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Dormancy removal of apple seeds by cold stratification is associated with fluctuation in H_2O_2, NO production and protein carbonylation level

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ABSTRACT

Reactive oxygen (ROS) and nitrogen (RNS) species play a signaling role in seed dormancy alleviation and germination. Their action may be described by the oxidative/nitrosative "window/door". ROS accumulation in embryos could lead to oxidative modification of protein through carbonylation. Mature apple (*Malus domestica* Borkh.) seeds are dormant and do not germinate. Their dormancy may be overcome by 70–90 days long cold stratification. The aim of this work was to analyze the relationship between germinability of embryos isolated from cold (5 °C) or warm (25 °C) stratified apple seeds and ROS or nitric oxide (NO) production and accumulation of protein carbonyl groups. A biphasic pattern of variation in H_2O_2 concentration in the embryos during cold stratification was detected. H_2O_2 content increased markedly after 7 days of seeds imbibition at 5 °C. After an additional two months of cold stratification, the H_2O_2 concentration in embryos reached the maximum. NO production by the embryos was low during entire period of stratification, but increased significantly in germination *sensu stricto* (i.e. phase II of the germination process). The highest content of protein carbonyl groups was detected after 6 weeks of cold stratification treatment. Fluctuation of H_2O_2 and protein carbonylation seems to play a pivotal role in seed dormancy alleviation by cold stratification, while NO appears to be necessary for seed germination.

Introduction

Dormancy is a temporary block of viable seeds to complete germination. Dormant seeds cannot germinate even under environmental conditions favorable for the germination process. By contrast, completely non-dormant seeds have the capacity to germinate over the wide range of physical environmental factors possible for the plant genotype (Finch-Savage and Leubner-Metzger, 2006). Additionally, it has been proposed that dormancy should not be associated only with the block of germination. Even if the seeds germinate, abnormal seedlings may develop from seeds when dormancy was not completely removed. Stratification is an old and simple method of pre-germination seed treatment to break dormancy and to promote rapid germination. It is based on the simulation of the natural conditions that seeds endure before germination. Depending on the species, seeds need only warm or cold stratification or a combination of warm and cool treatments followed by warmth to germinate. Apple (*Malus domestica* Borkh.) seeds have often been used as a model to study deep embryonic dormancy, since apple embryos remain dormant even after isolation from the seed coat and endosperm. Their deep dormancy is removed by a three-month-long cold (5 °C) stratification leading to quick and uniform germination and also to growth of seedlings without any morphological deformation (Lewak, 2011 and references therein). Although the phenomenon of cold stratification has been well described, the mechanisms of chilling-dependent release from deep embryonic dormancy of seeds are only partially known, and from a biochemical point of view are based on data collected more than 20–30 years ago. The principal model still in use is focused on stratification-dependent alterations in the phytohormonal balance in embryo axes (Blake et al., 2002; Koornneef et al., 2002). Results obtained on apple seeds also favor a hormonal regulation (via giberelins, ABA and ethylene) of embryo dormancy alleviation (Rudnicki et al., 1972; Subbaiah and Powell, 1992; for review see Lewak, 2011). Bogatek et al. (1991) demonstrated that short term (6 h) fumigation with hydrogen cyanide (HCN), a product of hydrolysis of cyanogenic compounds accumulated in apple seeds, results in removal of embryo dormancy. Quite recently, it was shown that reactive oxygen (ROS) and nitrogen (RNS) species would also play a fundamental role in apple embryo dormancy breakage (Gniazdowska et al., 2010a,b). Short term (3 h) pre-treatment of dormant apple embryos with various donors of nitric oxide (NO) stimulated germination and growth of young seedlings. Moreover, we demonstrated that according to model of

Abbreviations: ABA, abscisic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DNPH, 2,4-dinitrophenylhydrazine; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluoresceine diacetate; HCN, hydrogen cyanide; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species.

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the "oxidative window" described by Bailly et al. (2008), the elevated ROS production in the embryos during NO- or HCN-mediated dormancy alleviation is necessary for seed transition from the dormant to the non-dormant state, and to accomplish the germination process (Gniazdowska et al., 2010a). Similarly, the hypothesis of the "nitrosative door", describing RNS levels necessary to terminate *sensu stricto* germination of the embryos, was proposed (Krasuska and Gniazdowska, 2012).

It is postulated that ROS production in dry seeds would result mainly from non-enzymatic reactions such as Amadori and Maillard or lipid peroxidation (Sun and Leopold, 1995). One consequence of ROS accumulation in the cell may be induction of posttranslational modifications of proteins. Among such modifications, the most frequently detected is carbonylation – formation of carbonyls groups (aldehydes or ketones). This modification can be formed by a derived metal oxidative (MCO) attack on amino acid such as lysine, arginine, proline, threonine, or may result from the addition of reactive carbonyl species (RCS), which are formed from the oxidation of carbohydrates or lipids (Dalle-Donne et al., 2003). Eichholtz et al. (1983) detected a gradual decrease in the amount of the most predominant axis polypeptides (possibly storage proteins) during apple seed stratification, accompanied by some alterations in soluble protein composition. It is currently believed that carbonylation not only alters protein activity, but predominantly increases their susceptibility to proteolytic attack (Dean et al., 1997; Vanita et al., 2008). Moreover, the specific modifications in the pattern of carbonylated proteins were detected in germinating sunflower (*Helianthus annuss*) (Oracz et al., 2007) and *Arabidopsis thaliana* seeds (Job et al., 2005; Rajjou et al., 2006), suggesting the important role of this modification in the regulation of seed dormancy and germination (for review see Arc et al., 2011).

In this work, we explored, for the first time, the relationship between the hydrogen peroxide (H_2O_2)/NO level in embryos and pattern of protein carbonylation during dormancy alleviation of apple seeds by cold (5 °C) stratification. We analyzed H_2O_2 concentrations, NO levels and fluctuations in concentrations of protein carbonyl groups in embryos isolated from seeds characterized by the various depth (degree) of dormancy, resulting from different periods of seed stratification at 5 °C. Moreover, to determine the impact of temperature during dormancy removal, experiments were also performed on seeds stratified at 25 °C, which is unable to remove deep embryonic dormancy of apple embryos (Côme, 1980/81; Lewak, 2011).

Materials and methods

Plant material

The experiment was carried out on apple (*Malus domestica* Borkh. cv. Antonówka) seeds harvested in 2009–2011. Apples were obtained from the Kordel fruit producer at Tarczyn (Poland). Dormant seeds were isolated from ripened apple fruits, dried at room temperature and stored in dark glass containers at 5 °C. The procedure for stratification was performed according to Lewak and Smoleńska (1968). Seeds were mixed with sterile quartz sand at 60% humidity and kept at 5 °C (cold stratification) or 25 °C (warm stratification) in darkness. The duration of warm or cold stratification was 70 or 90 days respectively, as seeds stratified in 25 °C for 90 days lost their viability. Seeds after 1, 7, 14, 21, 40, 70 days of stratification in both temperatures, and after 90 days of stratification only in 5 °C were used for determination. Every week, seeds and sand were mixed to avoid oxygen deprivation and sand humidity was maintained to be kept constant during the entire period of stratification.

Before the analyses, seeds were removed from the sand, rinsed in tap water and embryos were isolated from seed coats and endosperm. Then, isolated embryos were placed in distilled water for 1 h and used for biochemical determinations.

Germination tests

Apple embryos isolated from seeds after a range of (1–90 days) of cold and (1–70 days) of warm stratification were used for germination tests. Germination tests were performed on ø90 mm Petri dishes (20 embryos per dish) filed with filter paper moistened with 5 mL of distilled water. Experiments were performed for 7–8 days in a growth chamber (Sanyo MLR-35OH) at a temperature of 25/20 °C day/night, a 12/12 h photoperiod, and light intensity of 150 μmol PAR m^{-2} s^{-1}. Embryos were consider to have germinated when embryo axes were 2–3 mm long and exhibited characteristic gravitropic bending. Germination tests were done in three independent experiments, with 60 embryos used in each experiment.

Measurement of hydrogen peroxide (H_2O_2) concentration

The concentration of H_2O_2 in apple embryos was determined according to the method of Velikova et al. (2000) with some modifications, as described previously by Gniazdowska et al. (2010a). Apple embryos isolated from seeds stratified at 5 °C or 25 °C for 1–90 or 1–70 days, respectively, were used for determinations. Plant material (5–6 embryos, approximately 0.5 g) was homogenized in ice with 0.1% (w/v) cold TCA and then centrifuged at 15,000 × *g* for 15 min at 4 °C. The H_2O_2 concentration was measured spectrophotometricaly at 390 nm using Shimadzu UV-1700 spectrophotometer, in the assay mixture containing: 0.5 mL supernatant, 1 mL freshly prepared 1 M KI in 10 mM potassium phosphate buffer (pH 7.0) and 0.5 mL 10 mM potassium phosphate buffer (pH 7.0). Measurements of H_2O_2 concentration were done in three-four independent experiments, each in three replicates and expressed as μmol mg^{-1} FW.

Quantitative measurement of protein carbonyl groups

Protein carbonyl groups were determined according to the method of Levine et al. (1994) with some modifications, in embryos isolated from apple seeds stratified at 5 °C or 25 °C for 1–90 or 1–70 days, respectively. Embryos (0.3 g) were homogenized in 3 mL 0.1 M Tris–HCl (pH 7.0) containing 1 mM EDTA, 2% (w/v) PVPP, 5 mM DTT, 1% (w/v) protease inhibitor cocktail (Sigma P9599). After 15 min centrifugation 15,000 × *g* at 4 °C, the supernatant was filtered through the cotton wool and 1% (w/v) streptomycine sulphate was added. Then the supernatant was incubated in the dark for 20 min at room temperature. Aliquots of the supernatant containing 0.5 mg protein were incubated with 500 μL 10 mM 2,4-dinitrophenylhydrazine (DNPH) (Sigma) in 2 M HCl in the dark for 35 min at 37 °C. Blank samples (without DNPH) were incubated in 500 μL 2 M HCl. Proteins were precipitated 10 min with 500 μL 20% TCA and the pellets were washed 3 times with 1:1 (v/v) ethanol:ethyl acetate. After each washing step, the samples were centrifuged for 5 min at 10,000 × *g*. Washed pellets were dissolved in 6 M guanidine hydrochloride (Sigma G4505) in 2 M HCl. The absorbance of the supernatant was measured using a spectrophotometer (Shimadzu UV-1700) at 375 nm, and the concentration of protein carbonyl groups was calculated from the extinction coefficient for DNPH, $\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$.

Determinations of protein carbonyl groups were done in three-four independent experiments, each in three replicates and expressed as nmol mg^{-1} protein.

Protein detection

The protein concentration was measured according to Bradford (1976) using bovine serum albumin as standard.

Immunodetection of protein carbonyl groups

Embryo axes (0.03 g – about 25 axes) isolated from stratified apple seeds were homogenized in 1 mL 0.1 M Tris–HCl (pH 7.0) buffer containing 1 mM EDTA, 2% (w/v) PVPP, 5 mM DTT, 1% (w/v) protease inhibitor cocktail (Sigma P9599), 1% (w/v) streptomycine sulphate and 0.1% (w/v) Triton X-100. After centrifugation at 10,000 × *g* for 5 min at 4 °C, the collected supernatant was incubated with 10 mM DNPH in 2 M HCl (w/v). Blank samples were incubated in 2 M HCl, as described above. Incubation was prolonged 30 min in darkness at 26 °C. The reaction was stopped by adding 400 μL 20% TCA. After 10 min the samples were centrifuged for 5 min at 10,000 × *g* and wash 3 times with 0.2 mL 1:1 (v/v) ethanol:ethyl acetate. The pellet was dissolved in 1: 15 (v/v) 5 M thiourea in 0.1 M Tris–HCl (pH 7.0): 0.1 M Tris–HCl (pH 7.0).

For immunolocalization of protein carbonyl groups by dot blotting, extracts from embryo axes, containing 4 μg of proteins (diluted in the same volume), were put onto nitrocellulose membrane (Sigma Z670979). Immunodetection of carbonyl groups was done using the monoclonal primary antibodies (Monoclonal Anti-dinitrophenyl (DNP) antibodies, Sigma A2831) conjugated with the alkaline phosphatase at dilution 1:60,000. Visualization of carbonyl groups was performed using a buffer 100 mM Tris–HCl pH 9.5, 100 mM NaCl, 5 mM MgCl, 0.2 mM NBT, 0.21 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

The experiments were performed using axes of embryos isolated from seeds stratified at 5 °C or 25 °C for 1–90 or 1–70 days, respectively.

Densitometry analysis was performed using ImageJ software (National Institutes of Health, USA). Levels of modified proteins were plotted in the graphs as area under the curve. Assays were done in three independent experiments and their typical results are presented.

Detection of NO production by embryo axes

Production of NO by embryo axes was detected as described previously by Gniazdowska et al. (2010b). The experiments were performed using axes of embryos isolated from seeds stratified at 5 °C or 25 °C for 1–90 or 1–70 days, respectively. Additional tests were done on embryo axes isolated from seeds stratified for 70 days at 25 °C or 90 days at 5 °C cultured in a growth chamber for 1 day. Three isolated embryo axes (approximately 0.005 g) were washed 3 times in 10 mM buffer HEPES–KOH (pH 7.4) and then incubated 1 h in darkness at 20 °C with 50 μL 20 μM fluorescent marker 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Invitrogen D23844) in 10 mM buffer HEPES-KOH (pH 7.4). After incubation, axes were washed 3 times in 10 mM buffer HEPES–KOH (pH 7.4) and transferred to the cuvette containing 700 μL 10 mM HEPES–KOH (pH 7.4). Fluorescence was measured and recorded for 1000 s (excitation 495 nm, emission 515 nm) using a Hitachi F-2500 spectrofluorimeter.

All measurements were carried out in at least 6–7 replicates, and their exact reproducibility was confirmed. Fluorescence was normalized per milligram FW and expressed in fluorescence units.

Statistical analysis

Mean values were computed for each experiment and mean differences were calculated using analysis of variance (ANOVA) and Duncan's test. Standard deviations (SD) are also provided to indicate the variations associated with the particular mean values. Calculations were performed using the package agricolae (de Mendiburu, 2010) for the statistical freeware R version 2.14.2 (R Development Core Team, 2012).

Results

Cold stratification led to dormancy alleviation of apple seeds

Embryos isolated form dormant seeds stratified at 25 °C for shorter than 40 days did not germinate at all, even after 7 days of culture in warm temperature (Fig. 1, Table 1). Still, after 70 days of warm stratification, germination of embryos was very low and did not exceed 20% after 7–8 days of culture (Table 1, Fig. 1). Embryos isolated from seeds stratified for 90 days at warm temperatures did not germinate since they lost viability (data not shown). Therefore, all other biochemical determinations were done only using seeds stratified at 25 °C not longer than 70 days. In contrast, cold stratification resulted in seed dormancy removal, although the beneficial effect of imbibition at cold temperature was time-dependent. Three weeks of cold stratification did not lead to dormancy breakage, and after that time no one embryo was able to germinate (Table 1, Fig. 1). The prolonged stratification treatment resulted in stimulation of embryo germination. Embryos isolated from seeds stratified for 40 days at 5 °C germinated at 85% after 7–8 days of culture (Table 1, Fig. 1). The highest germination rate was observed for seeds stratified 70 days or longer, which germinated at 100% after 24 h of culture at 20/25 °C day/night (Table 1, Fig. 1).

Morphological abnormalities expressed as asymmetric growth and greening of cotyledons were observed after 4 days of culture. Warm stratification did not remove symptoms of apple seed dormancy (Fig. 1). Almost 80% of the embryos isolated from seeds

Table 1
Germination (%) of apple embryos isolated from seeds stratified in cold (5 °C) or warm (25 °C) temperature.

Culture period (days)	Stratification period (days)						
	Cold (5 °C)				Warm (25 °C)		
	21	40	70	90	21	40	70
1	0	0	0	95 ± 10a	0	0	0
2	0	0	90 ± 2a	100	0	0	0
3	0	0	90 ± 1a	100	0	0	0
4	0	35 ± 1c	100	100	0	0	0
5	0	80 ± 2b	100	100	0	0	5 ± 2f
6	0	85 ± 3b	100	100	0	0	10 ± 1e
7	0	85 ± 1b	100	100	0	0	20 ± 1d

Mean of values in a columns are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Average of the 3 replications ±SD.

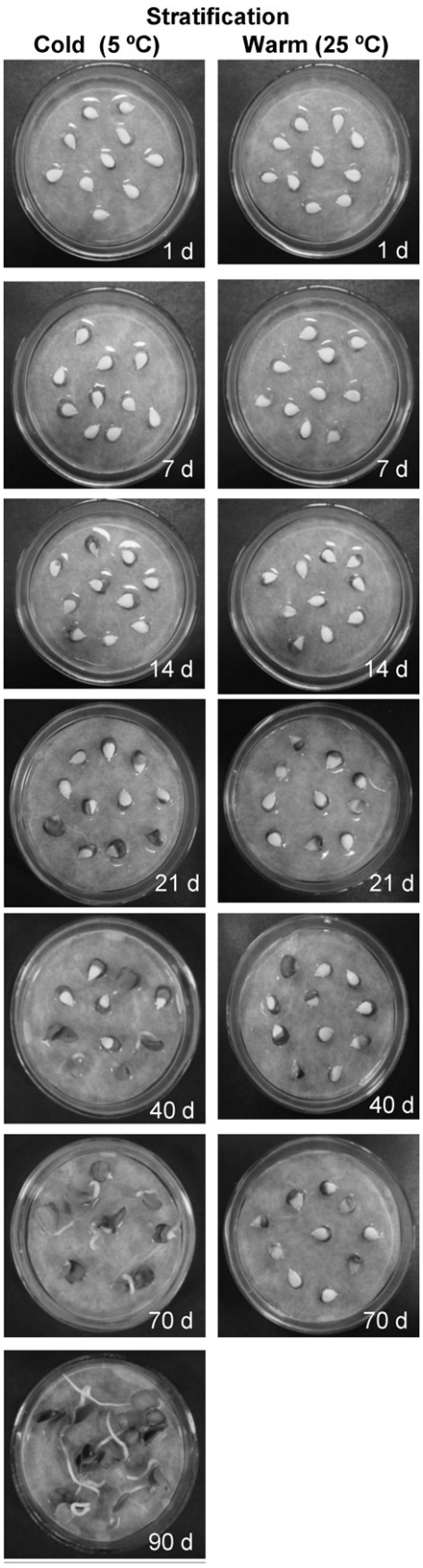


Fig. 1. Apple embryos or seedlings developed from embryos isolated from seeds stratified at cold (5 °C) or warm (25 °C) temperature for 1–90 days or 1–70 days, respectively. Culture of embryos was prolonged for 7–8 days at warm temperature (25/20 °C day/night) and 12 h photoperiod.

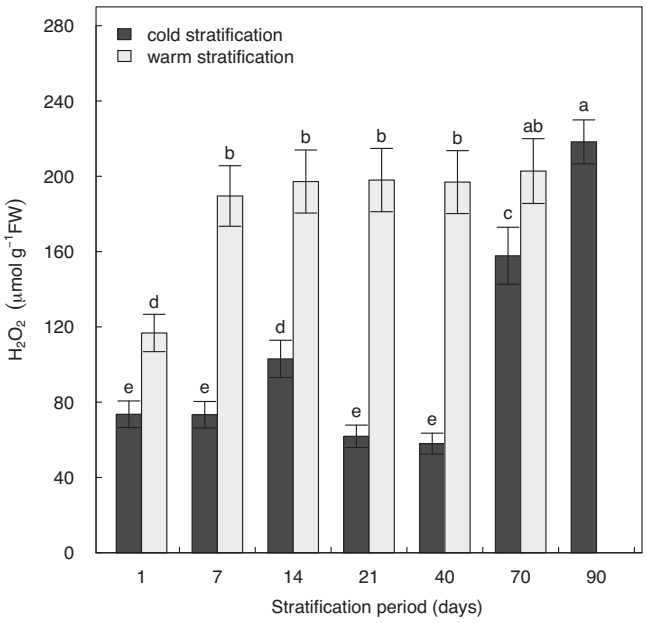


Fig. 2. Concentration of H_2O_2 in embryos isolated from seeds subjected to cold (5 °C) or warm (25 °C) stratification. Mean of values are statistically different at $p < 0.05$ when they share no common letter(s). The comparisons were made using the Duncan test. Average of the 3–4 experiments ±SD.

stratified at 25 °C for 40 days were abnormal, with asymmetric growth and greening of cotyledons (Table 2) Seedlings developed from germinating embryos isolated from seeds stratified at 5 °C longer than 70 days did not exhibit any morphological abnormalities (Table 2, Fig. 1). Morphological anomalies were observed only in non-germinating embryos obtained from seeds subjected to cold stratification for a period shorter than 40 days (Table 2, Fig. 1).

Seed dormancy removal by cold stratification involves fluctuation in embryo H_2O_2 concentration

Stratification at 25 °C resulted in a rapid increase in H_2O_2 concentrations in apple embryos. After first day of warm stratification, the H_2O_2 concentration reached value 120 μmol g^{-1} FW. It increased almost twice in next 7 days and was stable till the end of the treatment (Fig. 2). During cold stratification, a characteristic pattern in fluctuation of H_2O_2 concentration was detected (Fig. 2). At the beginning of the treatment (1–7 days), the H_2O_2 concentration in apple embryos was relatively low (below 80 μmol mg^{-1} FW). The first small maximum in H_2O_2 concentration (around 115 μmol mg^{-1} FW) was noted in embryos stratified for 14 days. After 21–40 days of cold treatment the H_2O_2 concentration decreased to the previous level (Fig. 2). The two-fold enhancement in H_2O_2 concentration in embryos was detected after 70–90 days of cold stratification, reaching a value similar to that observed in embryos stratified at warm temperature.

Soluble protein carbonylation seems to be not strictly involved in dormancy removal of apple seeds by cold stratification

The concentration of protein carbonyl groups was high in embryos isolated from seeds after the first day of warm stratification (Fig. 3A). It decreased insignificantly during the next two weeks, and then enlarged to a maximum after 21 days of warm treatment. Under prolonged stratification at 25 °C, the concentration of soluble protein carbonyl groups in embryos declined to a level of about 7 nmol mg^{-1} protein. In embryos isolated from seeds stratified at cold temperature, the concentration of protein

Table 2
Morphology of apple seedlings developed from embryos isolated from seeds stratified in cold (5 °C) or warm (25 °C) temperature, expressed as % of normally developed seedlings with both green and growing cotyledons.

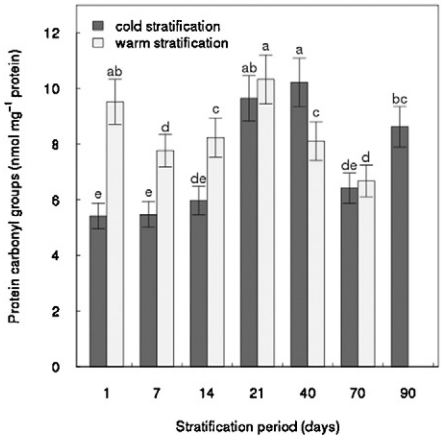
Culture period (days)	Stratification period (days)						
	Cold (5 °C)				Warm (25 °C)		
	21	40	70	90	21	40	70
4	5 ± 1e	35 ± 1c	90 ± 1a	100	0	0	0
5	5 ± 1e	75 ± 1b	100	100	0	5 ± 1e	5 ± 2e
6	10 ± 2e	80 ± 2b	100	100	5 ± 1e	10 ± 1e	10 ± 1e
7	10 ± 1e	80 ± 2b	100	100	10 ± 1e	15 ± 1d	20 ± 1d

Mean of values in a columns are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Average of the 3 replications ±SD determined at 4, 5, 6, 7 day of culture.

carbonyl groups was constant at the beginning of the treatment (for two first weeks) and significantly lower than that observed in warm stratified seeds (Fig. 3A). Drastic (double) enhancement of protein carbonylation was detected in embryos stratified at cold for 21–40 days. Then a transient drop off concentration of protein carbonyl groups was characteristic for embryos treated by cold stratification for 70 days. A prolonged period of chilling resulted in a subsequent increase in the protein carbonylation level (Fig. 3A).

Dot blot analysis of protein carbonyl group in embryonic axes isolated from seeds subjected to warm or cold stratification (Fig. 3B and C) generally exhibited a pattern similar to that observed for quantitative spectrophotometric measurements of protein carbonyl groups in the whole embryos (Fig. 3B and C), with some differences. In axes of warm stratified seeds, the level of the carbonyl group was highest at the beginning of the treatment (after 1 day) and after 21 days, and then decreased significantly, reaching

a



b

Treatment	Stratification period (days)						
	1	7	14	21	40	70	90
Cold stratification							
Warm stratification							

c

Treatment	Stratification period (days)						
	1	7	14	21	40	70	90
Cold stratification	11.6±0.8cd	13.5±1.0c	17,9±1.3b	12.2±0.9c	16,0±1.2b	9.6±0.7d	19.2±1.4a
Warm stratification	21.5±1.6a	17.6±1.3b	12.6±0.9d	20.0±1.5a	14,3±1.0bc	13,9±1.0bc	-

Fig. 3. (A) Concentration of carbonyl groups of proteins in whole embryos isolated from seeds subjected to cold (5 °C) or warm (25 °C) stratification. (B) Visualization of dot blot analysis of proteins carbonylation in extracts from embryonic axes isolated from seeds subjected to cold (5 °C) or warm (25 °C) stratification. (C) Carbonylated proteins remaining as estimated by densitometry analysis of immunoblots. Mean of values are statistically different at $p < 0.05$ when they share no common letter(s). The comparisons were made using the Duncan test. Average of the 3–4 replications ±SD.

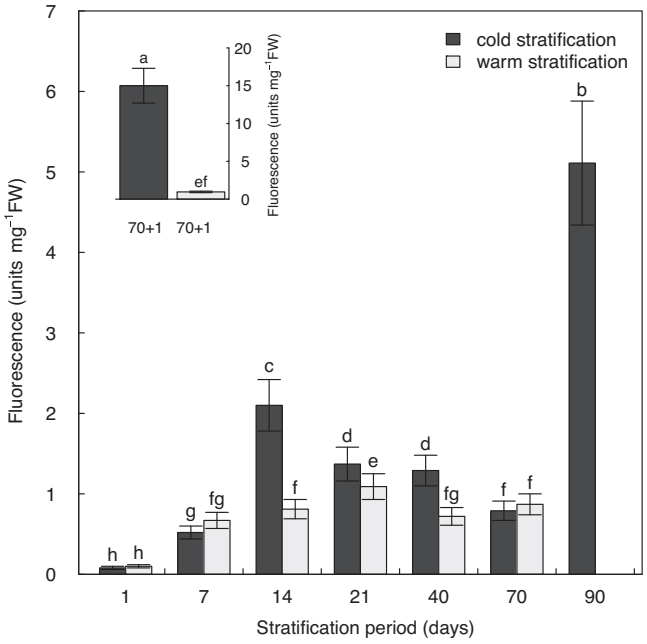


Fig. 4. Production of NO in embryo axes isolated from seeds subjected to cold (5 °C) or warm (25 °C) stratification just after stratification period. Inserted figure shows NO level in axes isolated from seeds subjected to cold (5 °C) or warm (25 °C) stratification for 70 days and then cultured for one day at 25/20 °C day/night photoperiod (70 + 1). Mean of values are statistically different at $p < 0.05$ when they share no common letter(s). The comparisons were made using the Duncan test. Average of the 6–7 replications ±SD.

a value of 13.9 after 70 days (Fig. 3C). By contrast, in axes of cold stratified seeds, a sinusoidal pattern of changes in carbonyl groups was detected. Two transient maxima: after 14 and after 40 days of treatment were noted, and finally, a huge increase in carbonyl was observed after 90 days in axes of non-dormant embryos (Fig. 3B and C).

Seed dormancy removal by cold stratification involves fluctuation in NO production in embryo axes

The production of NO from embryonic axes was measured as an increase of DAF-FM fluorescence within 1000 s (Fig. 4). During warm stratification, no significant changes in NO levels were observed. The NO value was around or less than 1 unit mg^{-1} FW. In cold stratified seeds, it increased gradually and reached a short-term maximum (2 units mg^{-1} FW) after two weeks of treatment (Fig. 4). Then it declined slightly until the 70th day, and did not differ significantly from that observed in axes of warm stratified seeds. The greatest enlargement of NO production was observed in axes isolated from non-dormant seeds after 90 days of stratification at 5 °C. These observations are similar to those of Gniazdowska et al. (2010b), who found that, in embryonic axes of warm or cold stratified apple seeds, using an NO scavenger (0.3 mM cPTIO) significantly lowered the NO detection level (data not shown).

One day of embryo culture (at 25/20 °C day/night) did not alter measurements of NO from axes of seeds stratified 70 days in warm temperature (Fig. 4). It was 0.955 ± 0.1 unit mg^{-1} FW. By contrast, the enormous increase in NO level was detected in axes isolated from seeds stratified for 70 days at 5 °C after the same period of culture in a growth chamber. It increased from around 0.8 to 15.0 units mg^{-1} FW.

Discussion

As described previously, all symptoms of deep physiological dormancy of apple seeds are released only by long-term cold stratification (for review see Lewak, 2011). Our data indicate that the duration of seed imbibition at temperature of 5 °C favorable for dormancy removal should be not less than 70 days. After that time, almost 100% of the embryos germinate and form seedlings without any morphological anomalies. By contrast, embryos isolated from seeds stratified for a time period shorter than 40 days are unable to germinate or to develop typical seedlings. Similar deformation of plant organs have also been observed for peach seedlings growing from seeds stratified for too short period of time (Martínez-Gómez and Dicenta, 2001). Apple embryos isolated from seeds subjected to warm stratification remained dormant independently of the treatment period. They did not germinate or germinated very slowly even after prolonged (70 days long) stratification at 25 °C. Moreover, all if any developed seedlings exhibited typical for dormancy morphological abnormalities. The hormonal theory describing mode of action of low temperature during stratification points to stimulation of gibberellins and ethylene synthesis accompanied by a decline in abscisic acid (ABA) concentration (Lewak, 2011 and references therein). Moreover, it was recently demonstrated that in cold stratified *Arabidopsis* seeds, up-regulated differentially expressed genes included in addition to oxidative pentose phosphate pathway also nitrogen and hormone (ABA, GA and ethylene) metabolism (Narsai et al., 2011; Weitbrecht et al., 2011). In addition to the well-known modification in phytohormone levels, in our experiment, the characteristic fluctuation in the concentration of other signaling molecules such as H_2O_2 and NO in apple embryos subjected to stratification were also noted. An enhanced concentration of H_2O_2 was detected in embryos isolated form seeds stratified at 5 °C for 14 days. This first peak in H_2O_2 production correlated well with one of the maxima of HCN emissions from the seeds, observed only during cold stratification (Dziewanowska et al., 1979) (Fig. 5A). Much more expressed enhancement of the H_2O_2 level occurred in apple embryos in the terminal phase of cold stratification, after 70–90 days. At this physiological stage, seeds lose their dormancy and become ready to germinate in the appropriate environmental conditions. This observation confirms the requirement for ROS production, expressed by the model of the "oxidative window", necessary to accomplish seed transition from dormancy to germination status. Moreover, our data showing the H_2O_2 level in warm stratified seeds suggests that it is not only the ROS concentration that governs dormancy alleviation, but rather a biphasic pattern of H_2O_2 fluctuation is needed. In warm stratified apple seeds, the concentration of ROS increased rapidly at the beginning of treatment, reaching a value nearly twice as high as that measured in embryos isolated from cold stratified ones. In contrast to chilling treatment, H_2O_2 accumulation in warm stratified embryos cannot be combined with emission of HCN, since emission of this gas does not occur in seeds subjected to stratification at 25 °C, even if cyanogenic compounds are accumulated in apple seeds (Dziewanowska et al., 1979). Therefore, it can be assumed that, with warm temperature the non-enzymatic reactions, e.g. such as Amadori and Maillard or lipid peroxidation, lead to sub-optimal, toxic accumulation of ROS. In some respects, this could be compared to the deterioration of sunflower (*Helianthus annus*) seeds associated with lipid peroxidation and decreases in enzymatic antioxidant activities resulting from accelerated aging (Bailly et al., 1996; Bailly, 2004). The elevated level of H_2O_2 in embryos of warm stratified seeds was associated with enhancement in protein carbonylation (Fig. 5B). A significantly lower concentration of protein carbonyl groups was detected in embryos obtained from seeds subjected to stratification at 5 °C. Similar to alterations in ROS concentrations, the

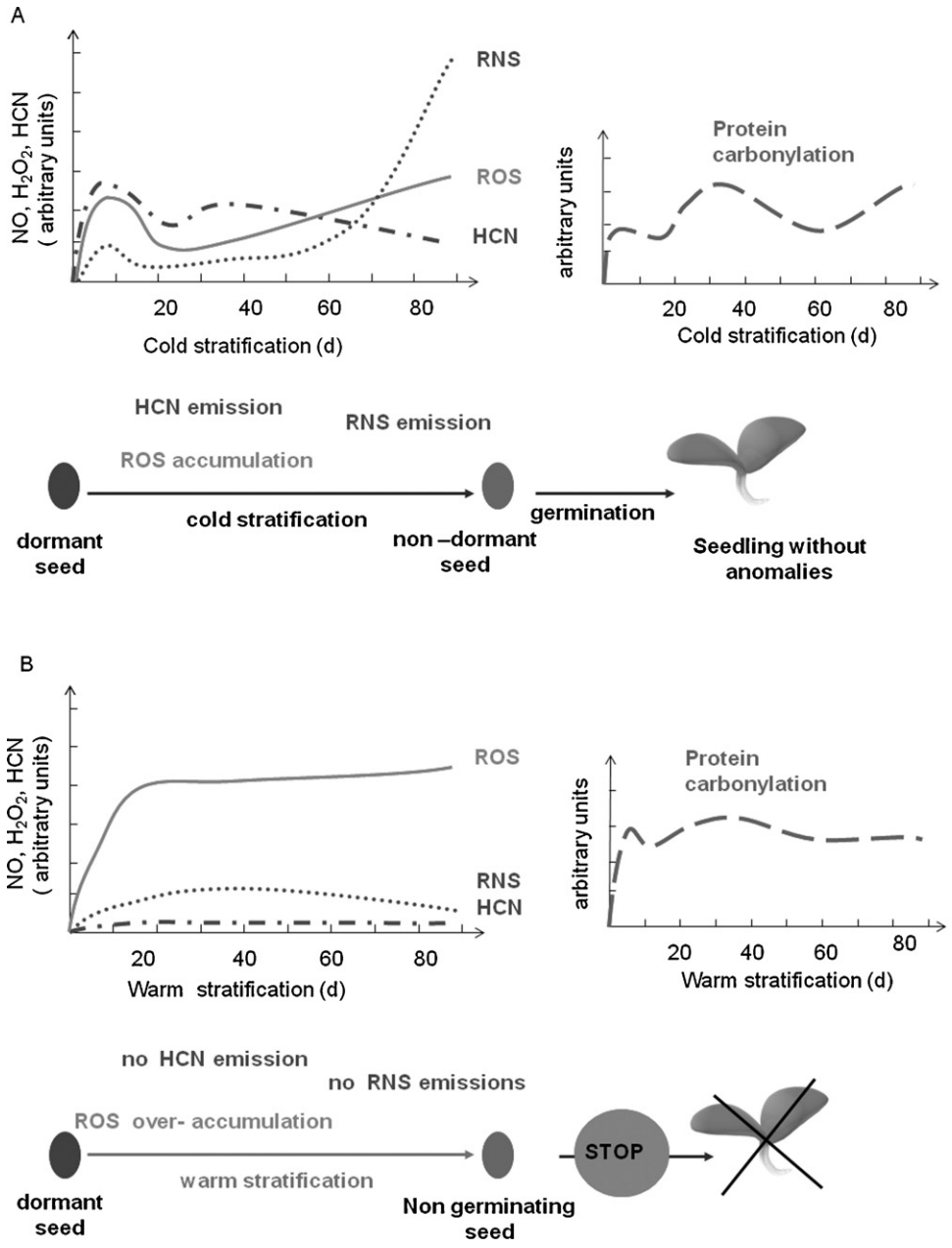


Fig. 5. (A) Scheme showing the interaction between ROS, RNS, HCN and level of protein carbonylation in dormancy alleviation by cold (5 °C) stratification. (B) In comparison, alteration in ROS, RNS, HCN and carbonylated protein croups in seeds subjected to warm (25 °C) stratification treatment, which does not lead to dormancy removal are presented. Data on HCN emission from stratified apple seeds are adopted from Dziewanowska et al. (1979).

process of protein carbonylation seems to show a biphasic characteristic. However, it did not strictly reflect periodic variations in H_2O_2 content in embryos (Fig. 5A). At the beginning of cold stratification (first two weeks), parallel alterations in the content of carbonylated proteins and H_2O_2 were noted. During the prolonged treatment, even with the terminal phase of the cold stratification period (70–90 days), the concentration of both H_2O_2 and protein carbonyl group was high (Fig. 5A). Job et al. (2005) observed that, during imbibition of viable *Arabidopsis* seeds, various carbonylated proteins were accumulated. This oxidative modification was not equally distributed among proteins, but only distinctive ones acted as its target. Therefore, the authors suggested that the specific changes in protein carbonylation patterns are probably required for counteracting and/or utilizing the production of ROS caused by recovery of metabolic activity in the germinating seeds. It was demonstrated that in dry seeds, possibly during the maturation process, 12S cruciferins were predominantly modified by carbonylation and then selectively degraded in the catabolic phase of seed germination. It may also be that in cold stratified seeds, in which HCN emission occurs particularly in the initial phase of treatment, distinct proteins are carbonylated. What is more, Oracz et al. (2007) demonstrated that release of dormancy in sunflower embryos in the presence of HCN or methylviologen (ROS donor) was associated with the appearance of specific carbonylation patterns. In contrast, protein carbonylation in apple seeds subjected to warm stratification probably is nonspecific, and may be similar to the reaction observed in plants under heat stress (Sundaram and Rathinasabapathi, 2009) or age-related disorders detected in animal and mammalian cells (Nyström, 2005) or plants (Johansson et al., 2004).

Carbonylation as oxidative modification depends on the redox potential in the cell, and it can therefore be influenced not only by ROS (directly) but also (indirectly) by RNS (Davies, 2005; Rinalducci et al., 2008). RNS are known to induce specific protein modifications such as S-nitrosylation, leading to thiol oxidation or nitration – NO_2 addition to tyrosine residue. S-nitrosylation is considered as a reversible posttranslational modification, taking part in rapid reaction to environmental conditions (Lindermayr et al., 2005; Palmieri et al., 2010; Astier et al., 2011). Lindermayr et al. (2010) proposed that protein S-nitrosylation may prevent its carbonylation. Pre-treatment of citrus plants (*Citrus aurantium*) by sodium nitropruside (SNP) lowered protein carbonylation induced by salinity stress (Tanou et al., 2009). In the present data set, we directed our attention to the possible existence of S-nitrosylated proteins in apple seeds although, until now, the presence of S-nitrosylated proteins has been confirmed only in *Antaris toxycaria* seeds (Bai et al., 2011). We showed enhanced NO emission from axes of embryos subjected to a 90 days long cold stratification (Fig. 5A). Moreover, 24 h imbibition of embryos isolated from chilling stratified seeds for 70 days led to elevation of NO emission, comparable to that observed during germination of non-dormant embryos (Krasuska and Gniazdowska, 2012). It could be mention also, that NO scavenger – cPTIO significantly (to only 15%) decreased germination of apple embryos isolated from non-dormant seeds, stratified at 5 °C for 90 days (Gniazdowska et al., 2010b), suggesting the crucial role of this molecule in the regulation of seed germination. High emission of NO during elongation of embryo axes in *sensu stricto* germination would result from its evolution from S-nitroso thiols (SNOs) e.g. S-nitrosoglutathione (GSNO), which may occur in the presence of reductants (ascobate or glutathione and Cu^{2+}) (Leterrier et al., 2011). Thioredoxin is also able to denitrosylate proteins, and is activated by ROS (Wu et al., 2011). Moreover it is known that thioredoxin takes part in redox regulation of protein metabolic status in germinating seeds (Arc et al., 2011).

The key role of NO in seed dormancy release is well documented (Bethke et al., 2007b). Some aspects of NO-mediated dormancy alleviation of apple embryos discussed in our previous work (Gniazdowska et al., 2007; 2010a; Krasuska and Gniazdowska, 2012) also focused on a strict interaction between ROS and RNS. Moreover, Liu et al. (2010) demonstrated that NO release by germinating *Arabidopsis* seeds depends on ROS, since it was enhanced by H_2O_2 application, and decreased by an inhibitor of H_2O_2 production. Perhaps the boost in H_2O_2 concentration observed in embryos after 90 days of cold stratification is required for NO synthesis. The impact of ROS accumulation in dormancy alleviation by cold stratification may also involve the regulation of hormonal balance. It is possible that H_2O_2 controls seed dormancy by activation of genes involved in ABA catabolism (*CYP707A*). ROS also regulate GA biosynthesis by stimulating expression of *GA3ox*, *GA20ox* genes in *Arabidopsis* (Liu et al., 2010). Moreover, it cannot be ruled out that NO participates in activation of *GA3ox* genes, as described in *Arabidopsis* seeds (Bethke et al., 2007a). In addition, similar alterations in transcription of genes encoding key enzymes in ABA and GA catabolic pathways were obtained in apple embryos (unpublished results).

Our data are in agreement with the concept of oxidative signaling in plant cells (Foyer and Noctor, 2005). It is believed, as stated by Rinalducci et al. (2008), that some oxidative and likely also nitrosative protein modifications, being specific and reversible, play a key role in normal cellular physiology. In this work we demonstrated the characteristic pattern of protein carbonylation and H_2O_2/NO production in seeds subjected to dormancy alleviation by cold stratification. More detailed experiments should be performed to identify molecular targets in embryonic axes that contribute to the seed transition from the dormant to the non-dormant stage.

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