

# Brassinosteroids increase winter survival of winter rye (*Secale cereale* L.) by affecting photosynthetic capacity and carbohydrate metabolism during the cold acclimation process

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**Abstract** The aim of the study was to verify the hypothesis that brassinosteroids would modify the cold acclimation process through the stimulation of photosynthesis and carbohydrate metabolism, and would consequently cause an increase in the plant's capacity for winter survival. Seedlings of two winter rye cultivars (winter-resistant and moderately winter-resistant types) were sprayed with 24-epibrassinolide (EBR) and then subjected to cold acclimation at 4 °C. The foliar hormone application was studied for its effects on two components of winter resistance: tolerance to frost; and resistance to snow mould. These were examined after medium (3 weeks) and long (6 weeks) terms of cold acclimation. Additionally, as a control group, the level of endogenous brassinosteroids was measured in non-treated plants. The maximum acquired snow mould resistance was observed in the EBR-treated plants after 3 weeks of cold acclimation, while the maximum frost tolerance was recorded after 6 weeks of cold acclimation, as compared with control. In the winter-resistant cultivars, the EBR-induced snow mould resistance coincided with a lower photosynthetic efficiency and a lack of change in their Rubisco activity and level of total soluble carbohydrates. The elevated EBR-induced frost

tolerance in the winter-resistant cultivars was associated with a carbohydrate metabolism that promoted a decrease in the monosaccharides of the leaves that stimulate photosynthetic efficiency and Rubisco activity. For both cultivars, the control plants developed frost tolerance between the third and sixth week of cold acclimation. However, the winter-resistant cultivar was characterised by a constant castasterone (CS) level, while the moderately winter-resistant one experienced a sharp increase in its endogenous CS after 3 weeks, and then after 6 weeks of cold acclimation.

**Keywords** Chlorophyll *a* fluorescence · Cold acclimation · Frost tolerance · Fructooligosaccharides · *Microdochium nivale* · Rubisco activity

## Introduction

Winter rye is one of the major cereals grown in Central and Eastern Europe, as it has low soil and water requirements and is resistant to frost. However, this crop is not sufficiently resistant to snow mould—a disease that affects many species of forage and cereal grasses. In Poland, snow mould is caused by a widespread fungal pathogen *Microdochium nivale* (Fr.) Samuels and Hallett, which inflicts damage and leads to serious yield losses and a decrease in the yield quality.

To survive the abiotic and biotic stresses of winter, crops have developed a mechanism of cold acclimation that typically takes place during the autumn and early winter (Lindén et al. 1999). In the winter, rye's cold acclimation is accompanied by the plant's capacity to maintain or increase its photosynthesis levels and its accumulation of soluble carbohydrates (Huner et al. 1993). High levels of

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soluble carbohydrates due to the carbon fixation that occurs during cold acclimation are frequently correlated with the survival of overwintering plants (Hurry and Huner 1991; Öquist et al. 1993). The genes involved in the sink functions and growth are controlled by high carbohydrate levels; whereas low carbohydrate levels promote photosynthesis and the mobilisation of reserves (Van den Ende and El-Esawe 2014).

It has been demonstrated in many reports on winter cereals that frost tolerance is not correlated with snow mould resistance, even though some physiological processes might be common to both phenomena (Gaudet et al. 2003; Gaudet and Kozub 1991; Iriki et al. 2001). Some components of snow mould resistance might be activated prior to the cold acclimation process (Ergon and Tronsmo 2006); however, cold acclimation at low but not freezing temperature allows for the optimal conditions for the plant to acquiring the maximum snow mould resistance (Gaudet et al. 1999; Pocięcha et al. 2010, 2013). This cold-induced snow mould resistance is also dependant on the plant's growth stage, as early-seeded plants that have achieved an optimal size before winter begins seem to be more resistant to snow mould than late-seeded plants (Gaudet et al. 2001). Another aspect of a plant's snow mould resistance involves the quantity and quality of its soluble carbohydrates. Specifically, the plants that accumulate more fructans than soluble sugars exhibit a greater snow mould resistance, as the snow mould fungi are less effective at metabolising fructan polymers than simple sugars (Gaudet et al. 1999).

A plant's response to cold is a complex process regulated by phytohormones, the level of which change at different stages of the plant's acclimation. During an early phase of acclimation, that follows an alarm phase, there is a decrease in the plant's growth inhibiting phytohormones (ABA) and an increase in the growth promoting phytohormones, like auxins, cytokinins and gibberelins. Brassinosteroids are steroidal plant growth promoting regulators, which control complex metabolic pathways in the plants development and vegetative growth (Fridman and Savaldi-Goldstein 2013; Holá et al. 2010). Brassinosteroids are also very important in the plant's protection against heat stresses, oxidative stress, drought and salinity (Bajguz and Hayat 2009; Janeczko et al. 2011; Fahad et al. 2015) and may be involved in increasing resistance to biotic stresses by inducing the systemic resistance process (Nakashita et al. 2003). At high temperatures, brassinosteroids also affect the plant's carbohydrate metabolism and photosynthesis. In cucumber leaves treated with EBR an increase in the concentrations of soluble sugars and starches was accompanied by an enhanced activity of the carbohydrate metabolism enzymes. At the same time increase in the quantum yield of PSII and Rubisco activity has been observed (Yu et al. 2004). Moreover, in tomato leaves EBR

alleviated photoinhibition-induced decrease in photosynthesis (Ogweno et al. 2010).

As far as we know, there is little information regarding the effects of brassinosteroids during the cold acclimation process, in which qualitative and quantitative changes in the plant's carbohydrates and level of photosynthetic capacity are essential.

Therefore, the aim of the present study was to verify the hypothesis that EBR would modify the cold acclimation process through stimulating photosynthesis and influencing the carbohydrate metabolism, and thus would increase the plant's capacity for winter survival. An attempt was made to investigate the effect of EBR on two components of winter resistance: resistance to snow mould; and tolerance to frost. The EBR-induced changes in the winter rye were examined in non-cold acclimated plants, and in plants subjected to 3 or 6 weeks of acclimation at 4 °C. To achieve the study's aim, we focused specifically on: chlorophyll fluorescence transients; Rubisco activity; and the level and composition of the carbohydrates. We also described the changes in the endogenous brassinosteroid levels during the plant's reactions while undergoing cold acclimation. All of the analyses were performed on a winter-resistant Dańkowskie Żłote cultivar and a moderately winter-resistant Stach cultivar.

## Materials and methods

### Plant materials and the experimental design

Two cultivars of winter rye (*Secale cereale* L.) were selected for our experiments. According to the Polish Research Centre for Cultivar Testing (COBORU) (2011), the Dańkowskie Żłote cultivar is winter-resistant, while the Stach cultivar is moderately winter-resistant. The seeds were sown in pots (20 plants per 20 cm pot) containing a mixture of soil:peat:sand (2:2:1 v/v/v). The pots (5 pots per treatment) were then kept for 3 weeks in a greenhouse at 18 °C (day/night) in natural light conditions during September/October, at a location of approximately 50°03'N lat., 19°55'E long. The plants were also supplied with a light intensity of 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD (with AGRO Philips sodium lamps) for up to 12 h photoperiods, and were fertilised weekly with a Hoagland solution. At the three-leaf stage, the plants were cold acclimated for 3 or 6 weeks at 4 °C and underwent an 8 h photoperiod with a light intensity of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. Before the cold acclimation, the plants were sprayed with 0.25 mg  $\text{dm}^{-3}$  of EBR. The stock solution of EBR (EBR, Sigma-Aldrich, Poznań, Poland) was prepared in 96 % ethanol using an ultrawave bath, and was diluted with distilled water to obtain the final concentration. Two independent

experiments conducted in the years 2013 and 2014 were designed in an entirely random fashion, and the results presented below combine the data obtained from both of these experiments.

### Artificial inoculations with *M. nivale* and an evaluation of the regrowth index

After undergoing cold acclimation, the plants were inoculated with the *M. nivale* mycelium isolate No. 39 from *Secale cereale* seeds, provided by Prof. Maria Prończuk from the Plant Breeding and Acclimatization Institute in Radzików (Poland). The inoculum's preparation and the inoculation were performed as described by Prończuk and Prończuk (1987). After 4 weeks of incubation with the inoculum, the plants were then uncovered, and after cutting were allowed to re-grow for 10 days at a temperature of 12 °C. The regrowth was evaluated using an arbitrary visual rating system (scored from 0 to 5) where 0 meant a dead plant with no signs of leaf elongation, and 5 meant a healthy plant with no visible symptoms of infection. An Average Regrowth Index (ARI) was also calculated from these ratings according to the formula:  $[(n \times 0) + (n \times 1) + \dots + (n \times 5)] \times N^{-1}$ —where 'n' is the number of plants corresponding to each disease rating (0–5), and 'N' is the total number of observations. The average ARI was calculated from 50 plants and was evaluated based on data from the two independent experiments. Decreased values of the ARI represent a decrease in the plant's resistance to the pathogen.

### Tests of freezing tolerance

The freezing tolerance was estimated by a modified version of Larsen's method (1978). First, cold-acclimated plants in pots were transferred to a growth chamber at a temperature of 4 °C. Next, the temperature was reduced at a rate of 3 °C h<sup>-1</sup> to reach -14 °C in dark conditions for a period of 6 h. Then the temperature was increased at a rate of 3 °C h<sup>-1</sup> to reach 4 °C, and the conditions for the cold-acclimation were recreated. Twenty-four hours later all of the plants were cut, and were transferred to an environment for their regrowth of: +12 °C, a 12-h photoperiod, and 200 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD. After 10 days, the regrowth was estimated using Larsen's (1978) visual score, where: 0—a completely dead plant, with no sign of leaf elongation; 1—a dead plant but where some leaf elongation c. 5 mm has occurred; 2—a dead plant but the leaves were elongated by 1–2 cm before dying; 3—a plant that is dying, but the leaves have elongated to 2 cm or more; 4—a plant that may die, as it has been growing but the inner leaves are brown; 5—a plant that may survive but is badly damaged, as the regrowth is generally discoloured and curled; 6—a plant that has survived but shows severe damage to c. 50 %

of the leaves; 7—a plant that is alive but some symptoms of freezing injuries are visible (i.e. some leaves are discoloured or deformed); 8—a plant where only the tops of some inner leaves are discoloured or deformed; and 9—a plant with no symptoms of injury.

### Efficiency of the photosystem II

The efficiency of the photosystem II was estimated based on the chlorophyll fluorescence measurements using a Plant Efficiency Analyser (PEA) (Hansatech Ltd. King's Lynn, UK). The measurements were carried out after allowing the leaves to adapt to darkness for 30 min. The phenomenological energy fluxes were calculated as follows: energy absorption by the antennas (ABS/CS = F<sub>m</sub>); the energy flux for trappings (the energy transferred to a reaction centre) (TR<sub>0</sub>/CS = F<sub>v</sub>/F<sub>m</sub> (ABS/CS)); the energy flux for electron transports (ET<sub>0</sub>/CS = (F<sub>v</sub>/F<sub>m</sub>) (1 - V<sub>j</sub>) F<sub>m</sub>); and the energy dissipation (the energy lost as heat) (DI<sub>0</sub>/CS = (ABS/CS) - (TR<sub>0</sub>/CS)), where CS is the cross section of the sample (Strasser et al. 2000). Moreover, the following measurements were also extracted from the PEA: Ψ<sub>0</sub>—the quantum yield of the electron transports (ET/TR); φE<sub>0</sub>—the quantum yield of the photochemical reactions (ET/ABS); and RC/CS<sub>m</sub>—the amount of active reaction centres per excited CS.

The measurement of a leaf's PSII efficiency were performed in 10 replications (1 leaf per replication) on the youngest fully developed leaves in a non-cold acclimated plants and after 3 and 6 weeks of cold acclimation in 10 replications (1 leaf per replication).

### Biochemical analyses

The isolation and identification of the brassinosteroids was performed according to a modified protocol described by Janeczko et al. (2010) and Swaczynová et al. (2007). Briefly, the plant material (2 g of fresh weight) was homogenised in 20 ml of 80 % cold methanol. After centrifugation, the supernatant was enriched with deuterium labelled brassinosteroids (internal standards) and passed through Strata X columns (Phenomenex, Torrance, CA, USA). The bound fraction was eluted with acetonitrile and was then evaporated to dryness. The level of brassinosteroids was determined by an ultra-high performance liquid chromatography with a tandem mass spectrometry (UHPLC-MS/MS).

The youngest fully developed leaves were collected for the analyses of the enzyme activities and their carbohydrate composition after 3 and after 6 weeks of the cold acclimation. The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO EC 4.1.1.39) activity was determined as described by Sharkey et al. (1991). Carbohydrate composition was

measured using the HPLC method as described by Pocięcha and Dziurka (2015). The content of sugars such as glucose, fructose, sucrose, 1-kestose, and nystose was also determined. Each carbohydrate composition assay was repeated 3 times on bulk samples, each of which contained 3 leaves from different plants for each treatment.

### Statistical analysis

The ANOVA normality assumption was verified by the Shapiro–Wilk test to validate the use of parametric testing. Also, a Levene test was used to check the homoscedasticity assumption. The data was analysed with a multi-factor analysis of variance (MANOVA), graphs were plotted using means and standard errors for each data point. A post hoc comparison was conducted using Duncan's multiple range test ( $p = 0.05$ ). All of the calculations were carried out using the STATISTICA 12.0 (StatSoft Inc., USA) software package.

## Results

### Frost tolerance and pathogen resistance

EBR, when applied exogenously before a plant's cold acclimation, will significantly improve the possibility for winter survival by affecting two components, i.e. the plant's frost tolerance and its snow mould resistance (Table 1). The degree of this positive effect on the frost tolerance is dependent on the cultivar and the duration of the cold acclimation. An increase in the pathogen resistance was observed after 3 weeks of cold acclimation, and the greatest improvement in the frost tolerance was recorded after 6 weeks of cold acclimation.

Both the control and the EBR-treated plants that were cold acclimated for 6 weeks were incapable of maintaining

**Table 1** The frost tolerance and pathogen resistance of the control and the EBR-treated winter rye plants, after 3 and 6 weeks of cold acclimation

Weeks of cold acclimation	Dańkowskie Złote		Stach	
	3	6	3	6
Pathogen resistance (points in scale 0–5)				
Control	2.84b	2.49b	1.99b	1.43b
EBR	3.85a	2.91b	3.04a	1.89b
Frost tolerance (points in scale 0–9)				
Control	3.26c	6.01b	2.80c	5.12b
EBR	4.51c	8.09a	2.58c	6.67a

The presented data are the mean values based on 50 replicates  $\pm$  SE. The mean values marked with the same letter within a parameter and a cultivar did not differ significantly according to Duncan's test, where  $p \leq 0.05$

their snow mould resistance at levels as high as the plants that were treated with EBR and cold acclimated for only 3 weeks. In the control plants, the pathogen resistance after 6 weeks of cold acclimation did not change when compared to those plants that were cold acclimated for only 3 weeks. But on the contrary, in the EBR-treated plants, those with a long (6 weeks) cold acclimation showed a lower pathogen resistance than the plants that had undergone a medium (3 weeks) cold acclimation. An increase in the frost tolerance was observed in all the treatments, which coincided with the cold treatment duration. After 3 weeks of cold acclimation, EBR did not enhance the plants' frost tolerance level; however, this parameter was significantly improved in the EBR-treated plants of both cultivars that were cold acclimated for 6 weeks.

### Efficiency of the photosystem II

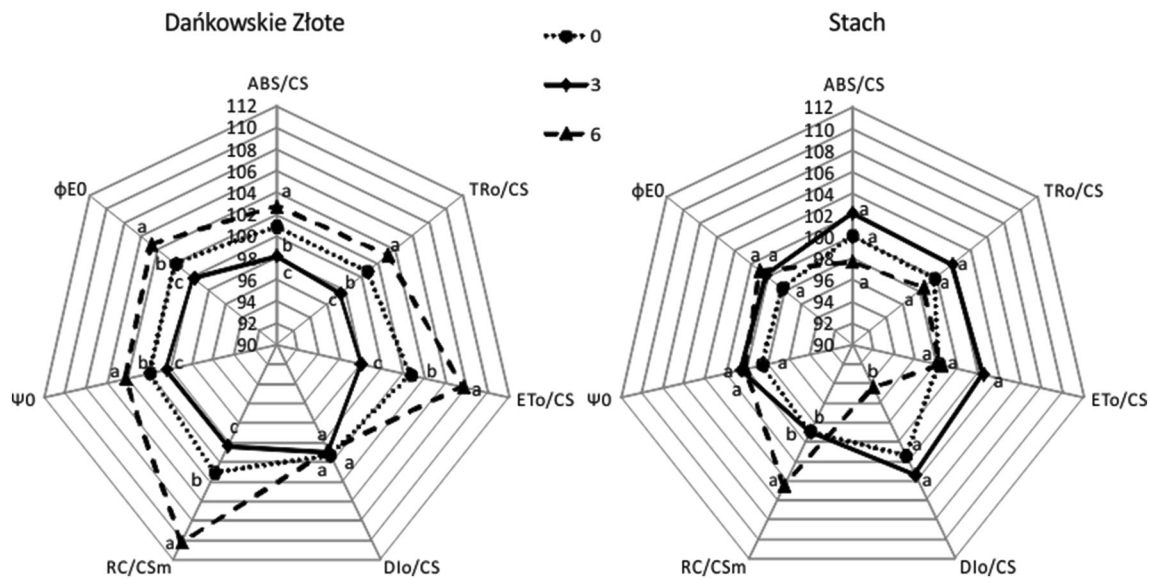
In both cultivars, EBR treatment induced changes in the photosynthetic efficiency that depended on the length of the plant's cold acclimation (Fig. 1). In the winter-resistant Dańkowskie Złote cultivar, 3 weeks of cold acclimation of EBR-treated plants induced a decrease in all the parameters, except for  $DI_0/CS$ , when compared to the plants that were non-acclimated and those that were cold acclimated for the longer period of 6 weeks. Contrary to this, after the long (6 weeks) cold acclimation, the EBR triggered an increase in all the investigated parameters except for  $DI_0/CS$ , when compared to the non-acclimated plants and the plants that were cold acclimated for only 3 weeks. To sum up, after 6 weeks of cold acclimation the EBR enhanced (and after 3 weeks of cold acclimation it lowered) the values of all parameters except for  $DI_0/CS$ . In the moderately winter-resistant Stach cultivar, the EBR did not affect the investigated parameters with two exceptions. After 6 weeks of cold acclimation, energy losses through heat ( $DI_0/CS$ ) were significantly lower, while the amount of active reaction centres  $RC/CS_m$  was significantly higher when compared to the non-acclimated plants and the plants that were cold acclimated for only 3 weeks.

### Endogenous brassinosteroid content

Only castasterone (CS) was detected in the winter rye leaves (Fig. 2). The content of the castasterone in the non-cold acclimated plants was the lowest, and this finding was similar for both cultivars.

In the Dańkowskie Złote cultivar, the castasterone level increased after the cold acclimation and was at a similar level after 3 and 6 weeks of this treatment. In the moderately winter-resistant Stach cultivar, cold acclimation resulted in a gradual increase in the castasterone content, which reached its highest level after 6 weeks at 4 °C.





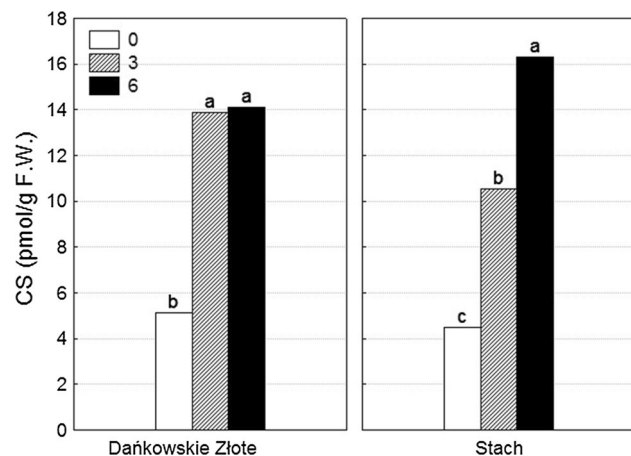
**Fig. 1** Phenomenological energy fluxes (ABS/CS, TR<sub>o</sub>/CS, ET<sub>o</sub>/CS, DI<sub>o</sub>/CS), the number of active reaction centres per excited CS (RC/CS<sub>m</sub>) and the energy transfer efficiency ( $\Psi_0$ ,  $\phi E_0$ ) in the leaves of the control and the EBR-treated non-cold acclimated winter rye plants, and the values after 3 and 6 weeks of cold acclimation. The presented

data (arbitrary units) are the mean values for the EBR-treated plants, expressed as percent of the control values based on 10 replicates. The mean values marked with the same letter within a parameter and a cultivar did not differ significantly according to Duncan's test, where  $p \leq 0.05$

**Composition of the soluble carbohydrates**

The content of individual carbohydrates in the plants depended on the duration of the cold acclimation and the treatment with the exogenous EBR (Table 2). In the non-cold acclimated control plants of the winter-resistant Dańkowskie Złote cultivar, glucose and fructose accounted for 31 % of the total soluble carbohydrates (TSC); and in the moderately winter-resistant Stach cultivar they

accounted for ca. 22 % of the TSC. The sucrose content was 12 % in Dańkowskie Złote and only 2 % in Stach. 1-Kestose content was ca. 2 % in the Dańkowskie Złote cultivar and 1 % in the Stach cultivar; while the EBR treatment mainly triggered qualitative changes that were manifested by a reduced content of monosaccharides and sucrose in favour of fructooligosaccharides (FOS), i.e. 1-kestose and nystose, that made up 5–6 % of the sugar pool in both cultivars. The EBR application also lowered the TSC level by ca. 40 % in the winter-resistant Dańkowskie Złote cultivar and by ca. 15 % in the moderately winter-resistant Stach cultivar.



**Fig. 2** The castasterone (CS) content in the leaves of the control non-cold acclimated winter rye plants, and the values after 3 and 6 weeks of cold acclimation. The presented data are the mean values based on five replicates. The mean values marked with the same letter within a cultivar did not differ significantly according to Duncan's test, where  $p \leq 0.05$

After 3 weeks of cold acclimation, the content of FOS in the TSC increased up to about 20 % in both cultivars, irrespective of their EBR treatment. However, after 6 weeks of hardening, this percentage varied in both cultivars. With 3 weeks of cold acclimation and the EBR treatment, the winter-resistant Dańkowskie Złote cultivar experienced a drop in its hexoses content and an increase in its sucrose level, when compared with the control plants. The total pool of sugars was also significantly greater in the control plants. After 3 weeks of cold acclimation, the plants of the moderately winter-resistant Stach cultivar treated with EBR contained fewer hexoses and less sucrose, and featured a lower TSC content than the control group.

In the winter-resistant Dańkowskie Złote cultivar that were cold-acclimated for 6 weeks, the content of monosaccharides was still lower in the EBR-treated plants

**Table 2** The carbohydrate composition and the total soluble carbohydrates (TSC) ( $\mu\text{g}/\text{mg}$  DW) in the leaves of the control and the EBR-treated non-cold acclimated winter rye plants, and the values after 3 and 6 weeks of cold acclimation

Weeks of cold acclimation	Dańkowskie Złote			Stach		
	0	3	6	0	3	6
Glucose						
Control	27.16d	36.69a	33.92b	16.76c	36.09a	27.66b
EBR	10.96f	31.92c	18.89e	11.65d	26.09b	18.64c
Fructose						
Control	12.08e	32.17b	36.10a	10.60e	30.87b	35.15a
EBR	8.48f	26.98c	22.93d	10.99e	22.02d	24.87c
Sucrose						
Control	14.90a	8.11c	10.97b	2.34f	17.95a	7.21d
EBR	4.76d	10.60b	11.18b	4.85e	13.79b	10.35c
Nystose						
Control	nd	13.32a	12.14a	nd	14.43a	7.69c
EBR	3.41b	11.33a	12.91a	4.35d	12.06b	11.90b
1-Kestose						
Control	1.88d	10.18b	13.59b	1.19e	17.86a	10.29c
EBR	3.30c	10.86b	10.23b	2.68d	14.54b	11.37c
Trehalose						
Control	nd	nd	nd	nd	tr	tr
EBR	nd	nd	nd	nd	nd	nd
Raffinose						
Control	nd	0.88a	0.72a	nd	1.18b	0.32c
EBR	0.05b	0.80a	0.71a	0.02d	1.46a	0.29c
TSC						
Control	125.87d	181.87b	218.02a	82.42d	194.42a	181.21a
EBR	79.79e	164.58b	188.35b	109.80c	166.75b	181.04a

The presented data are the mean values based on five replicates  $\pm$  SE. The mean values marked with the same letter within a parameter and a cultivar did not differ significantly according to Duncan's test, where  $p \leq 0.05$

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than in the control ones. In the EBR-treated Stach cultivar the content of monosaccharides was lower and the sucrose was higher than in the control groups. However, the EBR application did not affect the TSC content, which was the same in the experimental and the control groups.

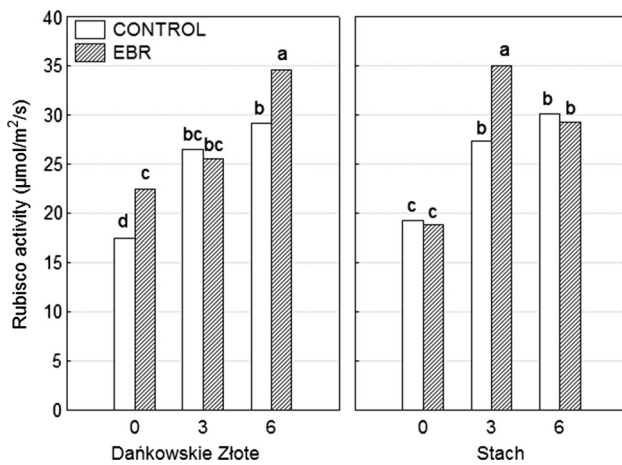
### Rubisco activity

In the moderately winter-resistant Stach cultivar the EBR mediated an increase in the Rubisco activity, which was observed after only 3 weeks of cold acclimation (Fig. 3). No changes in this parameter were found in the non-cold acclimated control plants and the plants that were cold acclimated for 6 weeks.

Contrary to this, no changes were found in the enzyme activity of the winter-resistant Dańkowskie Złote cultivar after 3 weeks of cold acclimation. However, the EBR did stimulate Rubisco activity in both the non-cold acclimated winter rye plants, and those that were cold acclimated for 6 weeks.

### Discussion

Although frost tolerance and snow mould resistance seem to be independent components of a plant's winter hardiness, the growth potential and photosynthetic capacity that lead to the accumulation of carbohydrates are important processes for the development of both of these traits. The study results indicate that the EBR induced changes in the photosynthesis and carbohydrate metabolism were associated with frost tolerance and with pathogen resistance, and were more pronounced in the winter-resistant plant cultivar. A beneficial effect from the exogenous EBR on snow mould resistance was observed in both cultivars after 3 weeks of cold acclimation, but after 6 weeks of the acclimation process this effect was not significant. Contrary to this, a significant increase in the plant's frost tolerance was only observed after 6 weeks of cold acclimation. The EBR-treated plants achieved higher levels of pathogen resistance after only 3 weeks of cold



**Fig. 3** The Rubisco activity in the leaves of the control and EBR-treated non-cold acclimated winter rye plants, and the values after 3 and 6 weeks of cold acclimation. The presented data are the mean values based on five replicates. The mean values marked with the same letter within a cultivar did not differ significantly according to Duncan's test, where  $p \leq 0.05$

acclimation than the control plants did after 6 weeks of hardening.

The lack of a correlation between EBR-induced snow mould resistance and frost tolerance may possibly indicate different physiological and genetic backgrounds. According to Gaudet et al. (2011), a long exposure of winter wheat to a temperature of  $-3\text{ }^{\circ}\text{C}$ , regardless of the presence or the absence of the snow mould pathogen, resulted in a reduction of the wheat's defence-related and carbohydrate metabolism-related transcripts, but did not influence level of a cold responsive (WCOR) transcript. Thus, the decrease in pathogen resistance in the EBR-treated plants after 6 weeks of cold acclimation may be attributed to a down-regulation of the pathogen defence related genes in the later phase of the cold acclimation. The longer cold acclimation increased the frost tolerance, probably thanks to the carbohydrate accumulation and the up-regulation of the genes related to frost tolerance. This hypothesis is further confirmed by a linear correlation between the freezing tolerance and the days of undergoing cold acclimation in rye grown at  $4\text{ }^{\circ}\text{C}$  (Fowler et al. 1996).

In the non-cold acclimated, EBR-treated winter-resistant cultivar, an increase in photosynthetic efficiency was followed by a decrease in the plants' sugar pool and an increase of Rubisco activity, indicating a stimulation of photosynthesis resulting from a decreased sugar level. The phenomenon of the stimulation of a plant's photosynthetic capacity and the activity of the Calvin cycle enzymes by brassinosteroids in no-stress conditions was described by some authors (Braun and Wild 1984; Hayat et al. 2000; Xia et al. 2009). This finding has also been confirmed in studies concerning the effects of short (3 days) periods of cold in *Arabidopsis* (Kagale et al. 2007).

After 3 weeks of cold acclimation in the winter-resistant and EBR-treated cultivar, the acclimation of the photosynthetic apparatus was accompanied by a decreased energy absorption by the antennas (ABS/CS) and a lower amount of energy transferred to the reaction centre (TR<sub>0</sub>/CS). However, no differences in the Rubisco activity and carbohydrate level were noticed. However, in the EBR-treated moderately winter-resistant cultivar, a protection of the photosynthetic apparatus was probably more important to the plant than maximising the photochemical efficiency, and this was reflected in a maximisation of the energy dissipation represented by an increased DI<sub>0</sub>/CS. After 6 weeks of cold acclimation, the winter-resistant EBR-treated cultivar showed an increase in all of the measured chlorophyll fluorescence parameters with no differences in the energy dissipation (DI<sub>0</sub>/CS). But at the same time, the moderately winter-resistant cultivar exhibited a higher number of active reaction centres (RC/CS<sub>m</sub>), combined with a much higher energy flux for energy dissipation from the PSII reaction centre, thus indicating a less effective energy conversion capability.

To survive in the winter and in freezing temperatures, winter cereals need to continue growing and developing at low temperatures. To achieve this, they need to maintain an appropriate level of photosynthetic efficiency that will provide the plant with energy necessary for growth and the adaptive changes. The most important of these adaptive adjustments include quantitative and qualitative changes in the plant's carbohydrates. In non-cold acclimated EBR-induced plants, mainly qualitative changes were observed. The control plants contained half of the shortest form of inulin trisaccharide 1-kestose, and did not contain any nystose or raffinose. However, irrespective of their genotype, EBR-treated non-acclimated plants and the 6 week cold acclimated plants contained more FOS than simple sugars, when compared with the control plants. Apart from supplying the plant tissues with hexoses, fructans can stabilise membranes by preventing ion leakages during drought or freezing. FOS and raffinose protect the cell membranes by inserting at least part of their polysaccharide into the lipid headgroup region of the membrane, thus improving its frost tolerance (Livingston et al. 2009). In our experiment, the 6 weeks of cold acclimation in the EBR-treated plants of both cultivars caused a decrease in monosaccharides. At the same time, the sucrose levels did not change in the winter-resistant cultivar and increased in the moderately winter-resistant cultivar. The EBR-induced winter-resistant cultivar underwent a reduction in simple sugars content after 6 weeks of cold acclimation, which stimulated photosynthesis, as an accumulation of the soluble carbohydrates in the leaves is associated with a decrease in the Rubisco activity and the other carbon reduction cycle enzymes (Worrell et al. 1991).

In our study, an increase in frost tolerance in the control plants was accompanied by a constant level of endogenous CS between the 3rd and the 6th week of the cold acclimation in the winter-resistant cultivar, and a considerable continuous increase in endogenous CS in the moderately winter-resistant cultivar. It indicates that increase of castasterone level after 3 and 6 weeks do not favor winter resistance since the effect of brassinosteroids is dependent on stage of growth. The content of the growth inhibitors and stimulants at different stages of winter hardening is an important feature for the development of frost resistance. According to Kosova et al. (2012), enhanced frost tolerance after a prolonged (21 day) exposure to cold is associated with a decline in the growth stimulants, such as cytokinins and auxins. Therefore, the lack of an increase in CS content (a growth stimulating hormone) after 6 weeks of cold acclimation in the winter-resistant cultivar may be related to its already higher frost resistance.

In summary, the pattern of changes observed after the EBR treatment in plants growing at a high temperature (non-cold acclimated) and at a low temperature (cold acclimated) was varied suggesting different control mechanisms involved in these responses. In the non-acclimated plants, the effects of the EBR were associated with carbohydrate metabolism, which promoted the synthesis of fructooligosaccharides from monosaccharides, and reduced the amount of TSC in the leaves that stimulate Rubisco activity. At cold the control mechanisms were dependent on the stage of growth. After 3 weeks of cold acclimation, the maximum acquired snow mould resistance for the EBR-treated winter-resistant cultivar, coincided with the stage of lowered photosynthetic efficiency. The maximum frost tolerance under EBR treatment was recorded after 6 weeks of cold acclimation in the winter-resistant cultivar and was accompanied by lower levels of TSC in the leaves, increased photosynthetic efficiency and increased Rubisco activity. Our study indicates that exogenous 24-epibrassinolide when applied before cold acclimation could modify this process which result in an enhanced rate of winter survival. In turn increased endogenous castasterone level at the latter stage of cold acclimation (6 weeks) is related to lower winter resistance. However, a more detailed study using brassinosteroids synthesis inhibitors or brassinosteroids deficient mutants is a challenge that should be addressed in the future.

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