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Lipid mobilization, gluconeogenesis and ageing-related processes in dormant walnut kernels during moist chilling and warm incubation

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Abstract

To understand the beneficial effects of cold conditions during the alleviation of dormancy of walnut (*Juglans regia* L.), lipid mobilization, gluconeogenesis and changes related to ageing were compared in dormant kernels incubated in the cold and under deteriorating warm conditions. Stratifying kernels at 5°C for 30 d enhanced their germination capacity, whereas warm-incubated (27°C) kernels turned rancid after 20 d and had reduced germination. Kernel imbibition was sufficient to bring about lipid mobilization, irrespective of temperature of incubation. Although imbibed kernels displayed high isocitrate lyase activity, starch and soluble sugar accumulation occurred only under cold conditions. Deteriorative 64 kDa fatty acyl-ester hydrolase activity declined in cold-stratified kernels. Cold treatment also led to reduced lipid peroxidation and hydrogen peroxide in kernels. The activity of $NADP^+$-isocitrate dehydrogenase, an NADPH-generating enzyme, declined in warm-incubated kernels. Thus, warm-incubated kernels undergo ageing associated with oxidative stress, but there are beneficial effects of cold stratification in preventing deteriorative ageing-related processes. Imbibition is sufficient to allow lipid mobilization to occur in dormant walnut kernels, although cold stratification accompanied by gluconeogenesis is essential for kernel germination.

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Keywords: acyl-ester hydrolase, cold stratification, isocitrate lyase, *Juglans regia*, lipid mobilization, seed ageing

Introduction

Mature seeds of many temperate tree species are dormant and only germinate after moistened seeds are exposed to cold (5°C, cold stratification). Changes in the metabolism or perception of plant hormones are frequently reported during such cold stratification (Corbineau *et al.*, 2002; Jacobsen *et al.*, 2002; Schmitz *et al.*, 2002). Cold stratification may also activate phytase (Andriotis *et al.*, 2004), lipases (Li and Ross, 1990a; Zarska-Maciejewska, 1992) and proteases (Forward *et al.*, 2001), whereas ultrastructural studies have shown a gradual decline of seed reserves during this treatment (Dawidowicz-Grzegorzewska, 1989; Wang and Berjak, 2000; Andriotis *et al.*, 2004).

Lipid mobilization during cold stratification is accompanied by the development of enzymatic activities related to gluconeogenesis, such as isocitrate lyase (Noland and Murphy, 1984; Li and Ross, 1990a), fructose 1,6-*bis*phosphatase (Li and Ross, 1988) and starch synthase (Li and Ross, 1990b), which facilitate accumulation of carbohydrates (Dawidowicz-Grzegorzewska, 1989; Li and Ross, 1990b). Lack of reserve mobilization and the inactivity of enzymes related to gluconeogenesis in warm-incubated, moistened dormant seeds led researchers to propose that metabolic inhibition (Ross, 1984; Lewak *et al.*, 2000) prevents dormant seeds from utilizing their food reserves, and cold conditions allow germination by activating hydrolases involved in reserve mobilization. However, further studies are needed because some results do not support this proposition (Downie and Bewley, 2000).

Incubating moistened seeds in warm conditions simulates accelerated ageing accompanied by oxidative stress, and finally leads to seed deterioration

(Li and Ross, 1990a; McDonald, 1999). Accordingly, in moistened seeds, cold conditions may have a beneficial role in the promotion of germination by activation of cellular repair mechanisms (Wang and Berjak, 2000) and suppression of oxidative damage.

Walnut kernels differ from other stratification-requiring tree seeds, in that storage protein mobilization occurs under both cold and warm conditions (Einali and Sadeghipour, 2007). However, under warm conditions, kernels deteriorate, whereas cold stratification leads to enhanced kernel germination. Thus, it was hypothesized that cold conditions direct the products of protein mobilization to unknown metabolic pathways needed for germination. Storage lipids constitute the major (about 70% dry weight) food reserve of walnut kernels (Savage *et al.*, 2001). Lipid mobilization and subsequent gluconeogenesis of the lipolytic products fulfil the major carbon and energy demands of seeds following germination (Cooper and Beevers, 1969), and may be important processes during seed dormancy removal by cold (Li and Ross, 1990a, b; Zarska-Maciejewska, 1992). To understand the beneficial effects of cold conditions in the alleviation of dormancy in walnut kernels, lipid mobilization and gluconeogenesis were studied during dormancy removal by cold, as well as seeds that deteriorated due to moist, warm conditions.

Materials and methods

Plant material, stratification protocol and germination studies

Freshly harvested seeds of Persian walnut (*Juglans regia* L.) were procured from the Gorgan Office of Natural Resources during October of 2004, 2005 and 2006. Kernels not older than 8 months after harvest were used for stratification studies. After soaking in tap water for 24 h, nuts were surface sterilized with 0.5% (w/v) sodium hypochlorite solution for 15 min, followed by washing four times in distilled water. To stratify kernels, every 10 d, lots of 75 nuts (in triplicates of 25) were wrapped in four layers of moistened cheesecloth covered with polythene bags and incubated at 5°C in a refrigerator for up to 60 d. The stratified and non-stratified nuts, the latter imbibed for 24 h only, were then transferred into sand, irrigated to keep them moist, and their germination was recorded for 40 d in a temperature-controlled culture room at 27°C in darkness. Non-stratified nuts kept at 27°C in sand are referred to as warm-incubated kernels. Kernels with an average radicle length of 10 mm were considered as germinated, and they were evident as bulges on the sand surface. After isolating kernels from both cold-stratified and warm-incubated nuts, axes and cotyledons were excised with a razor blade from those that did not show any visible sign of germination and were used for subsequent biochemical analyses.

Preparation of tissue homogenate

Cotyledonary or axial tissues were ground and homogenized in cold homogenization buffer, consisting of 0.1 M Tris buffer pH 7.5, 0.4 M sucrose, 10 mM KCl, 1 mM $MgSO_4$, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% (v/v) 2-mercaptoethanol and 0.6% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenization buffer also contained 0.1% (v/v) Triton X-100 when extracting isocitrate lyase. The ratio of homogenization buffer to tissue was 6:1 when extracting isocitrate lyase and $NADP^+$-isocitrate dehydrogenase, and 2:1 in other experiments. The homogenate was filtered through five layers of muslin cloth and centrifuged at 10,000 *g* for 30 min at 4°C. The oil body layer (top layer of the homogenate after centrifugation) was collected with a spatula and used for subsequent analyses. The total soluble protein (TSP) present in the 10,000 *g* supernatant was used for protein and enzymatic analyses. When using TSP for the assay of walnut kernel fatty acyl-ester hydrolase activity, PMSF and 2-mercaptoethanol were excluded from the homogenization buffer (Teissere *et al.*, 1995).

Assay of isocitrate lyase activity

Isocitrate lyase (EC 4.1.3.1) activity was assayed according to the method of Cooper and Beevers (1969) with minor modifications. The reaction mixture in a final volume of 1.2 ml consisted of 50 mM Tris buffer (pH 8.0), 5 mM $MgSO_4$, 50 mM 2-mercaptoethanol, 20 mM phenyl hydrazinium hydrochloride, 13 mM DL-isocitrate and aliquots up to 100 µl from the TSP fraction. The reaction was started following the addition of DL-isocitrate, and the increase in the absorbance at 324 nm, due to the formation of glyoxylate–phenylhydrazone complex, was recorded. Enzyme activity was expressed as nmoles glyoxylate produced per min per g fresh weight of tissue [nmol (min.g fresh weight)$^{-1}$], assuming an extinction coefficient (ε_{324}) of 17 (mM. cm)$^{-1}$ for the glyoxylate–phenylhydrazone complex.

Assay of $NADP^+$-isocitrate dehydrogenase activity

The activity of $NADP^+$-isocitrate dehydrogenase (EC 1.1.1.42) was determined as described by Chen *et al.* (1988) with slight modifications. The reaction mixture in a final volume of 1 ml consisted of 50 mM Tris buffer

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(pH 8), 5 mM $MgSO_4$, 0.2 mM DL-isocitrate, 0.1 mM $NADP^+$ and aliquots up to 100 µl from the TSP fraction as an enzyme source. The oxidation of isocitrate to α-ketoglutarate was started following the addition of enzyme extract, and the increase in absorbance at 340 nm, resulting from the reduction of $NADP^+$, was recorded for 5 min. Enzyme activity was expressed as nmoles isocitrate oxidized per min per g tissue fresh weight [nmol (min.g fresh weight)$^{-1}$], assuming an extinction coefficient (ε_{340}) of 6220 (M.cm)$^{-1}$ for the NADPH product (Tian *et al.*, 2005).

Fatty acyl-ester hydrolase activity assay

Fatty acyl-ester hydrolase (EC 3.1.1.1) activity was measured spectrophotometrically, according to the method of Winkler and Stuckmann (1979). Thirty milligrams of *p*-nitrophenyl palmitate (*p*NPP) were dissolved in 10 ml of isopropanol and mixed with 90 ml of 55 mM Tris buffer (pH 8.0), containing 0.11% (w/v) gum arabic and 0.23% (w/v) sodium deoxycholate. Freshly prepared substrate solution (2.4 ml) was mixed with aliquots (up to 100 µl) of TSP in a final volume of 2.5 ml. The enzyme-catalysed release of nitrophenyl anions at 37°C was monitored at 410 nm within 2–4 min after starting the reaction. Enzyme activity was expressed as the nmoles nitrophenyl anions released per min per g tissue fresh weight [nmol (min.g fresh weight)$^{-1}$], assuming an extinction coefficient (ε_{410}) of 15,000 (M.cm)$^{-1}$.

Other analytical methods

Extraction and quantification of total lipids from walnut kernels were carried out according to the method of Hara and Radin (1978). The defatted powder obtained following tissue total lipid extraction was used for extraction and measurement of starch, reducing and non-reducing sugars. Starch content was quantified according to the method of McCready *et al.* (1950). Reducing and non-reducing sugars were determined by the methods of Miller (1959) and Handel (1968), respectively. Soluble protein in the 10,000 *g* supernatant of the tissue extract was assayed by the Bradford (1976) method. The extent of lipid peroxidation was measured according to Du and Bramlage (1992). Tissue hydrogen peroxide content was quantified by the method of Jana and Choudhuri (1981).

Statistical analysis

Statistically significant differences at the 5% level were determined by the Duncan method and Nested Design Analysis (SAS software 2001, SAS Institute Inc., Cary, North Carolina, USA).

Results

Effect of cold stratification on germination of walnut kernels

Figure 1A shows the time course of germination of both cold-stratified and warm-incubated kernels. Warm-incubated kernels achieved 30% germination after 20 d culture at 27°C. Cold stratification of kernels for 30 d greatly enhanced their germination to more than 70% after a subsequent 20 d at 27°C, with lower enhancement after shorter cold periods. A maximum germination percentage of 45% was obtained for

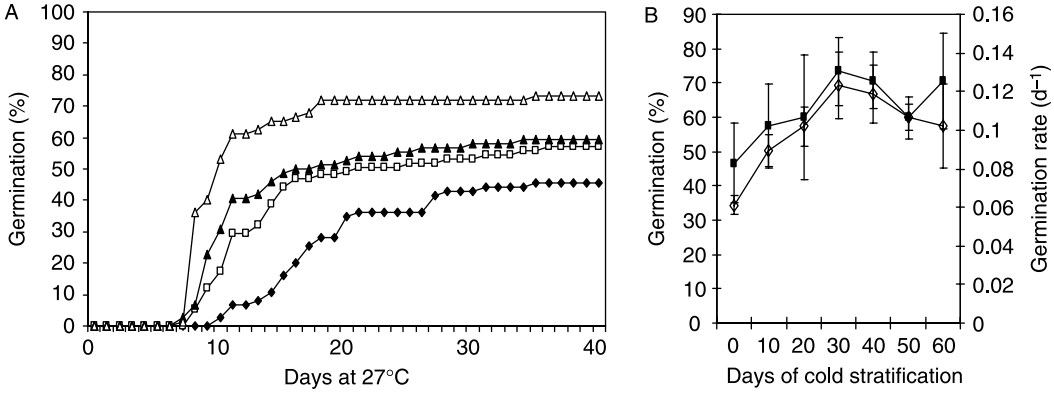

Figure 1. (A) Time course of germination at 27°C of walnut kernels non-stratified (closed diamonds) and cold stratified for 10 d (open squares), 20 d (closed triangles) and 30 d (open triangles). (B) Effect of stratification on germination percentage (closed squares) and germination rate (open diamonds) of walnut kernels. Every 10 d for a period of 60 d, seeds imbibed for 24 h were surface sterilized and incubated at 5°C. Moist-chilled seeds were then simultaneously transferred to a sand medium at 27°C, and germination was recorded for a period of 40 d. Each point represents the mean value of triplicate experiments, each consisting of 25 seeds ± SE.

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warm-incubated kernels that were kept moist for 40 d at 27°C. There were no significant changes in germination percentages of kernels cold stratified for more than 30 d. Kernels cold stratified for 30 d also displayed the highest germination rate (Fig. 1B). No germination occurred at 5°C during 60 d of cold stratification, and it started only after subsequently incubating kernels at 27°C.

Changes in total lipid content of cold-stratified and warm-incubated walnut kernels

Total lipids constitute about 65% of walnut kernel dry matter. Following imbibition, a significant decrease in lipid content occurred in cold-stratified cotyledons, but not in the embryonic axes (Fig. 2A). The rate of lipid mobilization was very high, i.e. 9.5 mg (g dry weight)$^{-1}$ in warm-incubated cotyledons (Fig. 2B), so that about 30% of the initial tissue lipid content was consumed within 20 d. In contrast, lipid content of axes did not change significantly.

Changes in starch and soluble sugar contents of cold-stratified and warm-incubated walnut kernels

The alleviation of apple seed dormancy is accompanied by activation of the gluconeogenesis pathway (Lewak *et al.*, 2000). Accordingly, changes in starch, non-reducing and reducing sugars were investigated in cold-stratified and warm-incubated walnut kernels (Fig. 3). Starch content of cotyledons increased significantly (about twofold) within 30 d of cold stratification, and from this time onward it remained more or less unchanged (Fig. 3A). The starch content of the embryonic axes remained unchanged throughout this period. Despite some alterations in starch content, changes were not significant in warm-incubated axes and cotyledons (Fig. 3B).

A twofold increase in non-reducing sugars occurred in cotyledons from 20 to 40 d of kernel incubation at cold temperatures (Fig. 3C). Cold-stratified axes showed fluctuations in non-reducing sugar content. Neither cotyledons nor axes of warm-incubated kernels accumulated non-reducing sugars (Fig. 3D).

The amount of reducing sugars remained significantly higher in cold-stratified kernels compared with warm-incubated ones (Fig. 3E, F). A significant decline in the amount of reducing sugars, by about 70%, occurred in cotyledons within the first 4 d in kernels incubated at warm temperatures (Fig. 3F).

Changes in walnut kernel fatty acyl-ester hydrolase activity in cold-stratified and warm-incubated walnut kernels

Fatty acyl-ester hydrolases are necessary to hydrolyse acyl-glycerols produced following lipase action in germinated oilseeds (Teissere *et al.*, 1995). This enzyme activity declined significantly in both cotyledons and axes of cold-stratified kernels (Fig. 4A), whereas it remained variably higher under warm conditions (Fig. 4B). Using α-naphthyl acetate as a substrate to detect fatty acyl-ester hydrolase of walnut kernels in the total soluble protein fraction after separation by SDS-PAGE (Gabriel, 1971), we found a 64 kDa protein,

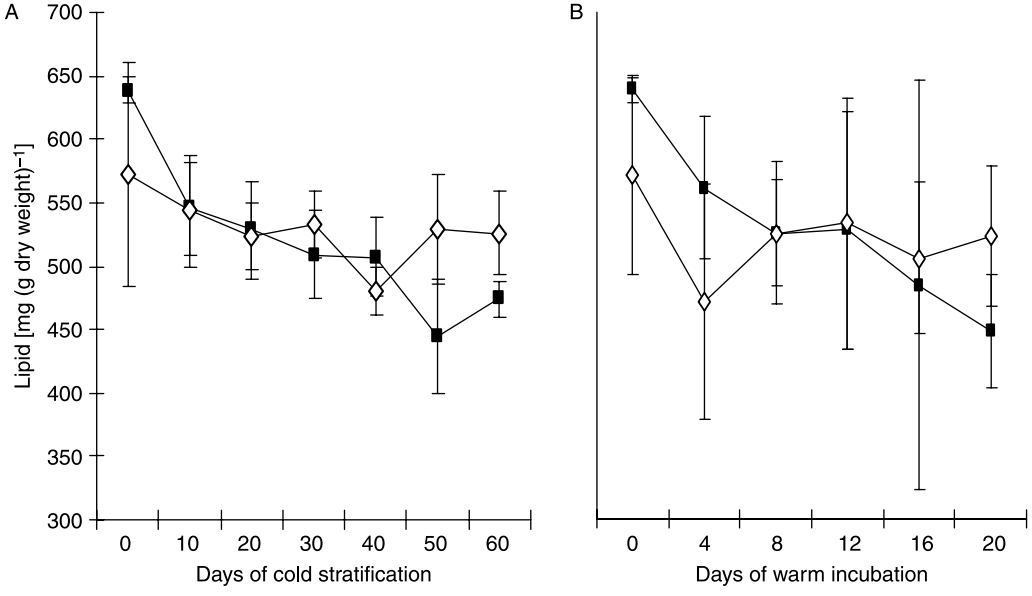

Figure 2. Changes in total lipid content of cotyledons (closed squares) and axes (open diamonds) of cold-stratified (A) and warm-incubated (B) walnut kernels. Each point represents the mean value of three separate extractions ± SE.

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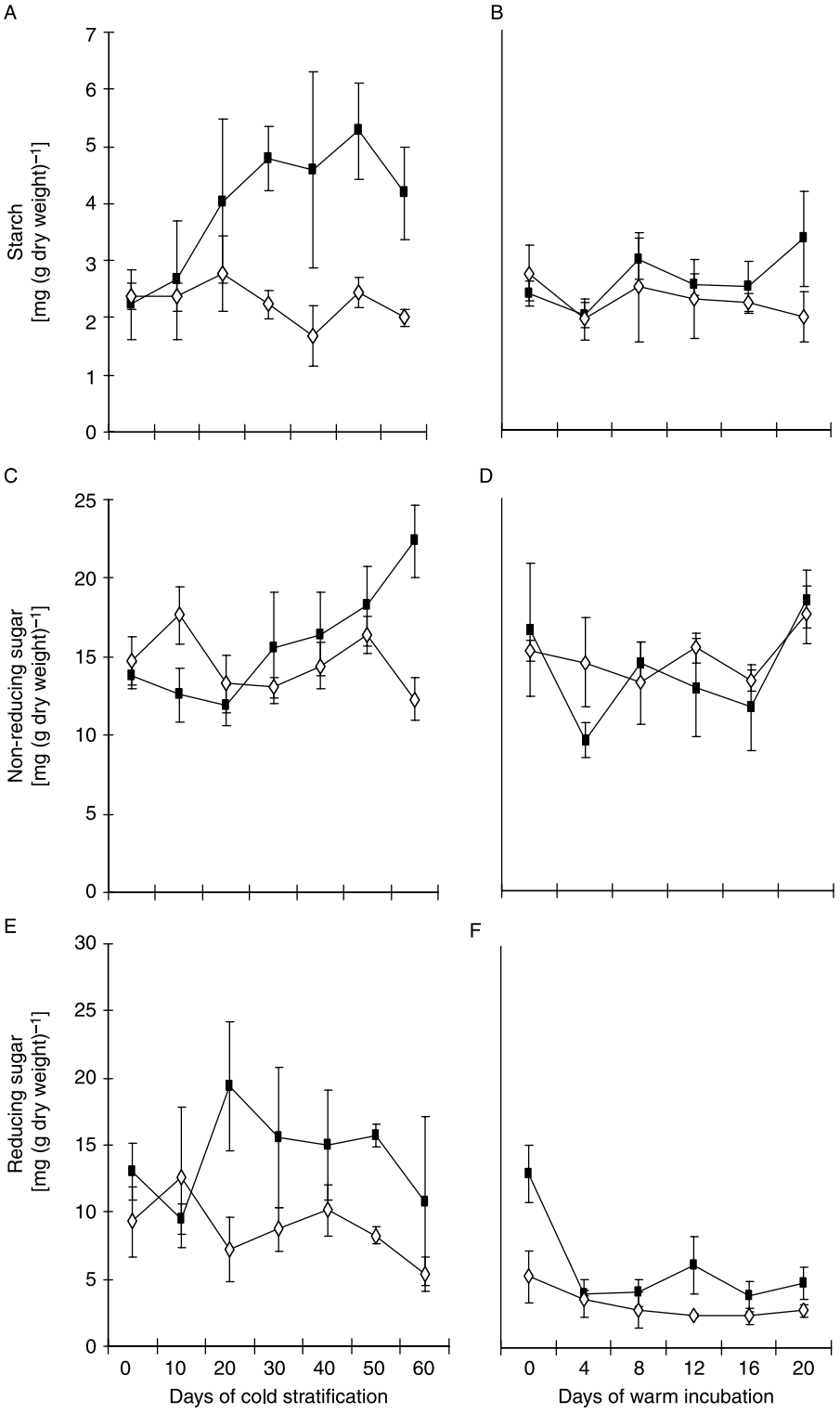

Figure 3. Changes in starch (A, B), non-reducing sugars (C, D) and reducing sugars (E, F) in cotyledons (closed square) and axes (open diamond) of walnut kernels during cold stratification (A, C, E) or warm incubation (B, D, F). The bars show standard errors of the means of the measured parameters of three separate tissue samples.

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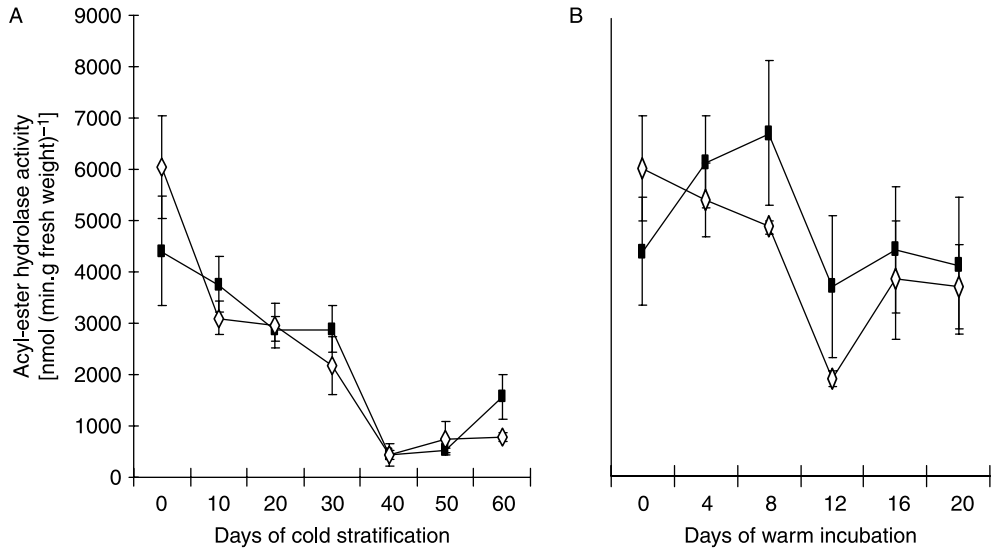

Figure 4. Changes in fatty acyl-ester hydrolase activity in cotyledons (closed squares) and axes (open diamonds) of cold-stratified (A) or warm-incubated (B) walnut kernels. Values are means of three separate extractions ± SE.

which displayed a more intense activity in extracts of warm-incubated kernels compared with those from cold-stratified ones (not shown).

Isocitrate lyase activity in cold-stratified and warm-incubated walnut kernels

The development of isocitrate lyase (ICL) activity is a marker of gluconeogenesis in both germinated and cold-stratifying oilseeds (Cooper and Beevers, 1969; Li and Ross, 1990a). Enzyme activity increased in cotyledons and axes of imbibed kernels, with maximum enzyme activity in the cotyledons after 50 d of cold treatment (Fig. 5A). ICL activity also developed in both cotyledons and axes of warm-incubated kernels, so that maximum enzyme activity occurred after 12–16 d (Fig. 5B).

Hydrogen peroxide contents in cold-stratified and warm-incubated walnut kernels

Seed deterioration is accompanied by the production of reactive oxygen species (McDonald, 1999). The beneficial effects of cold stratification on seed

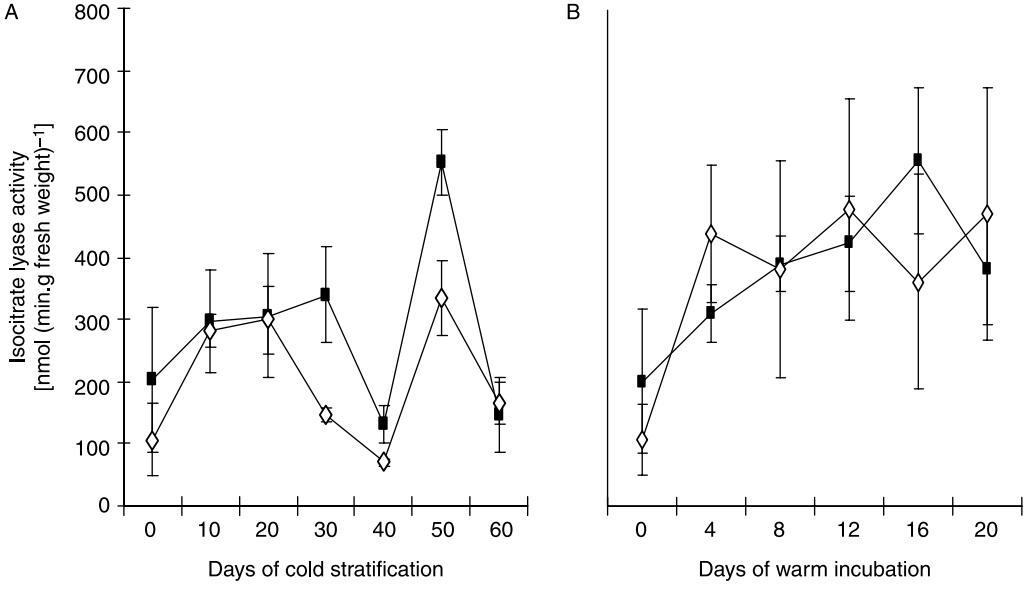

Figure 5. Changes in isocitrate lyase activity in cotyledons (closed squares) and axes (open diamonds) of cold-stratified (A) or warm-incubated (B) walnut kernels. Values are means of three separate extractions ± SE.

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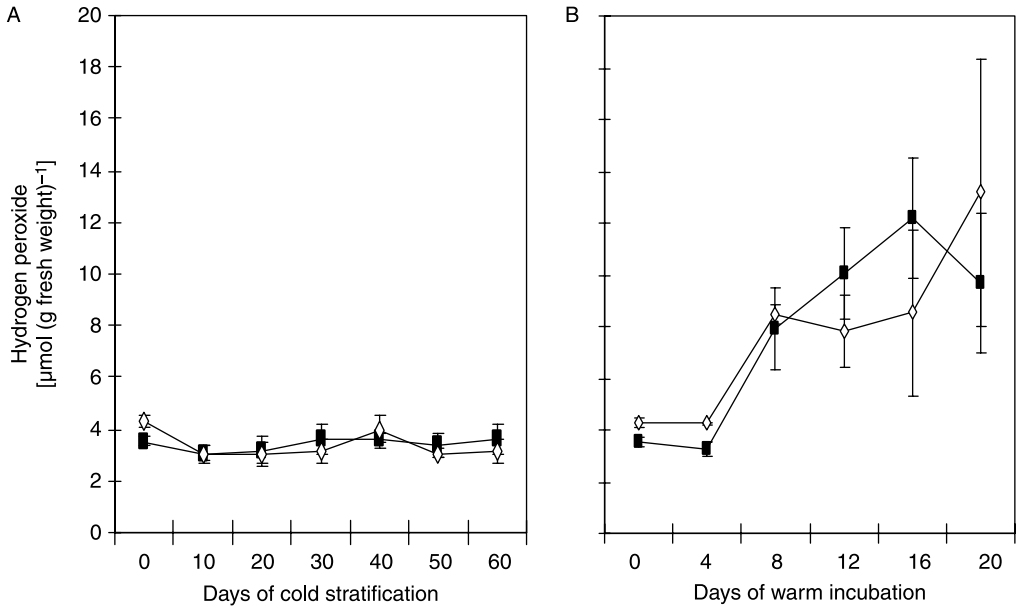

Figure 6. Changes in the hydrogen peroxide contents of cotyledons (closed squares) and axes (open diamonds) of cold-stratified (A) or warm-incubated (B) walnut kernels. Each point represents the mean value of three separate extractions ± SE.

germination (Wang and Berjak, 2000) might include prevention of a build-up of reactive oxygen species such as hydrogen peroxide. Hydrogen peroxide contents remained constant during cold stratification of kernels (Fig. 6A), whereas in warm-incubated kernels, extensive hydrogen peroxide accumulation occurred after 4 d, and by 20 d was at least threefold greater (Fig. 6B).

Lipid peroxidation in cold-stratified and warm-incubated walnut kernels

Lipid peroxidation is an important factor in seed deterioration (McDonald, 2004). If a cold-stratification stimulus exerts its beneficial effects through the avoidance of membrane damage (Wang and Berjak, 2000), the extent of lipid peroxidation might be a good measure in evaluating tissue damage to both cold-stratified and warm-incubated walnut kernels. Means of lipid peroxidation in both cotyledons and axes were significantly greater in warm-incubated walnut kernels than in cold-stratified ones when they were evaluated for the whole period of incubation (Fig. 7).

NADP$^+$-isocitrate dehydrogenase activity in cold-stratified and warm-incubated walnut kernels

NADP$^+$-isocitrate dehydrogenase is a cytosolic enzyme in plant tissues (Palomo *et al.*, 1998). Its role in counteracting oxidative stress is well documented in animal cells (Kim and Park, 2003), and by producing reducing equivalents of NADPH, it may behave

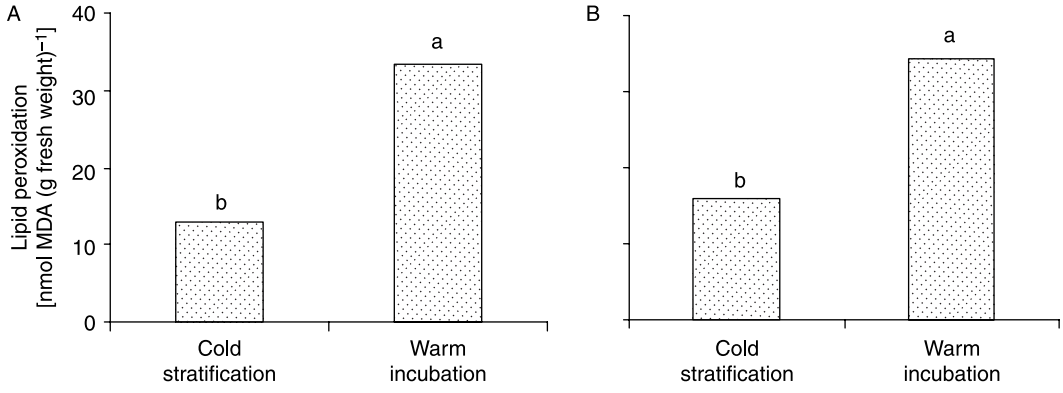

Figure 7. Comparison of lipid peroxidation between cold-stratified and warm-incubated walnut cotyledons (A) and axes (B). Means and significant differences for lipid peroxidation values were obtained following Nested Design Analysis of data obtained from cold-stratified and warm-incubated kernels for 60 d and 20 d, respectively. MDA, malondialdehyde.

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similarly in plants (Corpas *et al.*, 1999; del Rio *et al.*, 2002; Hodges *et al.*, 2003). Accordingly, changes in the activity of $NADP^+$-isocitrate dehydrogenase in walnut kernels were taken as a measure of their competence to generate reducing equivalents, and hence counteract oxidative stress. In imbibed axes and cotyledons, the mean enzyme activity was high, but showed no statistically significant changes during cold stratification for 60 d (Fig. 8A). Under warm conditions, $NADP^+$-isocitrate dehydrogenase initially increased by about 18% in cotyledons incubated for 8 d, but further incubation in the warm led to a significant decline of about 40% in both kernel tissues (Fig. 8B).

Discussion

Walnut kernels are not deeply dormant (Einali and Sadeghipour, 2007); in the absence of a cold stimulus, germination was between 20 and 45% (Fig. 1). Cold stratification enhanced kernel germination to 70% (Fig. 1), in agreement with data obtained by others (Kaur *et al.*, 2006; Sanchez-Zamora *et al.*, 2006). Since there was no germination in kernels during 60 d of stratification at 5°C, all the biochemical changes reported here are not a result of germination, and may be related to dormancy release.

Non-stratified kernels (i.e. warm-incubated) germinated after 10 d incubation at 27°C. Most of them retained white, sound-looking tissue for the first 20 d and 55% of them decayed thereafter. Accordingly, samples were taken from these kernels only up to 20 d of incubation, and to exclude any possible interference by germinative or post-germinative events, analyses were carried out exclusively in those not showing any visible signs of germination. Since cotyledons rather than axes appear to be the cold-perceiving organ in walnut kernels (Einali and Sadeghipour, 2007), the present discussion is based mainly on the changes in this organ.

Cold stratification is accompanied by gluconeogenesis of lipid reserves

Imbibition was sufficient to bring about mobilization of lipids (Fig. 2), and storage proteins (Einali and Sadeghipour, 2007) in walnut kernels, irrespective of the temperature of incubation, whereas the mobilization of food reserves in other stratification-requiring tree seeds commences only during cold treatment (Dawidowicz-Grzegorzewska, 1989; Li and Ross, 1990a; Zarska-Maciejewska, 1992; Wang and Berjak, 2000; Andriotis *et al.*, 2004). Lipid mobilization in cold-stratified walnut kernels was accompanied by an increase of isocitrate lyase activity (Fig. 5A) and the accumulation of starch and higher amounts of reducing and non-reducing sugars (Fig. 3A, C, E), which is consistent with the occurrence of gluconeogenesis. Carbohydrate accumulation and the development of isocitrate lyase activity have been reported in some tree seeds during the alleviation of dormancy by cold (Noland and Murphy, 1984; Dawidowicz-Grzegorzewska, 1989; Li and Ross, 1990a, b) as a prerequisite for germination.

Lipid mobilization in walnut kernels under warm conditions might be associated with a non-gluconeogenic operation of the glyoxylate cycle, as occurs in some oilseeds (Eastmond *et al.*, 2000; Eastmond and

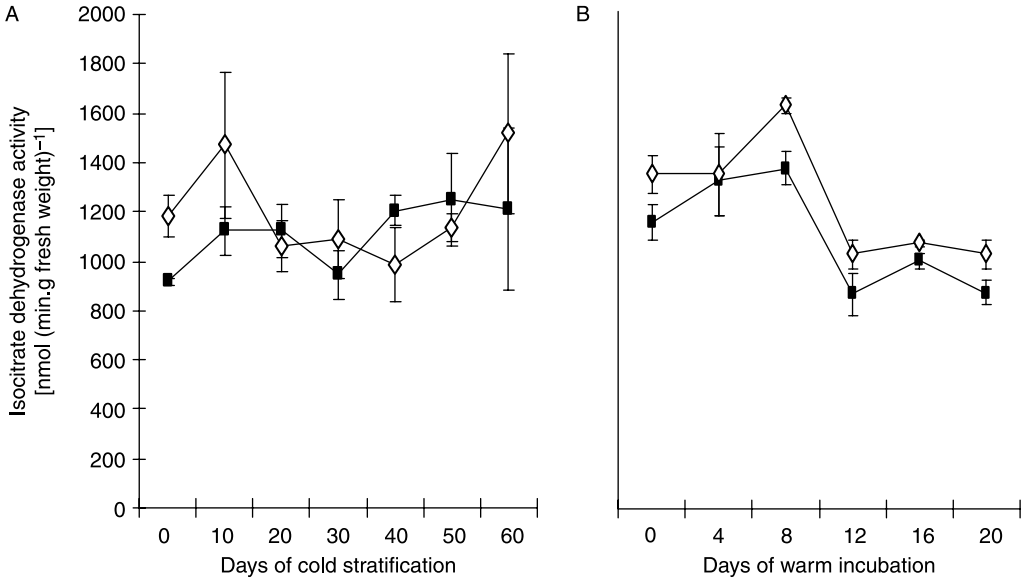

Figure 8. Changes in $NADP^+$-isocitrate dehydrogenase activity in cotyledons (closed squares) and axes (open diamonds) from cold-stratified (A) or warm-incubated (B) walnut kernels. Values are means of three separate extractions ± SE.

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Graham, 2001). Lack of starch accumulation under warm conditions (Fig. 3), despite isocitrate lyase activity (Fig. 5B), supports this idea. The diversion of lipolytic metabolites to respiratory pathways (Chia *et al.*, 2005) is expected in warm-incubated kernels.

Walnut kernel fatty acyl-ester hydrolase as a non-gluconeogenic enzyme

Two lipolytic activities are detectable in imbibed walnut kernels. One of them is a true lipase activity responsible for kernel lipid mobilization (unpublished data), and the other one is a fatty acyl-ester hydrolase activity that declined in cold-stratified kernels, but remained fairly level during warm incubation (Fig. 4A, B). SDS-PAGE revealed a 64 kDa acyl-ester hydrolase activity band with greater expression in warm-incubated kernels. However, this enzyme may not be involved directly in mobilization and gluconeogenesis of storage lipids and, rather, correlates with the deteriorative process in moistened kernels under warm conditions: (1) as lipid mobilization started in cold-stratified kernels, acyl-ester hydrolase activity declined (Fig. 4A); and (2) despite its higher activity in warm-incubated kernels (Fig. 4B), there was no accumulation of either starch or soluble sugars (Fig. 3). In addition, natural ageing of walnut kernels for 1 and 2 years abolished their germination capacity, but was accompanied by greater acyl-ester hydrolase activity (data not shown). Decreased seed viability of *Pinus sylvestris* L. due to natural ageing was correlated with changes in the composition of polar and non-polar lipids (Tammela *et al.*, 2000), implying the involvement of lipolytic enzymes. Acyl-ester hydrolases have also been implied as the initiators of other deteriorative processes, such as senescence in plant organs (Hong *et al.*, 2000; Thompson *et al.*, 2000).

Warm incubation accompanies the build-up of oxidative stress in walnut kernels

Warm-incubated walnut kernels undergo accelerated ageing (Einali and Sadeghipour, 2007) with a significant accumulation of hydrogen peroxide (Fig. 6), an indicator of oxidative stress leading to increased lipid peroxidation (Fig. 7), a process that would explain non-germinability and deterioration after 20 d in the warmth, since it is known as a primary cause of seed deterioration during ageing (McDonald, 2004).

A significant decline in the activity of $NADP^+$-isocitrate dehydrogenase from the mid-period of kernel incubation at warm temperatures (Fig. 8B) and the maintenance of this activity in cold-stratified kernels (Fig. 8A), implies that the regenerating capacity of reducing equivalents has been compromised. Thus, reduced germination of warm-incubated walnut kernels (Fig. 1) might result from failure to produce sufficient reducing equivalents to counteract oxidative stress. The role of cytosolic $NADP^+$-isocitrate dehydrogenase against oxidative stress has been well documented in animal cells (Kim and Park, 2003); a similar role has been implied in plants (Pastori and del Rio, 1997; Corpas *et al.*, 1999; Hodges *et al.*, 2003).

In conclusion, enhanced germination capacity of cold-stratified walnut kernels compared to the warm-incubated ones may be attributed partly to alterations in lipid metabolism. Lipid mobilization in cold-stratified walnut kernels is accompanied by increased products of gluconeogenesis. However, in warm-incubated kernels, the decline in lipid content is non-gluconeogenic and suggests deterioration, as it is accompanied by greater activity of a 64-kDa fatty acyl-ester hydrolase, increased lipid peroxidation, hydrogen peroxide accumulation and potential weakening of NADPH regeneration capacity. Whether these differences in walnut metabolism are the direct effects of cold conditions or associated with dormancy removal from walnut kernels needs further investigations. These data provide biochemical evidence in support of Wang and Berjak (2000), who consider that the beneficial effects of cold stratification of imbibed seeds are due to its ability to prevent deteriorative processes and activate cellular repair mechanisms. Furthermore, it refines the ideas suggested by earlier work (Einali and Sadeghipour, 2007) by showing that there is no block to lipid mobilization in walnut kernels after imbibition, and that cold stratification possibly promotes germination by enhancement of kernel gluconeogenic competence.

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References

Andriotis, V.M.E., Smith, S.B. and Ross, J.D. (2004) Phytic acid mobilization is an early response to chilling of the embryonic axes from dormant oilseed of hazel (*Corylus avellana* L.). *Journal of Experimental Botany* **56**, 537–545.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Chen, R.D., Le Marechal, P., Vidal, H., Jacquot, J.P. and Gadal, P. (1988) Purification and comparative properties of the cytosolic iso-citrate dehydrogenases (NADP) from

https://doi.org/10.1017/S0960258509306650 Published online by Cambridge University Press

pea (*Pisum sativum*) roots and green leaves. *European Journal of Biochemistry* **175**, 565–572.

Chia, T.Y.P., Pike, M.J. and Rawsthorne, S. (2005) Storage oil breakdown during embryo development of *Brassica napus* (L.). *Journal of Experimental Botany* **56**, 1285–1296.

Cooper, T.G. and Beevers, H. (1969) Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. *Journal of Biological Chemistry* **244**, 3507–3513.

Corbineau, F., Bianco, J., Garello, G. and Côme, D. (2002) Breakage of *Pseudotsuga menziesii* seed dormancy by cold treatment as related to changes in seed ABA sensitivity and ABA levels. *Physiologia Plantarum* **114**, 313–319.

Corpas, F.J., Barroso, J.B., Sandalio, L.M., Palma, J.M., Lupianez, J.A. and del Rio, L.A. (1999) Peroxisomal NADP-dependent isocitrate dehydrogenase. characterization and activity regulation during natural senescence. *Plant Physiology* **121**, 921–928.

Dawidowicz-Grzegorzewska, A. (1989) Degradation of protein and lipid bodies during dormancy removal in apple seeds. *Journal of Plant Physiology* **135**, 43–51.

del Rio, L.A., Corpas, F.J., Sandalio, L.M., Palma, J.M., Gomez, M. and Barroso, J.B. (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *Journal of Experimental Botany* **53**, 1255–1272.

Downie, B. and Bewley, J.D. (2000) Soluble sugar content of white spruce (*Picea glauca*) seeds during and after germination. *Physiologia Plantarum* **110**, 1–12.

Du, Z. and Bramlage, W.J. (1992) Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. *Journal of Agricultural and Food Chemistry* **40**, 1566–1570.

Eastmond, P.J. and Graham, I.A. (2001) Re-examining the role of the glyoxylate cycle in oil seeds. *Trends in Plant Science* **6**, 72–77.

Eastmond, P.J., Germain, V., Lange, P.R., Bryce, J.H., Smith, S.M. and Graham, I.A. (2000) Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. *Proceedings of the National Academy of Sciences, USA* **97**, 5669–5674.

Einali, A.R. and Sadeghipour, H.R. (2007) Alleviation of dormancy in walnut kernels by moist chilling is independent from storage protein mobilization. *Tree Physiology* **27**, 519–525.

Forward, B.S., Tranbarger, T.J. and Misra, S. (2001) Characterization of proteinase activity in stratified Douglas-fir seeds. *Tree Physiology* **21**, 625–629.

Gabriel, O. (1971) Locating enzymes on gels. *Methods in Enzymology* **22**, 578–604.

Handel, E.V. (1968) Direct microdetermination of sucrose. *Analytical Biochemistry* **22**, 280–283.

Hara, A. and Radin, N.S. (1978) Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry* **90**, 420–426.

Hodges, M., Flesch, V., Galvez, S. and Bismuth, E. (2003) Higher plant $NADP^+$-dependent isocitrate dehydrogenases, ammonium assimilation and NADPH production. *Plant Physiology and Biochemistry* **41**, 577–585.

Hong, Y., Wang, T.W., Hudak, K.A., Schade, F., Froese, C.D. and Thompson, J.E. (2000) An ethylene-induced cDNA encoding a lipase expressed at the onset of senescence. *Proceedings of the National Academy of Sciences, USA* **97**, 8717–8722.

Jacobsen, J.V., Pearce, D.W., Poole, A.T., Pharis, R.P. and Mander, L.N. (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiologia Plantarum* **115**, 428–441.

Jana, S. and Choudhuri, M.A. (1981) Glycolate metabolism of three submerged aquatic angiosperms during aging. *Aquatic Botany* **12**, 342–354.

Kaur, R., Sharma, N., Kumar, K., Sharma, D.R. and Sharma, S.D. (2006) *In vitro* germination of walnut (*Juglans regia* L.) embryos. *Scientia Horticulturae* **109**, 385–388.

Kim, S.Y. and Park, J.W. (2003) Cellular defense against singlet oxygen-induced oxidative damage by cytosolic $NADP^+$-dependent isocitrate dehydrogenase. *Free Radical Research* **37**, 309–316.

Lewak, S., Bogatek, R. and Zarska-Maciejewska, B. (2000) Sugar metabolism in apple embryos. pp. 47–55 *in* Viemont, J.D.; Crabbe, J. (Eds) *Dormancy in plants*. Wallingford, CAB International.

Li, L. and Ross, J.D. (1988) Fructose 1,6-bisphosphatase in seeds of *Corylus avellana*. *Phytochemistry* **27**, 1977–1980.

Li, L. and Ross, J.D. (1990a) Lipid mobilization during dormancy breakage in oilseed of *Corylus avellana*. *Annals of Botany* **66**, 50–505.

Li, L. and Ross, J.D. (1990b) Starch synthesis during dormancy breakage in oilseed of *Corylus avellana*. *Annals of Botany* **66**, 507–512.

McCready, R.M., Guggolz, J., Silviera, V. and Owens, H.S. (1950) Determination of starch and amylose in vegetables. *Analytical Chemistry* **22**, 1156–1158.

McDonald, M.B. (1999) Seed deterioration: Physiology, repair and assessment. *Seed Science and Technology* **27**, 177–237.

McDonald, M.B. (2004) Orthodox seed deterioration and its repairs. pp. 273–304 *in* Benech-Arnold, R.L.; Sanchez, R.A. (Eds) *Handbook of seed physiology.* New York, Haworth Press.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry* **31**, 426–428.

Noland, T.L. and Murphy, J.B. (1984) Changes in isocitrate lyase activity and ATP content during stratification and germination of sugar pine seeds. *Seed Science and Technology* **12**, 777–787.

Palomo, J., Gallardo, F., Suarez, M.F. and Canovas, F.M. (1998) Purification and characterization of $NADP^+$-linked isocitrate dehydrogenase from Scots pine. *Plant Physiology* **118**, 617–629.

Pastori, G.M. and del Rio, L.A. (1997) Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes. *Plant Physiology* **113**, 411–418.

Ross, J.D. (1984-) Metabolic aspects of dormancy. pp. 45–75 *in* Murray, D.R. (Ed.) *Seed physiology. Volume 2. Germination and reserve mobilization*. Sydney, Academic Press.

Sanchez-Zamora, M.A., Cos-Terrer, J., Frutos-Tomas, D. and Garcia-Lopez, R. (2006) Embryo germination and proliferation *in vitro* of *Juglans regia* L. *Scientia Horticulturae* **108**, 317–321.

Savage, G.P., McNeil, D.L. and Dutta, P.C. (2001) Some nutritional advantages of walnuts. *Acta Horticulturae* **544**, 557–563.

Schmitz, N., Abrams, S.R. and Kermode, A.R. (2002) Changes in ABA turnover and sensitivity that accompany dormancy termination of yellow-cedar (*Chamaecyparis nootkatensis*) seeds. *Journal of Experimental Botany* **53**, 89–101.

Tammela, P., Hopia, A., Hiltunen, R., Vuorela, H. and Nygren, M. (2000) Aging in *Pinus sylvestris* L. seeds: changes in viability and lipids. *Biochemical Society Transactions* **28**, 878–879.

Teissere, M., Borel, M., Caillol, B., Nari, J., Gardies, A.M. and Noat, G. (1995) Purification and characterization of a fatty acyl-ester hydrolase from post-germinated sunflower seeds. *Biochimica et Biophysica Acta* **1255**, 105–112.

Thompson, J., Taylor, C. and Wang, T.W. (2000) Altered membrane lipase expression delay leaf senescence. *Biochemical Society Transactions* **28**, 775–777.

Tian, J., Bryk, R., Itoh, M., Suematsu, M. and Nathan, C. (2005) Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis*: Identification of α-ketoglutarate decarboxylase. *Proceedings of the National Academy of Science, USA* **102**, 10670–10675.

Wang, B.S.P. and Berjak, P. (2000) Beneficial effects of moist chilling on the seeds of black spruce (*Picea mariana* [Mill.] B.S.P.). *Annals of Botany* **86**, 29–36.

Winkler, U.K. and Stuckmann, M. (1979) Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology* **138**, 663–670.

Zarska-Maciejewska, B. (1992) Lipolytic activity during dormancy removal in apple seeds. *Plant Physiology and Biochemistry* **30**, 65–70.

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