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ORIGINAL ARTICLE

Abscisic acid in preservation of *Taraxacum pieninicum* in the form of synthetic seeds in slow growth conditions

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Abstract

Taraxacum pieninicum Pawł. is listed as critically endangered species, for which currently applied protection methods are insufficient. The aim of this study was to investigate the possibility of *T. pieninicum* storage in the form of synthetic seeds under slow-growth conditions in combination with ABA treatment, as one of the ex situ protection methods of this species. The obtained results indicated that darkness was much more favorable condition for synseed storage and did not generate additional stress during cold exposure in contrast to the light conditions. The preculture of shoot tips on the medium supplemented with ABA led to a decrease in the shoots proliferation rate and inhibition of their growth. ABA clearly inhibited growth of the encapsulated shoot tips also during cold storage. Biochemical parameters showed that ABA effectively reduced the negative effect of the cold stress, what was found on the basis of analyzes of H_2O_2 and TBARS levels in the stored material. Moreover, synseeds stored under light conditions and treated with ABA exhibited decreased level of endogenous jasmonic acid what indicated interaction between those two phytohormones at a low temperature. The study also demonstrated that in vitro culture, cold storage and ABA treatment had no effect on the flowering process of this species after acclimatization to ex vitro conditions.

Key message

The aim of this research was to assess ABA effect on post-storage regrowth and the stress factors level in plants obtained after long-term storage of *Taraxacum pieninicum* synthetic seeds.

Keywords Chlorophyll · Cold · Hydrogen peroxide · Jasmonic acid · Proline · Soluble sugars · TBARS

Abbreviations

ABA	Abscisic acid
BAP	Benzylaminopurine
Chl	Chlorophyll
JA	Jasmonic acid
MDA	Malondialdehyde
NAA	Naphthalene acetic acid
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances

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Introduction

Taraxacum pieninicum belongs to the *Asteraceae* family and is a stenochoric species, occurring in only one site in the Central Pieniny in the Western Carpathians (Poland) (Piękoś-Mirkowa and Mirek 2009). This species is considered to be probably the oldest endemic species in Pieniny Mts. from the Paleogene (Puchalski et al. 2014). Creating collections in botanical garden as a method of its protection is not very effective due to the difficulty of keeping it in these areas. As the only species of the *Taraxacum* genus, it does not produce sesquiterpene lactones, such as taraxic acid and triterpenes, commonly referred to as taraxacin in older literature, which gives the leaf a bitter

taste (Schütz et al. 2006). Lack of biochemical protection means that it is eagerly eaten by various herbivore species. In addition, seeds lose their viability (Honek et al. 2005), therefore the in vitro method was considered appropriate for protection of this species. An efficient regeneration system for *T. pieninicum* has been developed (Trejgell et al. 2013), nevertheless maintaining cultivars in in vitro conditions is costly and carries the risk of contamination (Pence 2010; Cruz-Cruz et al. 2013). For these reasons optimization of the slow growth conditions is highly desirable to maximize time between passages. Lowering the temperature of *T. pieninicum* shoot tips culture allowed its storage for several months (Kamińska et al. 2018a), although increased survival of the explants was noted after encapsulation (Kamińska et al. 2018b). The possibility of extending the storage time of encapsulated explants by using abscisic acid as a growth retardant was evaluated in this paper.

Growth retardants are synthetic or natural chemical compounds that can cause structural changes or affect plant life processes by direct effect on their hormonal balance. Synthetic retardants that inhibit plant elongation growth are usually gibberellin antagonists that affect their metabolism (Espindula et al. 2009). Natural growth retardants include phytohormones, as abscisic acid (ABA) and jasmonic acid (JA), which show diverse physiological activity (Heinrich et al. 2012).

Endogenous and exogenous ABA is considered as auxins and gibberellins antagonist, leading to inhibition of cell division and elongation growth (Humplík et al. 2017; Lorrai et al. 2018). In conditions of drought, salinity or low temperature stress, this compound promotes closing stomata to reduce water loss by transpiration (Kim et al. 2010), affects the change in the morphology of the root system (Sah et al. 2016) and activates many genes encoding enzymes responsible for the biosynthesis of osmoregulatory substances and LEA-type proteins (Fujita et al. 2011).

The greatest importance in the reaction of plants to abiotic stress is attributed to abscisic acid. The role of ABA in acclimatization to cold is confirmed by increased freezing tolerance in plants growing at room temperature treated with ABA (Palva et al. 2002). In addition, the involvement of ABA in the activation of genes expression related to cold acclimatization has been documented many times. In our previous studies, we have shown the possibilities of storing *T. pieninicum* synseeds and the usage of jasmonic acid as a growth retardant (Kamińska et al. 2018a, b). The aim of this research was to assess the effect of abscisic acid on the condition of plants obtained after long-term storage of *Taraxacum pieninicum* synthetic seeds. Verification of the ABA influence on *T. pieninicum* stored explants was based on the alterations in post-storage regrowth, biochemical stress factors, endogenous ABA and JA content.

Materials and methods

Plant material and culture conditions

Seeds of *T. pieninicum* were obtained from the collection of the Polish Academy of Sciences Botanical Garden—Center for Biological Diversity Conservation in Powsin. The seeds were sterilized with 70% ethanol for 30 s, followed by 20% sodium hypochlorite solution for 20 min. After this time, the seeds were washed four times with sterile distilled water and were transferred onto MS medium (Murashige and Skoog 1962) without plant growth regulators. MS medium was supplemented with 3% sucrose, solidified with 0.8% agar and adjusted to pH 5.75–5.8 prior to autoclaving for 20 min at 121 °C.

Shoot tips of 12-day-old seedlings were isolated and transferred onto proliferation medium to induce the process of development of axillary buds and their proliferation. MS medium (see above) supplemented with 1.1 µM benzylaminopurine (BAP) and 0.14 µM naphthyl-1-acetic acid (NAA) was used for shoots multiplication (MS_{BAP}). Cultures were kept in a growth room at 26 ± 1 °C, under continuous light with a quantum irradiation intensity of 80 µmol m^{-2} s^{-1} (optimal growth conditions). After 4 weeks of culture, obtained axillary buds were isolated and transferred on fresh growth medium with the same composition.

Synthetic seeds production

Individual shoot tips after isolation from the culture of *T. pieninicum* axillary shoots were placed on a Petri dish containing 3% sodium alginate dissolved in liquid MS medium. A single explant covered with alginate solution was added dropwise to a 100 mM calcium chloride solution. Polymerization of alginate was carried out for 25 min with gentle stirring of the calcium chloride solution. Using sterile strainer, the calcium chloride solution was removed and the obtained synthetic seeds were rinsed with sterile distilled water.

Abscisic acid treatment

Two methods of ABA exposure were used: a four-week shoots preculture in the optimal growth conditions on MS_{BAP} medium supplemented with ABA preceding encapsulation (further indication in the paper—preculture) and addition of ABA to the alginate solution during shoot tips encapsulation (inside). For both experiments four concentrations of ABA were used: 19 µM, 38 µM, 57 µM and 76 µM. After preculture proliferation rate (number

of shoots per explant) and visual evaluation of the shoots growth were additionally assessed.

Cold storage conditions

Synseeds were placed on 50 mL MS medium without plant growth regulators in polycarbonate culture boxes (Magenta™ vessel, Sigma-Aldrich). Storage was carried out for 3, 6 or 9 months at 4 ± 1 °C in conditions of continuous light with a reduced quantum irradiation intensity to 40 µmol m^{-2} s^{-1} or in the dark. Synseeds non-treated with ABA were used as a control.

Post-storage regrowth

Directly after cold-storage a visual evaluation of the shoots and roots length was performed, then alginate coat was gently removed, leaves were collected for biochemical analyzes and isolated shoot tips were transferred onto MS_{BAP} medium to the optimal growth conditions. The proliferation rate was assessed after 4 weeks of 2 subcultures. For rooting shoots after 2nd subculture were transferred onto MS medium without plant growth regulators for 4 weeks. Rooting ability was investigated in terms of percent shoots able to form roots, number of roots per shoot and root length. Roots were gently washed with sterile distilled water and then plantlets were transferred to pots containing sterile vermiculite and sand (1:1 v/v). The pots were covered with a transparent lid to maintain humidity (60–70%). Plantlets grown at 23 ± 1 °C under white fluorescent light (80 µmol m^{-2} s^{-1}) for 4 weeks after which they were transferred to the pots containing sterile soil and then were transferred to the field conditions. Survival rate and ability to flower of the plantlets were noted in the next year after acclimatization (April/May).

Biochemical analyzes

Directly after cold-storage leaves of stored explants were analyzed in terms of chlorophyll, H_2O_2, TBARS, soluble sugars, free proline and endogenous phytohormone (ABA, JA) content. Leaves from stored explants non-treated with ABA were used as a control. Additional non-stored plant tissue from optimal growth conditions was used for phytohormone analyzes. Chlorophyll, H_2O_2, soluble sugars and proline content was also determined in leaves after 4-weeks preculture on medium supplemented with ABA under optimal growth conditions. Chlorophyll was extracted from 20 mg of plant tissue in 96% ethanol and determined by the method of Nair and Chung (2015). To determine the total chlorophyll content (a and b) the formula developed by Lichtenthaler (1987) was used:

$$\mathrm{Chl_{a+b}}\left(\mu g\ mg^{-1}\right) = \left[5,24 \cdot A_{664,2} + 22,24 \cdot A_{648,6}\left(\mu g\ mL^{-1}\right)\right] \cdot 10\ mL/m_{sample}(mg)$$

Hydrogen peroxide was determined according to Velikova et al. (2000). For extraction 100–200 mg of leaves was used and determination of H_2O_2 was based on the iodide-reduction method with 1 M KI solution. TBARS level was estimated following the protocol described by Song et al. (2011). Sample for this analysis contained approximately 50 mg of leaves. Free proline was determined spectrophotometrically using method based on a reaction with ninhydrin (Bates et al. 1973). Extraction and determination of total soluble sugars was carried out by the phenol–sulfuric acid method (PSA) described by Dubois et al. (1956). Glucose was used to prepare standard curve. Endogenous level of abscisic acid and jasmonic acid was determined by using mass spectrometry combined with liquid chromatography (LC–MS). Analysis was carried out for explants stored for 9 months on MS medium without ABA and treated with this phytohormone at concentrations of 38–76 µM. Sample contained 50–100 mg of leaves homogenized in liquid nitrogen (LN). For the extraction of phytohormones 80% methanol (v/v) was added. In this step also small amount of antioxidant butylhydroxytoluol (BHT), 5 ng of deuterated ABA (d_6ABA) and 10 ng deuterated JA (d_5JA) as internal standards were added. The mixture was shaken overnight (at least 18 h). After incubation, the samples were centrifuged and methanol was evaporated. To remove chlorophyll, the samples were acidified to pH 2 by the addition of hydrochloric acid. After centrifuged the extracts, the supernatant was subjected to solid phase extraction (SPE) using silica packed columns (Discovery® DSC-18 SPE Tube). The columns were activated with 100% methanol and conditioned with formic acid. The applied samples were purified with 1 M formic acid and a solution of 1 M formic acid in 20% methanol (v/v). Elution was performed using 80% methanol (v/v). Before analysis samples were concentrated 7.5 times and centrifuged. For phytohormones determination the LCMS-8045 tandem mass spectrometry (Shimadzu Corp.) was used. Chromatographic separation was carried out on a Kinetex® 2.6 µm XB-C18 100 Å reverse phase column (150 × 2.1 mm). Water with 0.1% formic acid (v/v) (A) and methanol with 0.1% formic acid (v/v) (B) were used as the mobile phase. The separation was carried out in a linear gradient of 40–90% (v/v) methanol for 7 min at a flow rate of 0.3 mL/min at 30 °C. In mass spectrometry, the samples were subjected to negative electrospray ionization (ESI) and ions were fragmented by collision-induced dissociation (CID). The ionization voltage was − 3 kV. Analysis of individual phytohormones was based on multiple reactions monitoring (MRM).

Data collection and statistical analysis

The results presented in this paper were expressed as the mean ± standard error. Parameters from preculture

(proliferation rate), storage (survival) and post-storage regrowth (proliferation rate and rooting ability of the shoots) were evaluated for 16 explants in each variant in three replicates. The leaves of survived explants from each vessel were grounded in LN together and biochemical analyzes were evaluated for 3 samples (1 representative sample from all of the explants stored in 1 vessel). Each biochemical test was performed in 3 biological and 3 technical replicates. The normality of the distribution of obtained results was tested by the Shapiro–Wilk test. In the case of data with non-normal distribution, the significance of differences was determined using non-parametric Mann–Whitney U test. Data with normal distribution and obtained in biochemical analyzes were analyzed using Tukey's test. In addition, the significance of the impact of the studied factors on the stored plant material and their interactions on the accumulation of selected chemical compounds was analyzed using two- and three-way ANOVA.

Results

Preculture with ABA in optimal conditions

The obtained results indicated that ABA, at all tested concentrations, significantly decreases shoot proliferation rate in comparison to the control (18.9 ± 1.6 shoots per explant). Proliferation rate of ABA-treated shoots ranged from 10.1 ± 1.4 (19 µM ABA) to 3.3 ± 0.4 (76 µM ABA) shoots per explants (Fig. 1a). In addition, it was observed that increasing ABA concentration slightly decreased shoots length (Fig. 1b).

ABA treatment during cold-storage

Preliminary observation indicated that exposure to ABA reduced growth of the shoots obtained during synseed storage in comparison to the control, both after 3 and 6 months of storage (Figs. S1-S2) and after 9 months, where a particularly significant reduction of shoot length occurred in the presence of 76 µM ABA inside artificial endosperm in both light and dark conditions (Fig. 2). In addition, shoots stored in the dark in all tested ABA variants did not form roots, regardless of the ABA concentration. However, when storing synseeds under light conditions ABA-treated shoots during preculture were able to form roots during cold storage for 6 and 9 months, but their elongation was inhibited (Figs. 2, S1-S2).

Regrowth of the stored explants under optimal conditions

After cold-storage the survival and shoots proliferation ability under the optimal growth conditions were analyzed. After 9 months storage treatment with ABA did not affect explants survival (Fig. 3). Over 75% of synseeds remained viable,

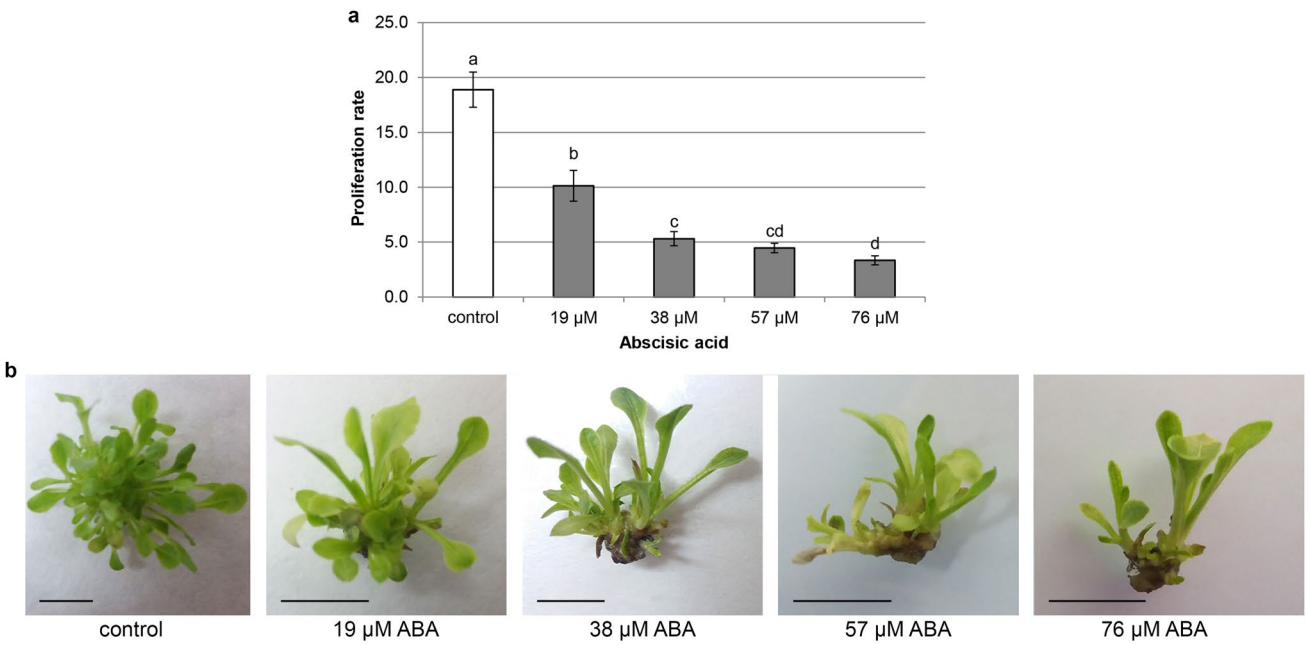

Fig. 1 The effect of ABA on *T. pieninicum* shoot proliferation in the optimal growth conditions: proliferation rate (**a**) and phenotype of shoots cluster (**b**) on MS medium supplemented with 1.11 µM BAP and 0.14 µM NAA and different ABA concentration after 4 weeks of the culture. Bar = 1 cm. Means with different letters are significantly different followed by Kruskal–Wallis test at $p \leq 0.05$

Fig. 2 *Taraxacum pieninicum* shoots obtained from synthetic seeds during 9 months storage at 4 °C under light conditions (**a**) and in the dark (**b**) after different treatments with ABA. Bar = 1 cm

regardless of the ABA treatment. Light conditions during storage decreased synseeds viability, although differences to the dark were not statistically significant in ABA-treated explants. Similar results were obtained after 6 months storage. Reduced survival of the synseeds treated with ABA was observed only after 3 months storage particularly under light conditions and when ABA was added inside synseed structure (Fig. S3).

The light conditions during cold-storage had the most significant impact on the post-storage proliferation ability of the explants under optimal growth conditions. Higher proliferation rate was indicated after storage in the dark in comparison to the light conditions, significant differences were noted for most of the used variants. After 9 months of storage ABA-treated synseeds remained comparable proliferation ability to the control stored in the light (7.6 ± 1.2 shoots per explant) and dark (13.7 ± 1.4) conditions (Fig. 4a). The method of ABA exposure was significant mostly after shorter storage time (Fig. S4). Similar relationships were observed after the second post-storage subculture (Fig. 4b).

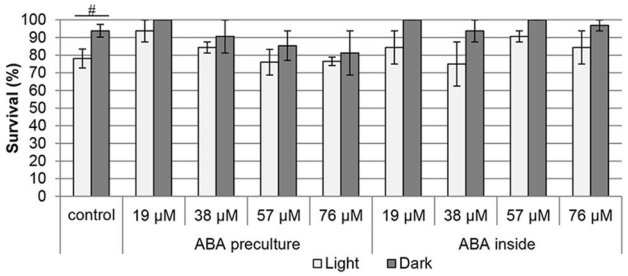

Fig. 3 Survival of the encapsulated shoot tips of *T. pieninicum* after 9 months storage 4 °C. Statistically significant differences assessed using Kruskal–Wallis test at $p \leq 0.05$: to the control plants in the same light conditions are denoted *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$); between light conditions are denoted $^{\#}$($p < 0.05$), $^{\#\#}$($p < 0.01$), $^{\#\#\#}$($p < 0.001$); between ABA treatment in the same light conditions are denoted $^{\Diamond}$($p < 0.05$), $^{\Diamond\Diamond}$($p < 0.01$), $^{\Diamond\Diamond\Diamond}$($p < 0.001$)

In comparison to the control, prior ABA treatment did not inhibit shoots proliferation ability. The highest proliferation rate in 2nd subculture was noted for shoots obtained from synseeds containing 38 µM ABA inside and stored in the dark for 9 months (23.7 ± 1.8; Fig. 4b) and also for 6 months (21.3 ± 1.7; Fig. S5).

The last stage of *T. pieninicum* post-storage regeneration was rooting shoots on the MS medium. Regardless of the storage variant, the shoots were able to root (Fig. 5), except for the few ABA variants stored for 3 and 6 months (Fig. S6). The highest percentage of rooted shoots was obtained after storage for 9 months in the dark with 76 µM ABA inside synseed (Fig. 5a). ABA treatment did not reduce the number of roots developing from the shoots obtained after storage, regardless of the storage time (Figs. 5b, S7), but inhibited roots elongation, regardless of its exposure method and concentration (Figs. 5c, S8). However, ABA did not affect the ability of the regenerants to acclimatize. All plantlets transferred to *ex vitro* conditions were able to acclimatize. The morphological appearance of the regenerants treated even with highest ABA concentration did not differ to the control plants and plantlets were able to flower after transfer to the field conditions (Fig. 6).

Biochemical analyzes

After 4-weeks preculture on medium supplemented with ABA in the optimal growth conditions it was indicated that ABA significantly decreased chlorophyll content in the multiplicated microshoots (Fig. 7a). The usage of the highest ABA concentration (76 µM) significantly increased accumulation of H_2O_2 (Fig. 7b) but in none of the used concentration ABA affected soluble sugars accumulation (Fig. 7c). Treatment of the explants with ABA in the two highest concentrations (57 µM and 76 µM) significantly increased proline accumulation in the leaves (Fig. 7d).

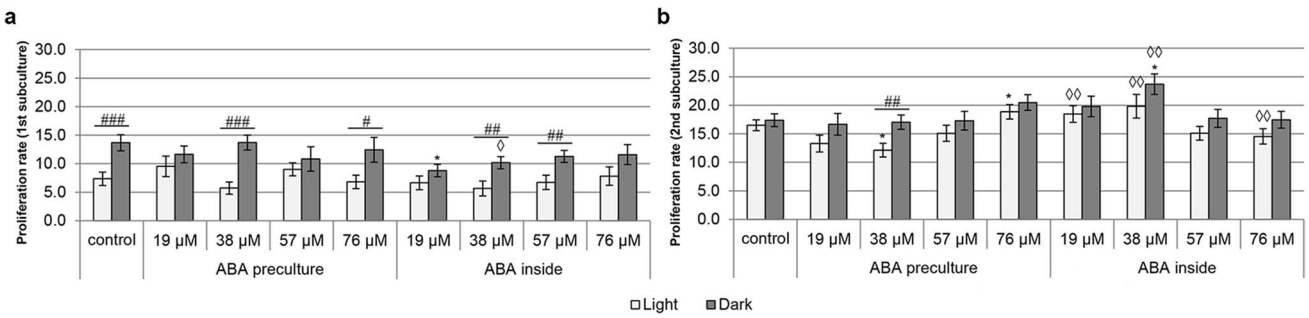

Fig. 4 The effect of light conditions and ABA-treatment on *T. pieninicum* proliferation rate of shoots during regrowth on MS medium with 1.11 µM BAP and 0.14 µM NAA in the 1st (**a**) and 2nd (**b**) subculture after 9 months storage at 4 °C. Statistically significant differences assessed using Kruskal–Wallis test at $p \leq 0.05$: to the control plants in the same light conditions are denoted *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$); between light conditions are denoted $^{\#}$($p < 0.05$), $^{\#\#}$($p < 0.01$), $^{\#\#\#}$($p < 0.001$); between ABA treatment in the same light conditions are denoted $^{\Diamond}$($p < 0.05$), $^{\Diamond\Diamond}$($p < 0.01$), $^{\Diamond\Diamond\Diamond}$($p < 0.001$)

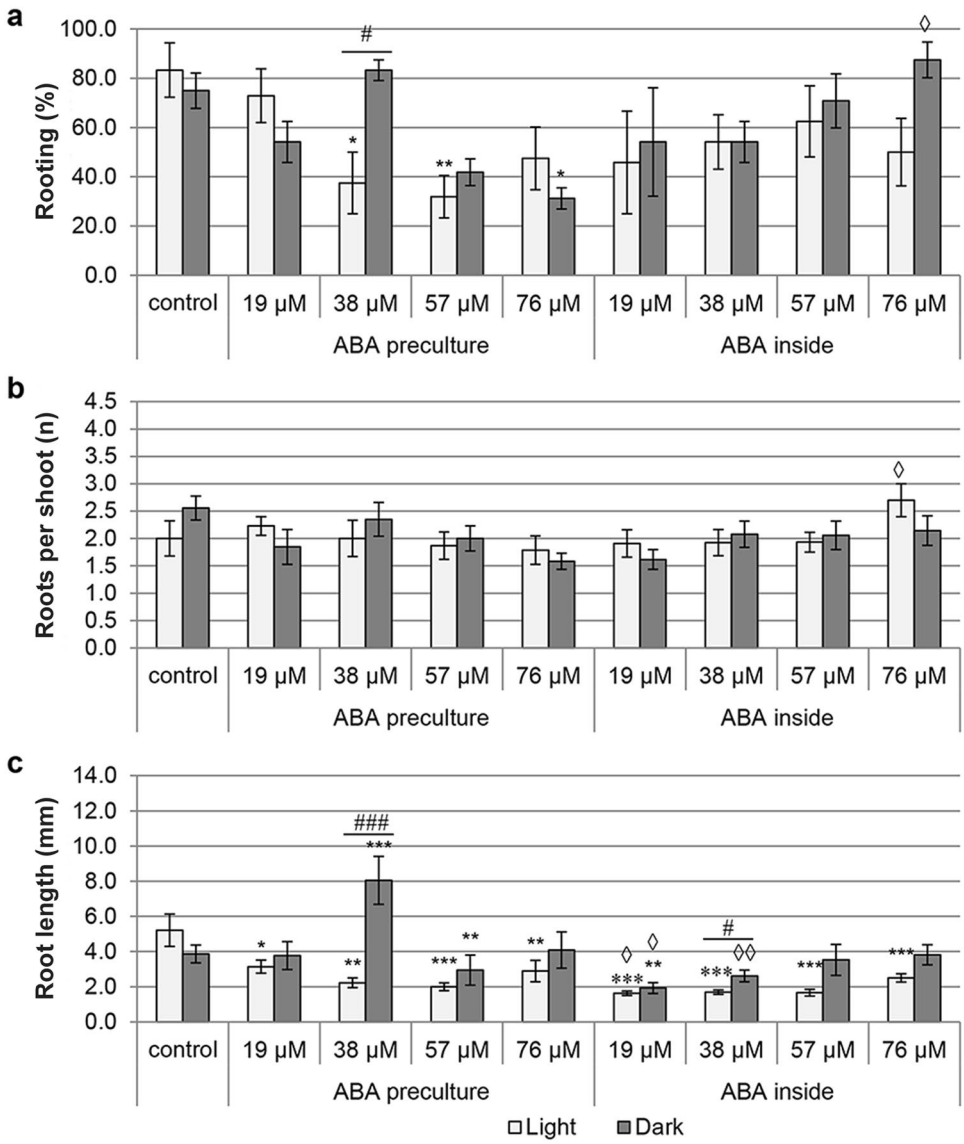

Fig. 5 The effect of light conditions and ABA-treatment on *T. pieninicum* rooting ability of shoots on MS medium after 9 months storage at 4 °C assessed by % of rooted shoots (a), number of roots per rooted shoots (b) and length of roots (c). Statistically significant differences assessed using Kruskal–Wallis test at $p \leq 0.05$: to the control plants in the same light conditions are denoted *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$); between light conditions are denoted #($p < 0.05$), ##($p < 0.01$), ###($p < 0.001$); between ABA treatment in the same light conditions are denoted ◊($p < 0.05$), ◊◊($p < 0.01$), ◊◊◊($p < 0.001$)

Fig. 6 Flowering *T. pieninicum* shoots in the field conditions, regenerated and acclimated after storage in the form of synthetic seeds with 96 µM ABA inside for 9 months at 4 °C under the light conditions (**a**) and in the dark (**b**)

After 9 months of storage the highest chlorophyll content was determined in control plants (Fig. 8a). The same results were obtained after 6 months of cooling, while after 3 months the highest chlorophyll accumulation was reported in leaves of shoots obtained from synseeds containing ABA in artificial endosperm (Fig. S9). Two-way ANOVA indicated that ABA application method had a significant effect on chlorophyll content after 3 and 6 months of storage, while ABA concentration had no significant effect regardless of the storage time (Table 1).

Increasing ABA concentration in the preculture treatment led to an increase in the accumulation of the hydrogen peroxide in the plant tissue after 9 months of storage, what was particularly evident in the material stored under light conditions (Fig. 8b). A similar effect was noted for explants stored in capsules containing ABA under light conditions. In the dark ABA inside synseed had no effect on H_2O_2 levels. Furthermore, after storage in the dark in each ABA-treated variant there were no significant differences compared to the controls. A three-way ANOVA showed a significant individual effect after 9 months of storage of ABA treatment method, ABA concentration and light conditions on H_2O_2 accumulation (Table 2). However, the interaction of these factors did not significantly affect analyzed parameter. Preculture with ABA also increased H_2O_2 accumulation after 3 months storage (Fig. S10a). However statistically significant differences to the control was noted only for tissue stored in the dark. ABA addition inside synseed had no effect on H_2O_2 accumulation after 3 months storage. After 6 months of storage preculture with ABA and addition of ABA to the synseeds stored in the dark reduced H_2O_2 accumulation (Fig. S10b).

Directly after storage, TBARS content was also determined as a marker for lipid peroxidation. After 9 months of storage ABA inhibited TBARS accumulation in comparison to the control, especially when synseeds were stored under light conditions (Fig. 8c), although increasing ABA concentration significantly increased accumulation of determined compound. Three-way ANOVA showed not only significant effect of the light conditions on TBARS content, but also ABA concentration and method of its exposure (Table 3). After shorter storage time ABA did not indicated significant effect on TBARS content in plant tissue (Fig. S11).

The accumulation of soluble sugars depended on all analyzed variation factors (Fig. 8d; Table 4). After 9 months of storage under light conditions a significantly higher than in control plants level of soluble sugars was determined in explants treated with 38 µM ABA and 57 µM ABA during preculture. Increased sugar accumulation was also shown in leaves from variant where ABA

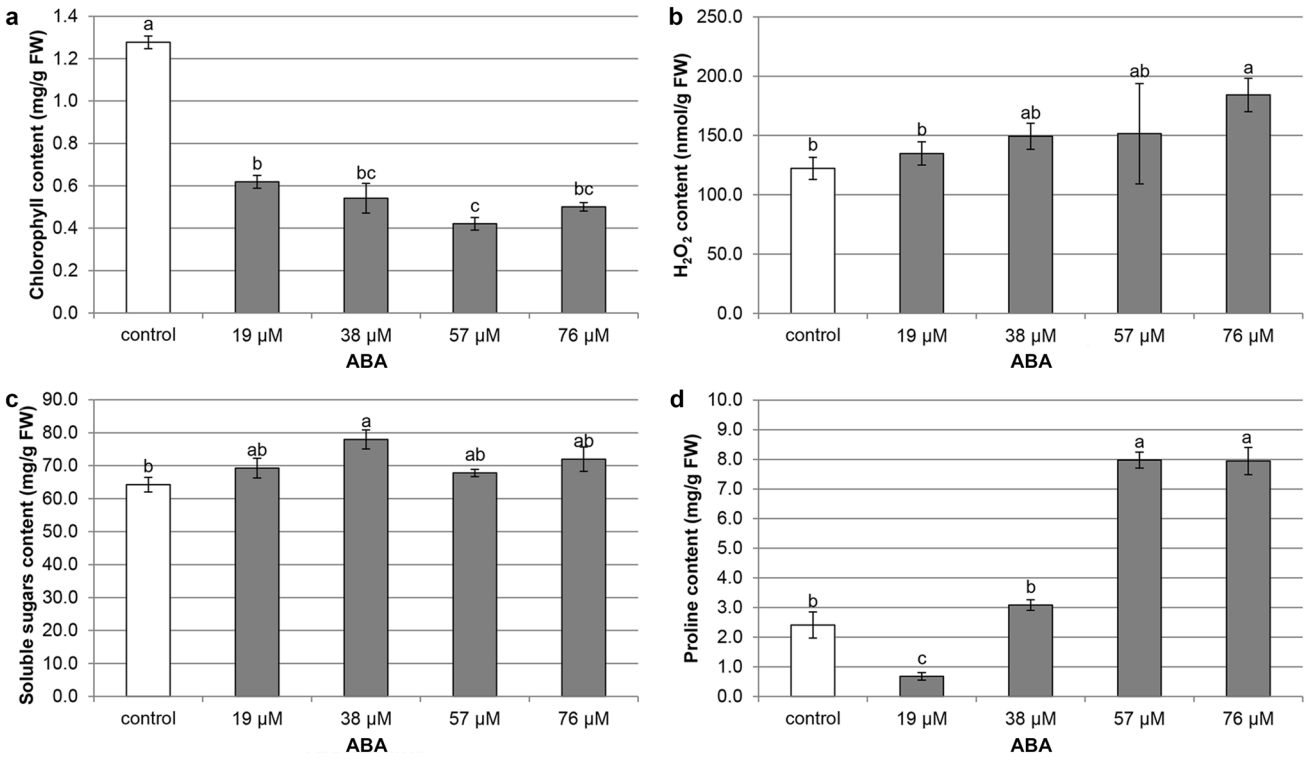

Fig. 7 Chlorophyll (**a**), H_2O_2 (**b**), soluble sugars (**c**) and proline (**d**) content in *T. pieninicum* shoots after 4-week preculture on medium MS supplemented with 1.11 µM BAP and 0.14 µM NAA and different concentration of ABA. Means with different letters are significantly different followed by Tukey test at $p \leq 0.05$

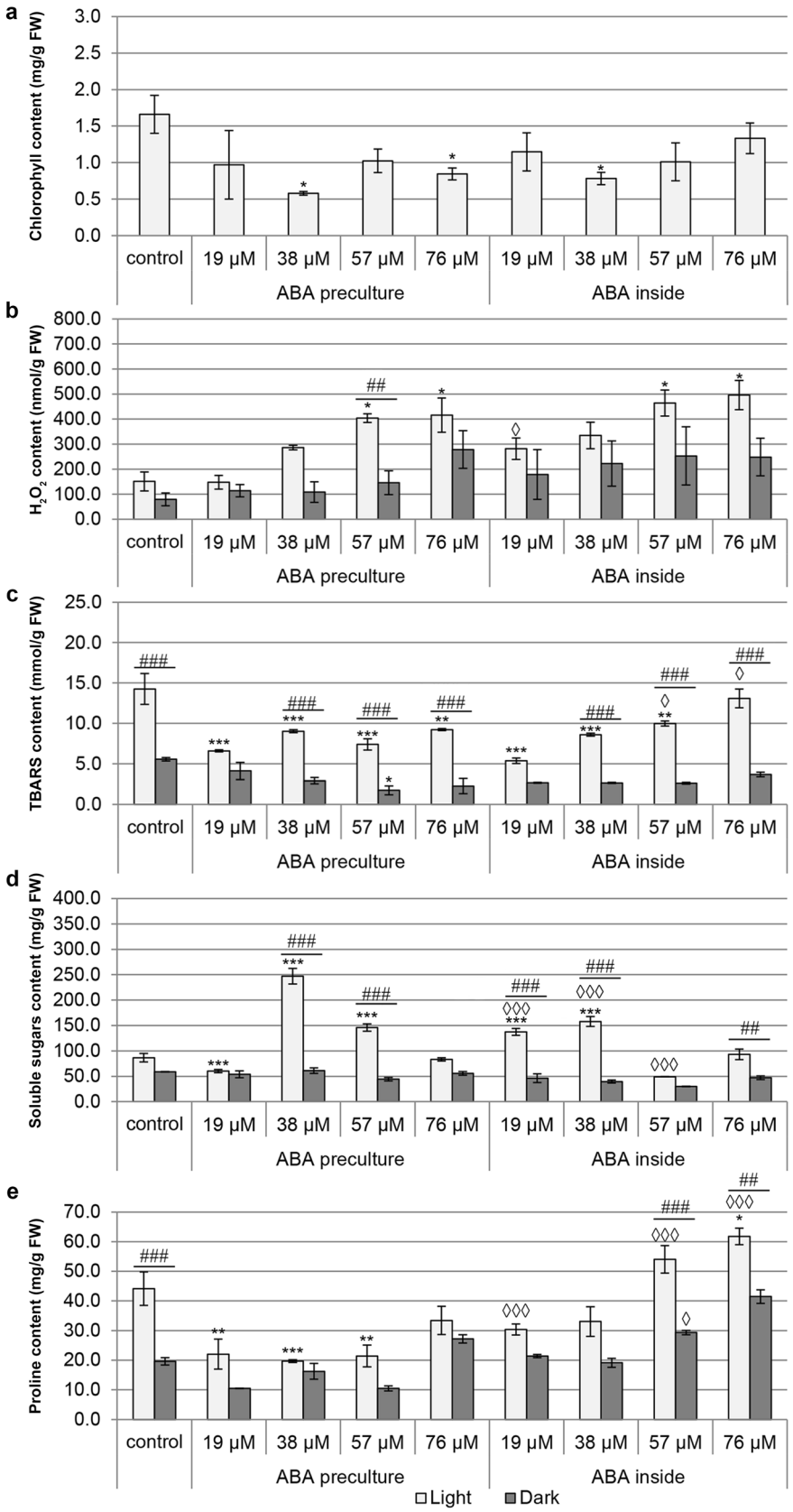

Fig. 8 The effect of the light conditions and ABA-treatment on accumulation of chlorophyll (**a**), H_2O_2 (**b**), TBARS (**c**), soluble sugars (**d**) and proline (**e**) in *T. pieninicum* shoots directly after 9 months storage at 4 °C (**a**). Statistically significant differences assessed using Tukey test at $p \leq 0.05$: to the control plants in the same light conditions are denoted *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$); between light conditions are denoted $^{\#}$($p < 0.05$), $^{\#\#}$($p < 0.01$), $^{\#\#\#}$($p < 0.001$); between ABA treatment in the same light conditions are denoted $^{\Diamond}$($p < 0.05$), $^{\Diamond\Diamond}$($p < 0.01$), $^{\Diamond\Diamond\Diamond}$($p < 0.001$)

Table 1 Analysis of the interaction of ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on chlorophyll content in *T. pieninicum* leaves performed by two-way ANOVA (α = 0.05)

Factors	3 months		6 months		9 months	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
ABA treatment (A)	60.44	< 0.001	5.40	< 0.05	2.43	n.s
ABA concentration (B)	1.25	n.s	1.61	n.s	1.90	n.s
Interaction						
A × B	2.22	n.s	0.26	n.s	0.57	n.s

Table 2 Analysis of the interaction of the light conditions, ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on H2O2 content in *T. pieninicum* leaves performed by three-way ANOVA (α = 0.05)

Factors	3 months		6 months		9 months	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
ABA treatment (A)	12.00	< 0.01	6.07	< 0.05	5.22	< 0.05
ABA concentration (B)	13.45	< 0.001	7.72	< 0.001	6.40	< 0.01
Light conditions (C)	0.71	n.s	60.66	< 0.001	25.66	< 0.001
Interactions						
A × B	7.95	< 0.001	3.65	< 0.05	0.26	n.s
A × C	0.30	n.s	51.77	< 0.001	0.07	n.s
B × C	0.38	n.s	4.88	< 0.01	1.27	n.s
A × B × C	1.84	n.s	7.45	< 0.001	0.47	n.s

Table 3 Analysis of the interaction of the light conditions, ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on TBARS content in *T. pieninicum* leaves performed by three-way ANOVA (α = 0.05)

Factors	3 months		6 months		9 months	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
ABA treatment (A)	0.13	n.s	5.76	< 0.05	6.23	< 0.05
ABA concentration (B)	0.47	n.s	1.89	n.s	13.35	< 0.001
Light conditions (C)	44.12	< 0.001	104.28	< 0.001	460.35	< 0.001
Interactions						
A × B	4.47	< 0.01	0.14	n.s	11.33	< 0.001
A × C	0.16	n.s	0.01	n.s	3.77	n.s
B × C	0.13	n.s	0.49	n.s	18.83	< 0.001
A × B × C	4.49	< 0.01	0.14	n.s	1.30	n.s

Table 4 Analysis of the interaction of the light conditions, ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on soluble sugars content in *T. pieninicum* leaves performed by three-way ANOVA (α = 0.05)

Factors	3 months		6 months		9 months	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
ABA treatment (A)	17.99	< 0.001	75.18	< 0.001	30.35	< 0.001
ABA concentration (B)	8.41	< 0.001	4.88	< 0.01	67.94	< 0.001
Light conditions (C)	11.00	< 0.01	183.50	< 0.001	476.95	< 0.001
Interactions						
A × B	6.11	< 0.01	43.31	< 0.001	42.59	< 0.001
A × C	8.16	< 0.01	29.57	< 0.001	2.99	n.s
B × C	6.56	< 0.01	7.68	< 0.001	59.67	< 0.001
A × B × C	0.47	n.s	29.88	< 0.001	32.98	< 0.001

was inside synseeds structure at a concentration of 19 µM and 38 µM and synseed were stored also under light conditions. After 6 months of storage ABA inhibited accumulation of soluble sugars in tissue cooled in both light conditions, while after 3 months ABA did not show any significant effect on analyzed parameter (Fig. S12).

The level of accumulated proline depended on the method of ABA treatment, its concentration and light conditions

during storage (Fig. 8e; Table 5). After 9 months of storage proline level increased with an increase of ABA concentration inside synseeds structure, especially in light-stored explants. In contrast, the material treated with 19–57 µM ABA during preculture accumulated significantly less proline under light conditions than control plants. Similar results were obtained after 6 months of storage, but ABA did not affect proline accumulation after the shortest storage time (Fig. S13).

In response to the cold stress, endogenous ABA level increased significantly in shoots obtained from the synseeds non-treated with ABA stored under light conditions (38.0 ± 4.0 ng/g FW; Fig. 9a), while in the dark ABA accumulation after cooling (5.8 ± 2.6 ng/g FW) was comparable to the non-stored control (4.9 ± 0.6 ng/g FW; data not shown in the figure). The explants stored under light conditions and exposed to ABA at a concentration of 38 µM and 76 µM (both ABA-treatment) accumulated less endogenous ABA compared to the cold-stored control, while in the dark 38 µM ABA stimulated accumulation of endogenous ABA in the explants. Treatment with higher ABA concentration during storage in the dark did not affect the endogenous level of the phytohormone compared to the stored control and in addition determined level was comparable to non-stored control.

Three-way ANOVA showed that the endogenous ABA level depended on ABA treatment method, its concentration and the light conditions (Table 6). A significant correlation between the examined factors was noted only for the ABA concentration and light conditions. Like ABA, the level of JA increased significantly in control shoots after cold-storage under the light conditions (187.4 ± 25.0 ng/g FW) in comparison to the non-stored control (31.2 ± 3.2 ng/g FW) (Fig. 9b). It was showed that ABA treatment, irrespective of its concentration and method of application, led to a statistically significant decrease in the level of JA in explants stored under light conditions. In shoots from all analyzed variants of synseed storage in the dark, the JA level was comparable to the control. Three-way ANOVA showed that light conditions had a significant effect on JA levels individually, as well as in correlation with ABA treatment method or ABA concentration (Table 6).

Discussion

Explant encapsulation used during storage of *T. pieninicum* is designed to protect plant tissue from low temperature damages and to physically reduce initial growth rate through

Table 5 Analysis of the interaction of the light conditions, ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on proline content in *T. pieninicum* leaves performed by three-way ANOVA (α = 0.05)

Factors	3 months		6 months		9 months	
	F value	*p* value	*F* value	*p* value	*F* value	*p* value
ABA treatment (A)	7.71	< 0.01	38.44	< 0.001	117.13	< 0.001
ABA concentration (B)	8.36	< 0.001	10.75	< 0.001	37.65	< 0.001
Light conditions (C)	60.82	< 0.001	60.15	< 0.001	69.02	< 0.001
Interactions						
A × B	4.62	< 0.01	8.27	< 0.001	3.93	< 0.001
A × C	15.77	< 0.001	11.88	< 0.01	10.43	< 0.01
B × C	4.72	< 0.01	1.50	n.s	2.10	n.s
A × B × C	2.23	n.s	0.42	n.s	1.31	n.s

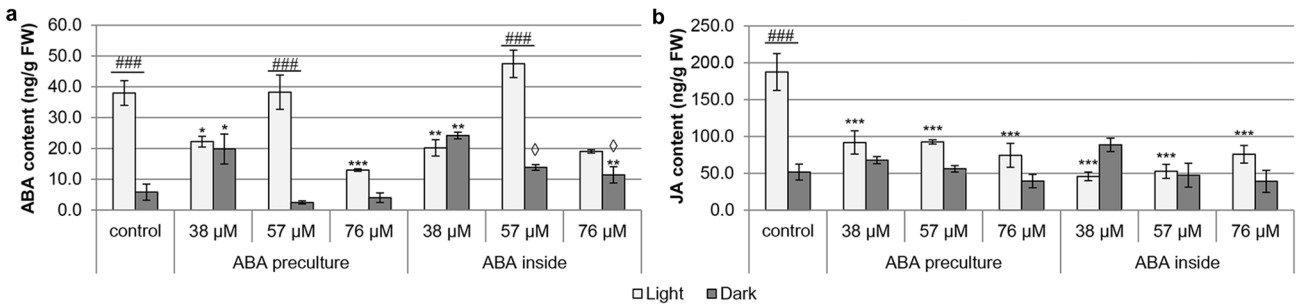

Fig. 9 The effect of the light conditions and ABA treatment on the content of endogenous ABA (**a**) and JA (**b**) in shoots of *T. pieninicum* obtained from artificial seeds after storage for 9 months at 4 °C; the significance of differences was assessed by the Tukey test at α = 0.05 and determined: to the control plants in the same light conditions are denoted *(p < 0.05), **(p < 0.01), ***(p < 0.001); between light conditions are denoted #(p < 0.05), ##(p < 0.01), ###(p < 0.001); between ABA treatment in the same light conditions are denoted ◊(p < 0.05), ◊◊(p < 0.01), ◊◊◊(p < 0.001)

Table 6 Analysis of the interaction of the light conditions, ABA treatment and its concentration during cold storage for 3, 6 and 9 months at 4 °C on endogenous ABA and JA content in *T. pieninicum* leaves performed by three-way ANOVA (α = 0.05)

Factors	ABA		JA	
	F value	*p* value	*F* value	*p* value
ABA treatment (A)	13.80	< 0.01	3.57	n.s
ABA concentration (B)	24.65	< 0.001	2.27	n.s
Light conditions (C)	74.14	< 0.001	6.04	< 0.05
Interactions				
A × B	2.63	n.s	1.25	n.s
A × C	1.02	n.s	6.25	< 0.05
B × C	42.54	< 0.001	4.29	< 0.05
A × B × C	0.23	n.s	2.38	n.s

an alginate coating (Kamińska et al. 2018a). Slowing the explants growth during storage is highly desirable due to the longer time without passage what reduce costs and the risk of contamination. The simplest method to slow down the growth of the plant material is to lower the culture temperature (Divakaran et al. 2006). Storage of synseeds at a reduced temperature was used to protect endangered species such as *Celastrus paniculatus* (Fonseka et al. 2019), *Ceropegia barnesii* (Ananthan et al. 2018), *Cymbidium aloifolium* (Pradhan et al. 2016), *Eclipta alba* (Salma et al. 2019), *Mondia whitei* (Baskaran et al. 2015), *Spilanthes acmella* (Sharma et al. 2009) and *Withania coagulans* (Rathore and Kheni 2017).

Morphological observations combined with biochemical analyzes have shown that light is a stress factor during storage of *T. pieninicum* synseeds, what was with agreement with our previous results also those obtained for non-encapsulated shoot tips of this species (Kamińska et al. 2016). Many studies indicated numerous relationships between light stress and low temperature conditions. Light-induced stress may result from an imbalance between the absorption and usage of a light energy (Ensminger et al. 2006; Beck et al. 2007). Absorption of a large amount of light in low metabolic activity conditions resulted from a lowered environmental temperature may led to damage of D1 protein, the site of attachment of plastoquinone QB. The inability to transfer energy to the PS reaction center may lead in turn to ROS production (Roach and Krieger-Liszkay 2014). Furthermore, under cold conditions electron transport may be inhibited by changing the structure of the cell membrane (Ensminger et al. 2006) and by reducing the activity of photosystem II (Borawska-Jarmułowicz et al. 2014) what may increase stress on which plant tissue is exposed.

Storage of synseeds in the dark seems to be a better method due to a more effective growth inhibition in comparison to the light conditions. This was confirmed for *Hibiscus moscheutos* synseeds which might be stored in the dark at 5 °C for almost 20 months with 80% viability (West et al. 2006) and for *Splachnum ampullaceum* synseeds stored under the same conditions for 30 months with maintaining viability of 50% explants (Mallón et al. 2007). Better results after storing the plant material in the dark compared to a light conditions were also obtained for *Vitis berlandieri x V. riparia* synthetic seeds after 9 months of cooling (Benelli 2016).

In order to further growth reduction of shoots during *T. pieninicum* synseeds storage in this study natural growth retardant abscisic acid was examined. Results showed that ABA limited shoots growth during the preculture preceding storage, as well as during synseeds storage (inside treatment) in both light variants. This is a potential indicator of the possibility of extending storage due to slower nutrient compounds consumption. Numerous studies indicated the inhibitory effect of ABA in plant in vitro culture. ABA inhibited the elongation growth of *Citrus lemon* (Kotsias and Roussos 2001), *Oryza sativa* (Pattanagul 2011) and *Teucrium polium* shoots (Rabba'a et al. 2012) during in vitro culture.

Numerous reports suggested that the addition of ABA to the medium allow extension of the storage time of viable explants e.g., *Malus domestica* (Kovalchuk et al. 2009), *Tetrastigma hemsleyanum* (Peng et al. 2015) or *Lilium davidii* and *L. longiflorum* (Yun-peng et al. 2012). However, medium supplementation with ABA reduced the survival of adventitious shoots of *Vitis heyneana* (Pan et al. 2014). Also in *Physalis peruviana* the addition of ABA to the synseeds reduced the survival of nodal cuttings and inhibited their elongation growth (Yücesan et al. 2015).

An important step in the in vitro preservation of plant material is the possibility of their micropropagation after storage. Regardless of the ABA treatment and duration of synseeds cold-storage, the shoot tips of *T. pieninicum* have been propagated under optimal growth conditions on the medium supplemented with BAP, but this process was more effective after storage in the dark, what was also noted after storage of non-encapsulated shoots of *T. pieninicum* (Kamińska et al. 2016). Similar results were published by Hausman et al. (1994) storing shoot tips of *Populus tremula* x *P. tremuloides* for 3 months at 4 °C in the dark without influence on shoot proliferation rate in comparison to non-stored control. Piccioni et al. (1996) reported regrowth of encapsulated explants of M.26 apple rootstock after 2 and 4 months at 4 °C. Effective propagation after synseeds storage was also noted for *Olea europea* and *Quercus cerris* after 6 weeks at 4 °C (Micheli et al. 1998; Tsvetkov and Hausman 2005), *Corymbia torelliana* × *C. citriodora* and *Khana senegalensis* after 12 months at 14°C in the dark (Hung and Trueman 2012). ABA treatment did not affect *T. pieninicum* shoots ability to proliferate during post-storage regrowth. Similarly, addition of ABA to the medium did not affect the

proliferation rate of *Cedrus libani* explants after 6 months of cold-storage (Renau-Morata et al. 2006). Also, the preculture of *Castanea sativa* shoots on the medium supplemented with 5 – 10 µM ABA did not affect their survival, but highest ABA concentration reduced shoots ability to proliferate after storage at 4 °C (Capuana and Di Leonardo 2013). In turn, in the study of da Silva and Scherwinski-Pereira (2011) the addition of ABA into MS medium reduced survival and proliferation rate of *Piper aduncum* and *P. hispidinervum* after 6 months of storage.

Taraxacum pieninicum shoots were effectively rooted on MS medium without auxin. Only shoots obtained after 3- and 6-months storage in capsule containing highest ABA concentration did not root. However, root organogenesis occurred after transfer of shoots to the vermiculite-sand mixture in ex vitro conditions what was also reported for other species from the *Asteraceae* family, e.g. *Carlina acaulis* (Trejgell et al. 2009).

Storage of the plant material using in vitro cultures, due to the presence of cold stresses and growth regulators (especially cytokinins) during regrowth stage may lead to genetic variability. However, during shoots multiplication from shoot tips usually the DNA level remained stable (Clarindo et al. 2008). In our previous research we verified the genetic stability of the plants obtained after long-term storage of non-encapsulated shoot tips on medium supplemented with ABA. No differences were observed using flow cytometry, RAPD, ISSR and SCoT markers between regrown plantlets after storage and plants cultivated from seeds in a soil (Kamińska et al. 2016, 2020).

The metabolic content changes under the influence of a low temperature may be associated with (a) catalytic activity or stability of enzymes involved in biosynthesis or degradation of specific compounds; (b) biosynthesis of compounds resulting from cellular damage; (c) regulating the concentration of given compounds to maintain homeostasis or (d) biosynthesis and accumulation of compounds involved in developing tolerance (Janmohammadi 2012). Therefore, the levels of chlorophyll, H_2O_2, TBARS / MDA, soluble sugars, proline as well as ABA and JA were analyzed.

Hordeum vulgare and *Zea mays* explants stored under high light intensity and low temperature were found to increase chlorophyll and xanthophyll content in response to ABA additions, what would indicated the role of ABA in protection of photosystem II (Jia and Lu 2003). The results presented in this paper did not indicate the participation of ABA in this process in *T. pieninicum* during storage in conditions of reduced light intensity because a decrease in Chl content in explants was noted. Furthermore, decreased level of Chl was also observed after preculture with ABA in optimal growth conditions without exposure to the low temperature. The cold conditions during storage of plant material also reduced the Chl content in *Avena nuda* seedlings (Liu et al. 2013). However, short-term storage of synseeds did not affect its level in *T. pieninicum* shoots obtained under optimal growth conditions. Similar results were reported for *Rauvolfia tetraphylla* synseeds stored for 4 weeks at reduced temperature (Faisal et al. 2013).

Numerous scientific reports indicated that under reduced temperature conditions there is an accumulation of ROS that leads to oxidative stress in plant tissues. Hydrogen peroxide is the most stable form of ROS, and its accumulation is considered as a direct response of plant cells to a low temperature (Carvajal et al. 2015). An increase in H_2O_2 content was noted after few hours of storage at 4 °C in *Vitis vinifera* (Rooy et al. 2017). In *A. thaliana* callus cells, the maximum increase in H_2O_2 accumulation was observed after 2 days at 4 °C, but from the 5th day of cooling, the level of H_2O_2 decreased (O'Kane et al. 1996). It was assumed that after the cessation of cold stress, the level of ROS decreases to the level occurring in optimal conditions (Einset et al. 2007). Excessive ROS accumulation can lead to chlorophyll degradation (Kavi Kishor and Sreenivasulu 2014), lipid peroxidation (Bhattacharjee 2005), and even to induction of programmed plant cell death (Desikan et al. 1998). Under stress conditions, level of endogenous ABA increase in plant tissue, what is one of the elements of the signal transductions pathway leading to an increase in chlorophyll photooxidation resistance (Pospíšilová et al. 2009) and activation of antioxidant enzymes (Jiang and Zhang 2001). Application of exogenous ABA may also reduce the effects of stress on plant tissue. However, in the case of *T. pieninicum* no such effect was found. A significant increase in H_2O_2 accumulation has been shown in the light, while ABA may not be involved in the production or neutralization of ROS in this species. However, high concentration of ABA increased H_2O_2 level in *T. pieninicum* leaves grown under optimal growth conditions.

The consequence of ROS accumulation may be a peroxidation of membrane lipids which is denoted by the accumulation of malonodialdehyde (Liu et al. 2013; Rooy et al. 2017). *T. pieninicum* explants stored in the light accumulated significantly more TBARS / MDA than in the dark. In addition, its level in plant tissue increased with prolonging storage time. The ABA application did not affect the level of the tested parameter after 3 and 6 months of storage. However, after 9 months significantly less TBARS/MDA was determined in plant tissue treated with ABA than in the control, especially after storage under light conditions, what may indicated the involvement of ABA in lipid peroxidation inhibition mechanisms. Similar results were obtained for *Saccharum officinarum* (Huang et al. 2015) and *Vitis vinifera* (Karimia et al. 2016), where foliar application of 50–200 µM ABA led to a decrease in TBARS content under reduced temperature conditions.

An increased content of soluble sugars during acclimation to cold has been observed for many species, including *A. thaliana* (Klotke et al. 2004), *Triticum aestivum* (Kamata and Uemura 2004), *Hordeum vulgare* (Tabaei-Aghdaei et al. 2003) and *Solanum tuberosum* (Folgado et al. 2013). Tognetti et al. (1990) proposed that the increased accumulation of sugars in plant tissue enhanced resistance of the species to a low temperature. After 3 months of storage of *T. pieninicum* no effect of ABA on the accumulation of soluble sugars in tissue was noted. Additionally, treatment of explants with ABA during preculture and prolonged storage for up to 9 months in the dark also did not affect the level of accumulated sugars compared to controls. In contrast, literature reports point to a relationship between ABA accumulation and soluble sugars under cold stress (Rooy et al. 2017).

Accumulation of proline in the tissues of cooled plant material has proven to be of key importance in the process of cold acclimation, what has been shown for many species, including *Saccharum officinarum* (Rasheed et al. 2010), *Vitis vinifera* (Rooy et al. 2017) and *Avena nuda* (Liu et al. 2013). However, the increased proline accumulation does not necessarily indicate a high tolerance to coldness or freezing of a given species, but it is necessary to adapt the tissue to a condition that may lead to a disruption of its growth (Rooy et al. 2017). In *Quercus rober* proline abolished the inhibitory effect of cold on the growth of embryogenic tissue (Gleeson et al. 2004), while in *Cicer arietinum* proline stimulated the accumulation of sucrose and chlorophyll in plants exposed to low temperature (Kaur et al. 2011). Accumulation of proline did not depend on storage time in *T. pieninicum.* Higher proline content was determined in light-stored tissue than in the dark, what confirmed that light combine with the low temperature causes severe stress in this species. Treatment of ABA explants did not significantly affect proline accumulation during storage in the dark. However, the stimulating effect of ABA on proline accumulation was determined in shoots obtained from synseeds containing 76 µM ABA in artificial endosperm and stored for 9 months under light conditions. Also, high ABA concentration resulted in increased proline accumulation after preculture under optimal growth conditions. Numerous studies indicated that ABA led to increased proline accumulation (Kavi Kishor and Sreenivasulu 2014), such a relationship was noted for example for *Triticum aestivum* (Hou et al. 2010).

Exposure of *T. pieninicum* tissue to the low temperature led to a significant increase in endogenous ABA and JA only after storage under light conditions. It is well-known that the level of endogenous ABA in plant material can be controlled by light (Xu et al. 2014; Seo et al. 2009). In our study reduced shoot proliferation after storage under light conditions may probably resulted from the high levels of endogenous ABA in tissue. However, no significant differences between light conditions during storage and ABA accumulation were noted. It seems that the response of this species to cold-storage under continuous light is more complex and does not depend only on the accumulation of ABA in tissue. Treatment of explants with ABA did not lead to an increase in endogenous ABA accumulation in tissue compared to the cooled control. Under low temperature conditions, an increase in endogenous ABA levels was noted in *Oryza sativa* (Oliver et al. 2007) and *Vitis vinifera* (Rooy et al. 2017). In *Actinidia deliciosa* endogenous ABA levels increased until the second month of cooling at 0 ± 0.5 °C. However, longer storage led to a decrease in ABA accumulated levels (Yang et al. 2013). In *Arabidopsis* exposure to JA led to a decrease in ABA accumulation, while exogenous ABA under abiotic stress stimulated JA biosynthesis (Adie et al. 2007; Fan et al. 2009; Wang et al. 2018). However, treatment of *T. pieninicum* tissue with ABA led to significantly lower JA content after light storage.

Summing up the results, it can be stated that treatment with ABA reduces the effects of cold stress in *T. pieninicum* tissues stored in the light, what was manifested by reduced H_2O_2 levels in shoots subjected to the preculture on medium supplemented with ABA (19–57 µM). In addition, significant reduction of membrane lipid peroxidation was noted after tissue treatment with ABA before and during cold-storage. No stimulating effect of treatment with ABA on the level of chlorophyll and the proliferation rate of cooled shoots was observed. Signal transduction pathways of ABA and JA are interrelated because treatment of ABA tissue significantly reduced JA content. ABA and JA are involved in the protective mechanisms of plant tissues against low temperature stress, however, processes associated with acclimatization to the cold are multifactorial, and the studied phytohormones are not the only factors affecting the expression of genes associated with the plant's response to cold. Potential participation in this process might be also attributed to ethylene (Zhao et al. 2014), gibberellic acid (Richter et al. 2013), brassinosteroids (Singh et al. 2012), salicylic acid (Dong et al. 2014), ROS and cytosolic Ca^{2+} (Chinnusamy et al. 2007).

Conclusions

In conclusion, light combined with low temperature is a stress factor during storage of *T. pieninicum,* regardless of the ABA exposure. Treatment with abscisic acid of *T. pieninicum* explants limits their growth, which contributes to the extension of their storage time at reduced temperature at least to 9 months and alleviates the effects of cold stress. Shoots of *T. pieninicum* after cold storage and ABA treatment are able to regrowth effectively under optimal growth

conditions, while field acclimatized regenerants after storage and propagation in vitro are able to flower.

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Author contributions MK designed and carried out all the experiments, analyzed the data and wrote the manuscript, JK helped in analysis of endogenous phytohormones level and AT helped in analysis of the data and in writing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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