensitivity to that steroid. On the other hand, an increased content of BR in cold hardening together with an increased plant sensitivity to BR may additionally confirm some significance of these steroids for the acclimation of winter wheat plants to low temperatures.

In our experiment, progesterone was also bound in the cytoplasmic fraction, which suggests the presence of some putative receptors of the steroid in the cytoplasm of wheat cells. Cytoplasm progesterone receptors are well known in mammals. In plants, the nature and structure of these sites as yet remains unknown and requires further studies. The concentration of progesterone binding sites in the cytoplasm decreased in five of the six tested cultivars during cold hardening. Only in the cytoplasm of cv. Smuga was the concentration of the progesterone binding sites increased after cold hardening. This same phenomenon was described earlier for the progesterone binding sites in the

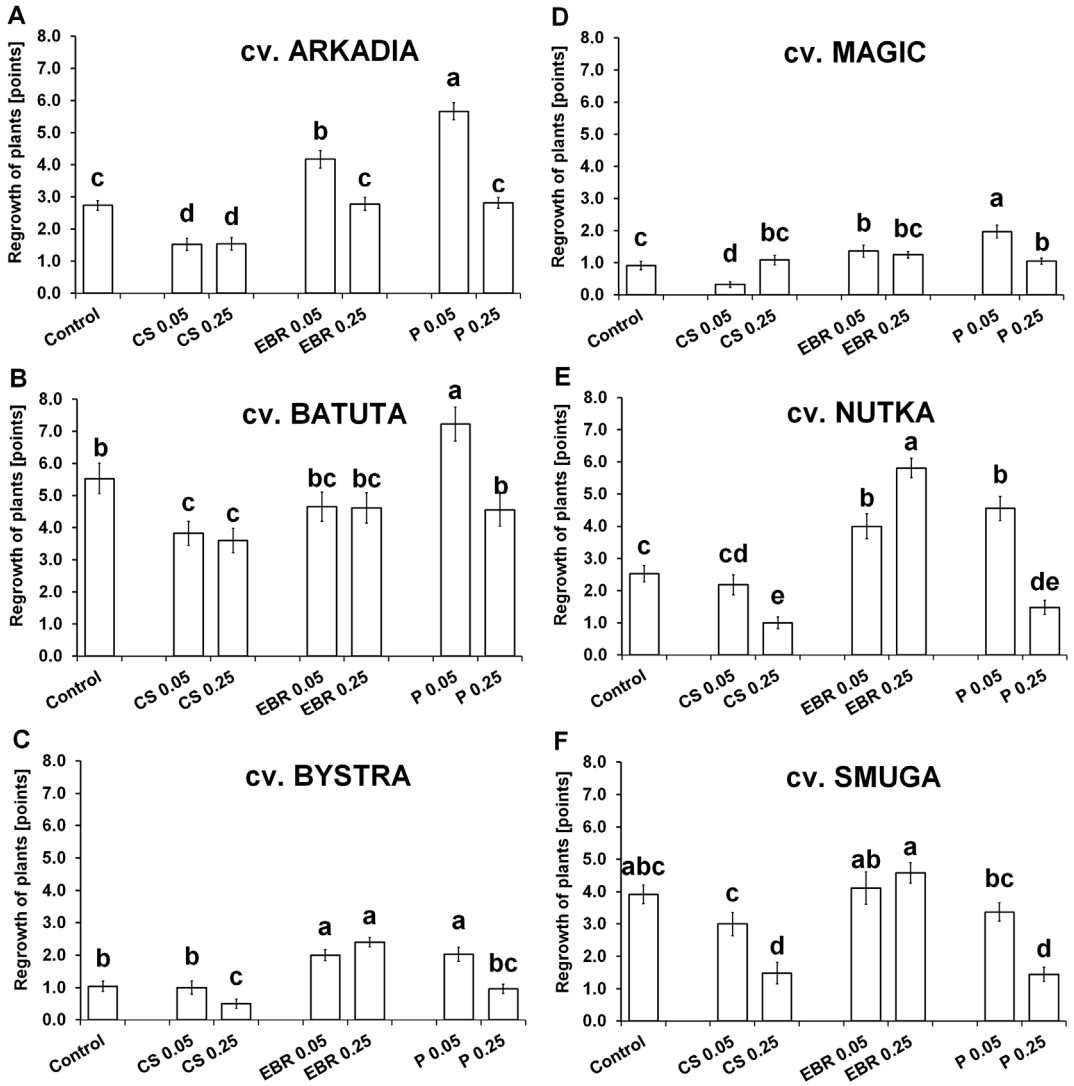

Fig. 4. Frost tolerance (expressed as plant regrowth after frost) of six cultivars of winter wheat that had been pretreated with steroid regulators and cold hardened (A–F); CS – castasterone, EBR – 24-epibrassinolide, P – progesterone in concentrations 0.05 and 0.25 mg l^{-1}. *Values (± SE) marked with the same letters are not significantly different according to the Duncan test ($P \leq 0.05$).*

cytoplasm of cv. Kobiera after eight weeks of growth at cold (Janeczko et al., 2008).

We did not find any connection between the level of frost tolerance and the concentration of progesterone binding sites in cold-hardened (nor in the not cold-hardened) winter wheat cultivars.

4.3. The effect of brassinosteroids and progesterone on frost tolerance of winter wheat

As was mentioned in the Methods, according to COBORU, the winter wheat cv. Smuga and cv. Batuta were classified as having a high winter survival rate, cv. Arkadia and cv. Nutka as having rather moderate winter survival rate while cv. Bystra and cv. Magic as having low winter survival rate. Although frost tolerance is only part of winter survival, the data obtained in our experiment are in agreement with the data from COBORU. Cv. Smuga and Batuta showed the best regrowth after frost while Bystra and Magic showed the weakest (Fig. 4 A-F, Fig. 5A–H). Data confirmed also earlier author's findings, although the regrowth of plants in experiment Janeczko et al. (2018b) was respectivelly higher for tested cultivars (Bystra, Smuga and Nutka) than observed in current studies, probably due to little different experimental model.

Pretreatment with steroids changed the frost tolerance partly in a cultivar-, concentration- and steroid structure-dependent manner (Fig. 4A–F). Brassinosteroid castasterone generally lowered the frost tolerance or did not change it at all. The most improved frost tolerance was observed for the 24-epibrassinolide. However, the effect of the hormone was not observed in the highly frost-tolerant cultivars (cv. Smuga and cv. Batuta). Four other cultivars reacted well to EBR. EBR at a lower concentration (0.05 mg l^{-1}) improved the frost tolerance in cv. Bystra, cv. Magic, cv. Nutka and cv. Arkadia, while at a higher concentration, it also improved in cv. Bystra and cv. Nutka. In these two cultivars, EBR improved the frost tolerance from 30% to more then 50% compared to the EBR-untreated controls. Improvement of frost tolerance by brassinosteroid (24-epibrassinolide) is in agreement with our earlier findings for winter rye and perennial ryegrass (Pociecha et al., 2016, 2017). Observed a decrease in frost tolerance by the second BR - castasterone - indicates that concentration of CS could be not well fitted. Simultaneously, it may indicate the chemical structure of BR is also important here for the physiological effect. The structure-activity dependency of BR is well known. The 7-oxalactone types of BR are considered to be more active than the BR without this oxygen bridge in the B-ring. The CS molecule lacks the oxygen that is inserted in ring B while EBR is a 7-oxalactone type steroid (Fig. 1). The second difference

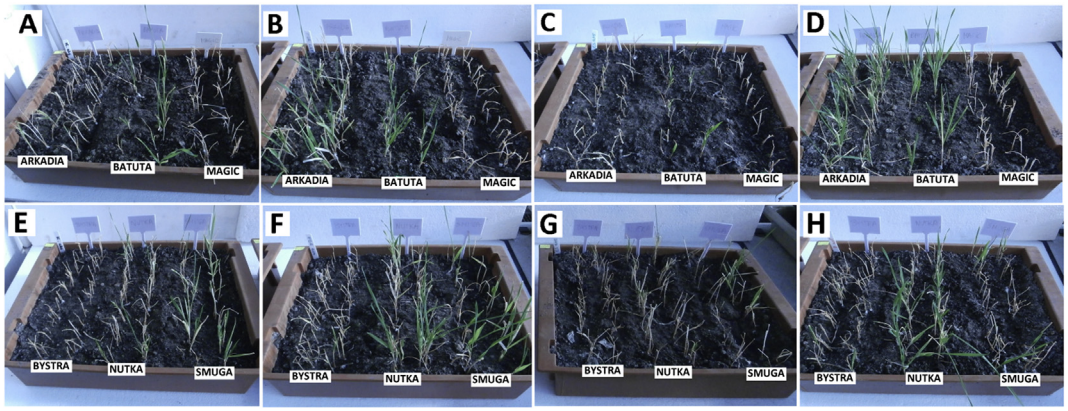

Fig. 5. Exemplary photographs illustrating the positive effect of the steroid regulators 24-epibrassinolide and progesterone and the negative effects of castasterone on the regrowth of six cultivars of winter wheat after they had been exposed to frost (−12 °C). A,E − control; B,F – plants that had been pretreated with 24-epibrassinolide (0.05 mg l^{-1}); C,G – plants that had been pretreated with castasterone (0.05 mg l^{-1}); D,H – plants that had been pretreated with progesterone (0.05 mg l^{-1}).

is the spatial orientation of the methyl group that is attached to C-17. The BR structure is important for their interaction with receptors and signalling. The role of BR-signalling in the process of cold hardening and frost tolerance was confirmed by a study on the model plant - *Arabidopsis thaliana* L. mutants with impaired BR-signalling (Eremina et al., 2016). Compared to the wild type, the BR-hypersignalling line *35S:BRI1-GFP (BRI 1oe)* mutants demonstrated a higher frost tolerance. In turn, *bri1-301* and *bri1-1* mutants with disturbances in BR-signalling exhibited a drastically reduced frost tolerance. Moreover, the BR-hypersignalling *A. thaliana* mutant (*BRI1oe*) had less ion leakage (membrane permeability) after freezing at −10 °C (6 h) than the wild type (Eremina et al., 2016). In another studies, the *A. thaliana bri1-116* (−/−) mutant (BR insensitive null allele of the BR receptor BRI1) had about a 50% higher ion leakage than the Col-0 wild type after 24 h exposure to 0 °C (Qu et al., 2011). This brings us to a possible second mode of BR action, which could be the direct impact of BR on the cell membranes. Changes in the cell membrane structure and its physicochemical properties are a crucial part of plant acclimation to low temperatures. An increase in the fluidity of membranes as a result of an increase in the content of unsaturated fatty acids or the accumulation of selected sterols in membranes is a well-known mechanism of plant acclimation to low temperatures. A higher degree of membrane fluidity limits low temperature-induced cell membrane damage. We suspect that the phenomenon of the BR interaction with cell membranes that leads to their higher degree of fluidity (in a manner similar to sterols) results in a better tolerance to low temperatures may have occurred in these experiment. Especially that our simultaneous studies confirmed accumulation of endogenous brassinosteroids in chloroplasts of wheat (Filek et al., 2019). To get more details about this phenomenon, we performed experiment 4 the results of which are discussed in a further part of this work.

Third tested steroid – progesterone improved frost tolerance if applied at lower concentration (0.05 mg l^{-1}). A higher concentration of progesterone (0.25 mg l^{-1}) had no effect or even lowered the frost tolerance. Th activity of progesterone in the alleviation of cold stress in maize was confirmed earlier (Erdal and Genisel, 2016). Progesterone also improved the chilling tolerance of chickpea seedlings and lowered the freezing point from −4 to −5.5 °C (Genisel et al., 2013). The plants that had been exposed to cold and treated with progesterone manifested less damage of the cell membranes than the cold-treated control (Genisel et al., 2013). Our experiment showed the protective activity of progesterone on the winter wheat plants exposed to frost. The effect of progesterone was only slightly dependent on the cultivar but the steroid was more effective at a low concentration. We suspect that the effect of progesterone was the result of the stabilisation of the cell membranes during the hardening process, which was one of reasons for the smaller injuries of the frost exposed plants manifested by their better regrowth. In experiment 4, in model studies, we verified the effect of progesterone on cell membranes using Langmuir bath studies of the interaction of progesterone with the lipid monolayers that had been isolated from different wheat cultivars growing at 20 and 5 °C.

4.4. Physiological/biochemical activity of steroid regulators during cold hardening of winter wheat

4.4.1. Regulation of CO_2 assimilation and sugar production

The accumulation of sugars during cold hardening is a well-known process for osmoprotective purposes and to build up reserves. Carbohydrate metabolism in plants is regulated by hormones including BR. BR-deficient barley mutants accumulated less sucrose (Janeczko et al., 2016). The application of BR stimulated the production pf soluble carbohydrates during the cold hardening of winter rye (Pociecha et al., 2016) and perennial ryegrass in which the most abundant sugar was sucrose (Pociecha et al., 2017). BR stimulate the activity of the enzymes that are engaged in sucrose biosynthesis (Yu et al., 2004). Moreover, progesterone also stimulates sugar production in plants (Erdal and Dumlupinar, 2011). Our results regarding accumulation of transcripts of small subunit of Rubisco, Rubisco activase, Rubisco protein accumulation and activity as well as sugar accumulation are presented on Fig. 6 A-J. The inhibitors of BR and progesterone biosynthesis unexpectedly did not change sugar accumulation in cold conditions but the additional supplementation with EBR and progesterone (treatments BRZ + EBR and TRIL + P) increased the accumulation of soluble sugars. This was accompanied by a decrease in the Rubisco activity (Fig. 6 G and H), which is known as the phenomenon of “feedback inhibition” and was observed earlier in plants (Roh and Choi, 2004). It is still unknown if the effect of the Rubisco decrease was temporary or not. In our previous studies on a frost-tolerant cultivar of perennial ryegrass, the Rubisco activity was lower after EBR pre-treatment and after three weeks of cold hardening but increased after six weeks of cold hardening compared to the untreated plants (Pociecha et al., 2017).

The concentrations of carbohydrates may affect the expression of photosynthetic genes such as *Rbc*S. A decrease of sugars may lead to the activation of gene expression and an increase in the photosynthetic capacity, and conversely, the subsequent increase in sugar concentrations in the leaves triggers the repression of photosynthesis (Krapp et al., 1993; Van Oosten and Besford, 1994, 1995). The latter situation may occur when the output exceeds the plant's capacity to metabolise or export sugars. The different genes that are involved in photosynthesis can be however regulated in different ways. For example, supplying

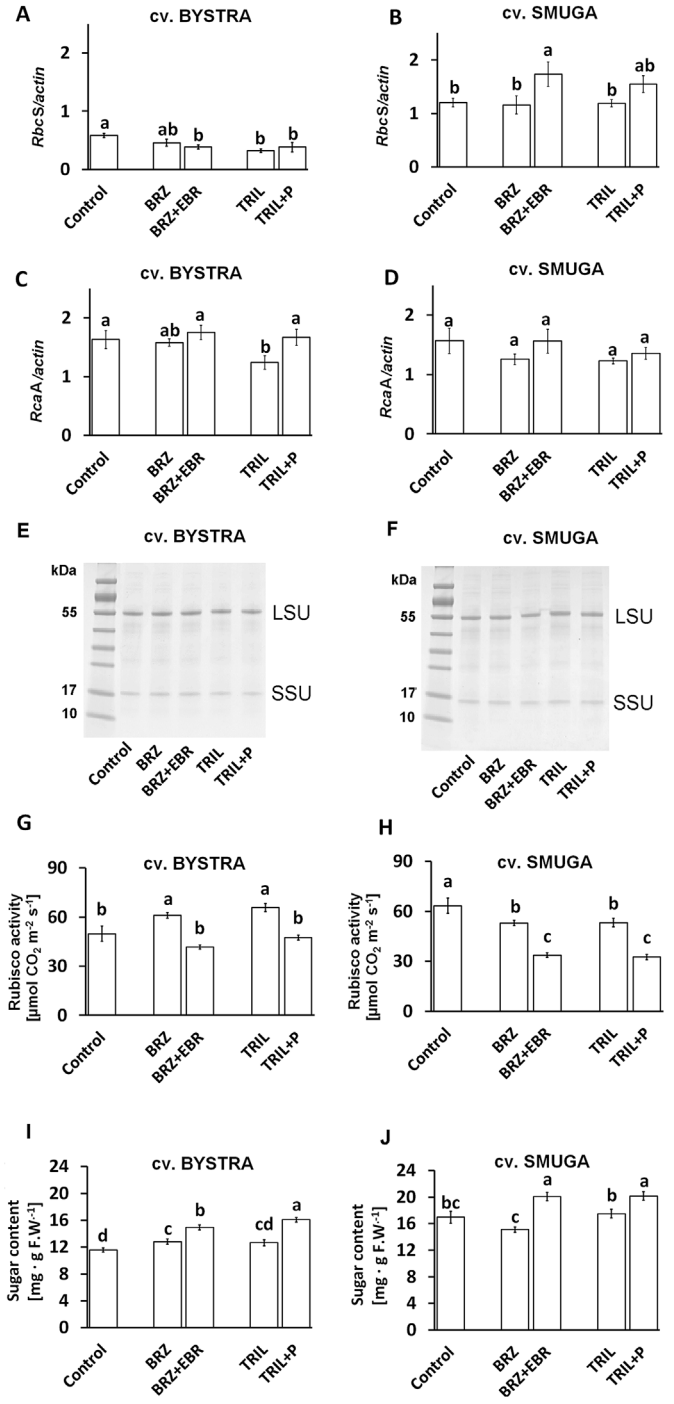

Fig. 6. Accumulation of the transcripts of the small subunit of Rubisco (*Rbc*S; A, B) and Rubisco activase (*Rca*A; C, D), accumulation of Rubisco small (SSU) and large (LSU) protein subunits (E, F), Rubisco activity (G, H) and soluble sugar content (I, J) in two cold-hardened cultivars of winter wheat (cv. Bystra, cv. Smuga) with an induced deficiency of brassinosteroids and progesterone and after supplementation with these regulators. Figs E and F: visualisation of LSU and SSU by Coomassie staining after SDS PAGE of the winter wheat leaf soluble extracts; exemplary gels. BRZ – brassinazole (brassinosteroid biosynthesis inhibitor treated plants), EBR – 24-epibrassinolide supplementation, TRIL – trilostane (progesterone biosynthesis inhibitor treated plants), P – progesterone supplementation. *Values (± SE) on figures A-D and G-J marked with the same letters are not significantly different according to the Duncan test (P ≤ 0.05).*

the leaf tissue with sucrose or glucose triggered a reduction in the abundance of *Rbc*S mRNA, but not *Rbc*L (Van Oosten and Besford, 1994). The phenomenon of the sugar-mediated regulation of *Rbc*S or *Rca*A might have been expected in our experiment, but the picture of obtained transcript accumulation pattern was not clear. There were cultivar-dependent differences in the accumulation of the transcripts of *Rbc*S or *Rca*A under the impact of BRZ and TRIL as well as BRZ + EBR and TRIL + P (Fig. 6A–D). Generally, it looks like the aforementioned "feedback inhibition" had little to do there and the observed effect was more likely a direct effect of EBR or progesterone (in treatments BRZ + EBR, TRIL + P) on the increased transcript accumulation of *Rbc*S in cv. Smuga or *Rca*A in cv. Bystra. BR-regulated higher accumulation of the transcripts of Rubisco or the Rubisco activase genes was already observed by Xia et al. (2009). There is however no data about the role of progesterone in this process so our results provide the first information. The results are especially interesting for cv. Bystra. Progesterone biosynthesis inhibitor decreased the accumulation of *Rca*A while supplementation with the steroid alleviated the effect, which may suggest a putative role of progesterone in the regulation of the expression of activase Rubisco in cold-hardened frost sensitive winter wheat cv. Bystra. Since only a statistically insignificant tendency was observed in frost tolerant cv. Smuga, caution is required for more general conclusions or the process is somehow connected to level of frost tolerance.

All of the changes in the *Rbc*S transcript accumulation that were observed in the plants were not accompanied by an accumulation of the small subunit of Rubisco (SSU), which may suggest the influence of posttranscriptional silencing as one of the regulatory mechanisms. The content of SSU (similarly as LSU) remained at the same level in all of the tested plants (Fig. 6 E and F).

To conclude, it appears that the model with to use of inhibitors did not allow a very clear explanation of the BR and progesterone mode of action on widely understood photosynthesis and sugar production during the process of cold hardening of winter wheat and the consequences for frost tolerance. We should however remember that used inhibitors do not reduce steroids to zero level and that there is still presence of endogenous steroids in plants treated with brassinozole or trilostane. As mentioned in Method part, brassinazole lowers BR content only by about 10 percent in cereals (Janeczko and Oklestkova, unpublished data) while trilostane lowers progesterone level about 50 percent (Janeczko et al., 2015). The transcript accumulation of *Rca*A and *Rbc*S and protein accumulation under EBR and progesterone treatment (treatments BRZ + EBR and TRIL + P) seems to be regulated at different levels. Although the genes that are involved in photosynthesis are most frequently regulated at the level of transcript accumulation, they are also regulated at the translation level (Pego et al., 2000). For the carbon metabolite-mediated mechanism of regulation of photosynthesis by EBR and progesterone, we can only say that the supplementation with these steroids to the plants of both cultivars (treatments BRZ + EBR and TRIL + P) did result in an increased content of sugar, which was accompanied by a decreased activity of Rubisco the most probably *via* "feedback inhibition".

4.4.2. Activity of SOD as an element of antyoxidant system

Cold is one of the factors that causes the overproduction of reactive oxygen species (ROS) and oxidative stress, among others, *via* disturbances in the metabolic processes occurred in different organelle (Sharma et al., 2012). The overproduction of ROS may cause lipid peroxidation (membrane injuries), and protein or DNA damage leading to the cell death. Plant cells respond to oxidative stress by changes in the activities of antioxidant defense system. SOD is one of the enzymatic antioxidants playing a major role in the scavenging of ROS. It belongs to the group of metalloenzymes and its function consist in the removal of superoxide radicals by forming O_2 and H_2O. This enzyme is also known to participate in regulation of various stress associated genes expression by manipulating the accumulation of H_2O_2. It has been already shown that in wheat, an increase in the SOD transcript and enzyme activity could be noted under different factors of abiotic stresses among them cold stress (Caverzan et al., 2016; Kumar et al.,

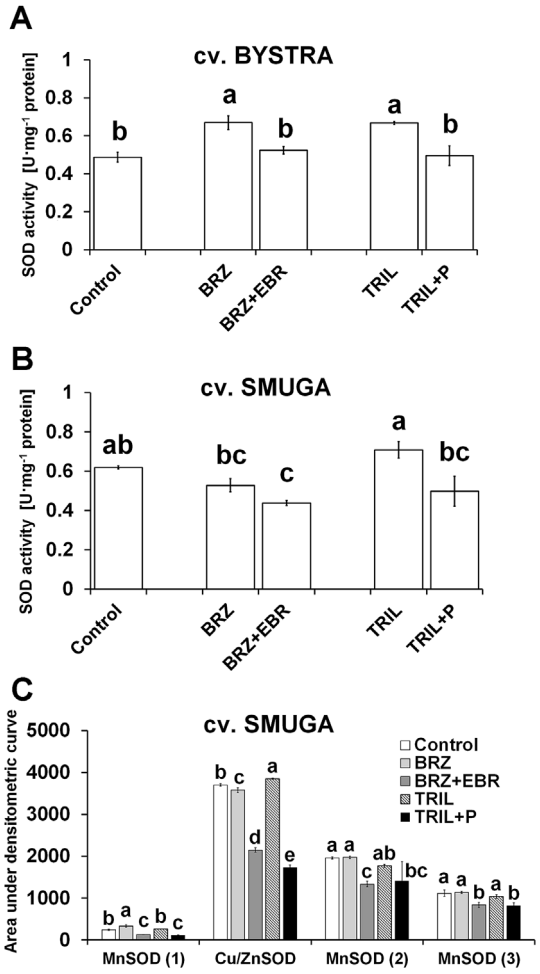

Fig. 7. Total activity of superoxide dismutase (SOD) that was measured spectrophotometrically in the leaf extracts of the cold-hardened winter wheat cv. Bystra (A) and cv. Smuga (B) with an induced deficiency of brassinosteroids and progesterone and after supplementation with these regulators. (C) Activity of particular SOD isoforms after the densitometric analyses of bands corresponding with SOD isoforms visualised on polyacrylamide gels in cv. Smuga with an induced deficiency of brassinosteroids and progesterone and after supplementation with these regulators. BRZ – brassinazole (brassinosteroid biosynthesis inhibitor treated plants), EBR – 24-epibrassinolide supplementation, TRIL – trilostane (progesterone biosynthesis inhibitor treated plants), P – progesterone supplementation. The activity of SOD isoforms in Figure C is expressed in arbitrary units – area under the densitometric curve. SOD from bovine erythrocytes (6.615 units·mg^{-1} protein) was treated as the reference. *Values (± SE) marked with the same letters are not significantly different according to the Duncan test ($P \leq 0.05$); figure C – separate statistical analyses for each isoform.*

2013). There are many factors that regulate the activity of antioxidant enzymes and steroid regulators may be among them. In our experiment, SOD activities were measured in wheat after three weeks of cold hardening. Plants treated before cold hardening with the steroid biosynthesis inhibitors (brassinazole and trilostane) usually had an increased SOD activity (Fig. 7 A and B). The exception was the BRZ-treated cv. Smuga in which no changes were found (Fig. 7 B). Supplementation with EBR and progesterone (BRZ + EBR and TRIL + P treated plants) reversed this effect. Detailed studies of the activity of particular isoforms generally confirmed the phenomenon (Fig. 7 C). SOD isoforms possessed different subcellular localization: MnSOD is found in the mitochondria and peroxisomes, FeSOD mainly in chloroplast while Cu/ZnSOD is associated with cytosol, peroxisomes, chloroplasts, mitochondria and apopolast. There are several data published so far concerning the identification of SOD isoforms in wheat (Huseynova et al., 2014; Leonowicz et al., 2018). In all of them FeSOD isoform was not detected. Similarly, in our studies we found the activity of three MnSOD (1-3) isoforms and one CuZnSOD while FeSOD was not identified (Fig. 7 C). The activity of MnSOD (1) increased with the use of BRZ compared to the control. Supplementation with EBR (BRZ + EBR) not only alleviated the effect but even further decreased the activity of the enzyme compared the control. No effect of the treatments was observed for MnSOD (2,3). The activity of Cu/ZnSOD was slightly decreased by BRZ and significantly (by about 50%) decreased in the plants that had been treated with BRZ + EBR. The use of TRIL resulted in an increased activity of CuZnSOD isoform when compared to the control while progesterone supplementation had an inhibitory effect on all isoforms identified in leaves of plants from cv. Smuga.

The increased activity of antioxidant enzymes is usually interpreted as a plant's reaction to the overproduction of ROS and the increased expression of many antioxidant enzymes is positively correlated with higher tolerance against abiotic stresses (Caverzan et al., 2016). According to our results, both EBR and progesterone seem to participate in regulating the antioxidant system in winter wheat during cold acclimation. Their deficit, which was induced in our experiment, in most cases caused plants to maintain higher activities of SOD (an especially clear picture was in cv. Bystra) until the end of the cold treatment. A mechanism of the influence of steroid regulators (for BR) on antioxidant enzymes was proposed by Zhang et al. (2010). According to the authors, the application of BR induces H_2O_2 production, which then activates ZmMPK5 (a mitogen-activated protein kinase). The kinase is involved in the self-propagation of apoplastic H_2O_2 by regulating the NADPH oxidase gene expression in the BR-induced antioxidant defense systems. The phenomenon, if it occurs in non-stress conditions (in our case steroids were applied before cold hardening), should immunise plants and make them better prepared for stress conditions. At the same time, a deficiency in the steroid content, which is induced by the exposure of plants to steroid biosynthesis inhibitors before stress, might cause some difficulties in maintaining the proper redox balance in cells that are exposed to cold, which was reflected in the still elevated activity of SOD after three weeks of cold hardening.

4.4.3. Plant stress hormone ABA

Plant hormones act in the connection network by controlling their own biosynthesis or transport. BR also participate in this network but little is known about the role of BR in controlling the level of other hormones in conditions of cold hardening. BR increase the accumulation of ABA at both low and high temperature, but studies have only been conducted in cell cultures. *Chorispora bungeana* cell cultures showed increased ABA content at temperature 0 °C and 4 °C compared to cultures at 25 °C, but cells that were cultured with 24-epibrassinolide accumulated twice as much ABA (Liu et al., 2011). Interestingly, barley BR biosynthesis mutants were characterised by a lower ABA content at a temperature of 20 °C (Janeczko et al., 2016). In our present studies on winter wheat, the content of ABA in cv. Bystra and cv. Smuga were very similar, 108 and 105 ng g D.W.$^{-1}$ (Fig. 8 A and B), which is on similar level as in our earlier findings (Janeczko et al., 2018b). No significant impact of the application of the inhibitors or supplementation with EBR and progesterone on the ABA content were observed in either cultivar.

4.5. Physicochemical properties of the membranes of winter wheat are modulated by steroid regulators (brassinosteroid, progesterone)

Examples of π vs the A isotherms for the studied monolayers are presented in Fig. 1 - supplementary materials. The course of the isotherms of both wheat cultivars that were obtained for the pure lipid monolayers on the water sub-phase indicated the formation of a liquid-expanding organization of the molecules. However, the isotherms of the plants that had been grown at 20 °C showed a steeper slope than those

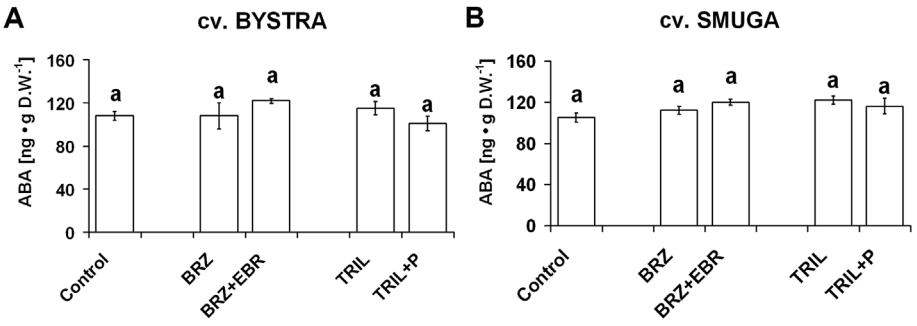

Fig. 8. ABA content in the leaf extracts of cold-hardened winter wheat cv. Bystra and cv. Smuga (A,B respectively) with an induced deficiency of brassinosteroids and progesterone and after supplementation with these regulators; BRZ – brassinazole (brassinosteroid biosynthesis inhibitor treated plants), EBR – 24-epibrassinolide supplementation, TRIL – trilostane (progesterone biosynthesis inhibitor treated plants), P – progesterone supplementation.

Table 2
The effect of 24-epibrassinolide (EBR) and progesterone (P) on changes the surface parameters of monolayers of plasmalemma lipids from leaves of seedlings of winter wheat (cv. Bystra and cv. Smuga) grown at 20 °C and 5 °C and calculated as the difference between values obtained in the steroid presence and the control (without steroids). A_{lim} – the limiting area per one molecule; π_{col} – collapse pressure; Cs^{-1} – maximal values of compression modulus. *Means values (± SE) marked with the same letters (separately for each steroid and temperature of growth) are not different according to Students's test (P ≤ 0.05).*

Wheat cultivar	Steroid applied	Surface parameters of monolayers modified by steroid presence					
		ΔA_{lim} [Å^2]		$\Delta\pi_{col}$ [mN/m]		ΔCs^{-1} [mN/m]	
		20 °C	5 °C	20 °C	5 °C	20 °C	5 °C
Bystra	EBR	0.8 ± 0.1^b	1.4 ± 0.1^b	0.1 ± 0.1^b	0.1 ± 0.1^b	7.3 ± 0.2^b	1.4 ± 0.1^b
Smuga	EBR	4.5 ± 0.2^a	5.9 ± 0.3^a	1.1 ± 0.1^a	0.5 ± 0.1^a	11.3 ± 0.3^a	9.0 ± 0.3^a
Bystra	P	2.0 ± 0.1^b	3.4 ± 0.2^a	0.1 ± 0.1^b	0.2 ± 0.1^a	3.5 ± 0.1^b	2.4 ± 0.1^b
Smuga	P	2.8 ± 0.1^a	2.6 ± 0.2^b	0.9 ± 0.1^a	0.3 ± 0.1^a	9.5 ± 0.2^a	5.3 ± 0.2^a

that were recorded at 5 °C, especially in the region of higher surface pressure, i.e. when the lipids were compressed into more condensed monolayers. The extrapolation of the steepest linear part of the isotherm at the end of compression to zero pressure allows a conclusion about the limiting area that is occupied by molecules in the monolayer to be drawn (A_{lim}) (Gaines, 1966). Thus, plants from lower temperature demonstrated a higher A_{lim} in the monolayers. The occurrence of a plateau at the π/A isotherms in the most condensed areas (close to 0) indicates the highest possible monolayer packing. Lower values of the maximum surface pressure (π_{col}) that characterised the plants that had been grown at 5 °C, but only very small differences connected with the presence of the steroid, were observed. Calculation of compressibility modulus ($Cs^{-1} = -\ d\pi/d\ln A$), provides information about the monolayer state (Davies and Rideal, 1963). Higher values of Cs^{-1} indicate that more condensed monolayers are obtained. Therefore, in the presented experiments, pure lipids generally formed the condensed monolayers and the interactions with EBR and progesterone resulted a weaker condensation of the monolayers. The detailed calculations of changes in the monolayer properties connected with the interaction of lipids with the studied steroids are presented at Table 2. Both of the studied steroids affected the increase of the distance between the molecules in the monolayers. A stronger effect (ΔA_{lim}) was observed for the plants that had been cultured at a lower temperature. EBR revealed larger changes in the Smuga monolayers, whereas progesterone action gave a similar increase of A_{lim} in both of the cultivars, but was slightly higher in Bystra at 5 °C. Differences in π_{col} between the pure monolayers and those that had been modified in the presence of steroids were very small and were usually not statistically significant. More evident dependences were found for the Cs^{-1} parameter. For both of the studied plants – larger changes were found when they were cultured at 20 °C rather than at 5 °C. The increase of ΔCs^{-1} parameter was especially evident for Smuga, and in the case of both steroids that were used.

The use of model lipid monolayers to characterise the interactions of molecules with biomembranes is more and more commonly used, especially in studies of the direct influence of selected substances on the membrane properties that are modified by growth conditions (Dufourc, 2008; Rudolphi-Skorska and Sieprawska, 2016). A precise description of the dependence between the structure of the monolayers that are prepared from separate lipids fractions (PL, MGDG and DGDG) and the diversity in the chemical properties of hormones was shown in our earlier studies (Filek et al., 2017). In these experiments, we concentrated on the possibility of interactions of EBR and progesterone with the polar lipids that are present in the plasmalemma, which is the membrane that is especially responsible for the transport of substances into/from the cells. In spite of the findings that the protein receptors and channels, which are specialised for the translocation of molecules between the external environment and the cytoplasm, the chemical structure of some compounds suggests the possibility of their direct transport through the lipid layer. The results that were obtained indicate that such an effect may accompany the interaction of EBR and progesterone with the plasmalemma of the studied wheat cultivars. The increase of the A_{lim} of the monolayers, regardless of the presence of EBR and progesterone (the hormones that interact with lipid membranes in different ways; mainly with the polar part of the membrane (progesterone) or with the hydrophobic part (EBR)), which was calculated as ΔA_{lim}, may confirm that both steroids are located in the membranes. Moreover, the increase of the ΔCs^{-1} values, which is a parameter that indicates the differences in membrane compressibility (resulting from stronger interactions between molecules in monolayers), indicates that both steroids are incorporated into the lipid layer and, like cholesterol, stabilise the interaction between the lipids - which may be important step for better frost tolerance (Dufourc, 2008). The greater changes of these parameters that are registered for EBR are presumably connected with the more hydrophobic structure of this substance, which provides the opportunity to localise in the vicinity of the fatty acids and to stabilise the structure of the membrane *via* van der Waals bindings. Such relationships may also explain the generally larger values of all of the parameters that were obtained for cv. Smuga, as the lipids of this cultivar are characterised by a higher fatty acid unsaturation than cv. Bystra (Filek et al., 2017), thereby allowing the better penetration and localization of the membranes by substances containing hydrophobic groups. The findings that the Smuga cultivar accumulates higher amounts of both steroids than cv. Bystra (Figs. 2 and 3A) confirm this

conclusion. Moreover, some of the similarities are also connected with the registered differentiation of concentration of steroids in the plants that had been cultivated at various temperatures as well as the modification of the structure of the membranes in the presence of these steroids (especially, the specific lipid domains)., sterols accumulated in lipid layers are included into the formation of liquid-ordered spaces in membranes (lipid "rafts") that are necessary for the course of important physiological processes such as signal transduction, asymmetric growth, cellular sorting, infection and temperature shocks (Beck et al., 2007). Thus, it may be concluded that the physicochemical properties of the plasma membranes (which to some extent stimulates the interaction with EBR and progesterone) are also responsible for the transport of these steroids through the plasma membranes.

Finally, separate problem is possible effect of tested steroids on fatty acid composition in membrane lipids, which can influence membrane properties. Cold hardening increased of participation of unsaturated fatty acids in membrane lipids of steroid-untreated cultivars Bystra, Nutka and Smuga (Filek et al., 2017). In present experiment we did not check the impact of steroids (BR, P) on composition of membrane lipids. Interestingly however in our earlier studies (Janeczko et al., 2009) we showed that in oilseed rape callus tissue BR application modified fatty acids composition of galactolipids and phospholipids in dependency on temperature (20 °C or cold). Changes of fatty acid content under BR treatment were also reported by (Li et al., 2012) for mango fruits stored at 5 °C. Level of unsaturated fatty acids in BR treated fruits was higher than in untreated fruits, simultaneously content of saturated fatty acids was lower after BR application. It can not be ruled out that some effect of BR or P on fatty acids composition of tested wheat (and additional increase of membrane fluidity) could also be one of reasons of increased tolerance to frost after application of these steroids. As for effect of steroids on membrane sterol composition (which is also important for membrane fluidity) in cold hardened plants, the effect of BR or P is not known but may be interesting direction for further studies.

5. Concluding remarks

Since frost is one of the major stress factors that causes damage to winter crops in Poland and in countries with similar climate conditions, studies on the mechanisms of frost tolerance are useful for breeding new cultivars that have a better tolerance to this stress factor. These studies also allow new regulators to be discovered, which can then be used for crop protection as agrochemicals to improve frost tolerance. The experiments performed in our research provided some new theoretical and practical information about role of steroid regulators in the frost tolerance mechanisms of winter wheat. Among results obtained in our studies the most important we can consider the following: (1) 24-Epibrassinolide (0.05 and 0.25 mg l^{-1}) increased the tolerance of winter wheat to frost while progesterone was effective only in the lower concentration. (2) During cold hardening the endogenous BR concentration increased, while progesterone or progesterone binding sites decreased. A higher amount of endogenous BR was accompanied by a higher level of frost tolerance of winter wheat. (3) The use of the inhibitors of brassinosteroid and progesterone biosynthesis did not allow a very clear explanation of the mode of action of brassinosteroid or progesterone on the widely understood photosynthesis and sugar production. 24-Epibrassinolide and progesterone seemed to regulate the activity of the SOD enzyme in winter wheat during cold hardening. The content of ABA was not affected by the steroid biosynthesis inhibitors or by the supplementation of plants with progesterone or 24-epibrassinolide. (4) Model studies on Langmuir bath suggested that 24-epibrassinolide and progesterone were incorporated into the lipid layer and might stabilise the interaction between lipids which could be important step for better frost tolerance.

Author contributions

Anna Janeczko – was author of the experiment idea, designed experiments and choose all other scientists to cooperation, extracted and purified samples to analysis of steroids, analysed and interpreted data, wrote of manuscript; Ewa Pociecha – made frost tests, measured Rubisco activity and SOD activity, Michał Dziurka – measured ABA content, Barbara Jurczyk – measured accumulation of transcripts, Marta Libik-Konieczny – made visualisation and identification of SOD isoforms on polyacrylamide gels, Jana Oklestkova and Ondrej Novak measured steroids on UHPLC-MS/MS, Maria Pilarska – measured accumulation of Rubisco, Maria Filek and Elżbieta Rudolphi-Skórska - made Langmuir bath studies, interpreted and discussed this data, Iwona Sadura - helped in measurements of SOD activity, Agata Siwek – made studies of radioligand binding.

Acknowledgment

These studies were financed by the National Science Centre (Poland) (project 2013/09/B/NZ9/01653). UHPLC-MS/MS analyses of endogenous steroids were additionally financed by project of the Ministry of Education, Youth and Sports CR,European Regional Development Fund-Project "Plants as a tool for sustainable global development" no. CZ.02.1.01/0.0/0.0/16_019/0000827.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.020.

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