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Suppression of mitochondrial dehydrogenases accompanying post-glyoxylate cycle activation of gluconeogenesis and reduced lipid peroxidation events during dormancy breakage of walnut kernels by moist chilling

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ABSTRACT

Cold stratification supposedly allows dormant seeds to germinate by removing their metabolic blocks. Nothing is known about the role of mitochondrial enzymes in metabolic regulation of seeds during dormancy release. As cold stratification removes walnut (*Juglans regia* L.) kernel dormancy, the activities of three mitochondrial enzymes i.e. pyruvate dehydrogenase complex (PDH), NAD^+-isocitrate dehydrogenase (NAD^+-IDH; EC 1.1.1.41), succinate dehydrogenase (SDH; EC 1.3.99.1) and the extra-mitochondrial glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) were compared in both cold-stratified (5 °C; 60 days) and warm-incubated (27 °C; 20 days) kernels. The kernel gluconeogenic competence was also assessed by measuring the activity of phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.49). These were then correlated to kernel lipid hydroperoxide contents and lipoxygenase (EC 1.13.11.12) activity. Lipid hydroperoxides and lipoxygenase activity were significantly greater in warm-incubated kernels. The mitochondrial NAD^+-IDH activity was indifferent between cold-stratified and warm-incubated kernels. The activities of PDH and SDH though undetectable in cold-stratified kernels, were relatively high under warm conditions. Succinate oxidation in cold stratified kernels however, was achieved by a glyoxysomal succinate oxidase activity formerly reported in monocotyledons. Furthermore, the kernel succinate oxidase, PEPCK and G6PDH activities were significantly greater at cold conditions. It was concluded that cold induced dormancy release of walnut kernels is associated with suppression of kernel mitochondrial respiration and oxidative stress and meanwhile it allows activation of oxidative pentose phosphate (OPP) and post-glyoxylate cycle gluconeogenesis pathways. The build-up of oxidative stress and toxic lipid hydroperoxide derivatives however, probably result in the compromised germination of warm-incubated walnut kernels.

1. Introduction

Seeds of many temperate woody species are dormant at maturity i.e. they cannot germinate under favorable environmental conditions. Damage to seed coat or its removal may promote embryo germination, however, in some other seeds the embryo is physiologically immature and hence germination or radicle emergence needs treatments other than seed coat removal. A common way to promote germination of seeds with dormant embryos is to incubate them for a defined period in a moist chilled condition; a treatment known as cold stratification. During stratification, the competency of embryos for germination is developed, thus after incubating seeds under suitable temperature-humidity conditions radicle emergence will occur (Hilhorst, 2007).

The alleviation of embryo dormancy by moist chilling is mediated by tissue hormonal alterations (Hilhorst, 2007). As a consequence, metabolic differences between non dormant cold-stratified seeds and those of dormant warm incubated ones are expected. The metabolic inhibition theory (Ross, 1984; Lewak,

Abbreviations: DTT, dithiothreitol; EDTA, ethylene diamine tetra acetic acid; G6PDH, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; LHP, lipid hydroperoxide; MDA, malondialdehyde; $NAD(P)^+$, β-nicotineamide adenine dinucleotide (phosphate); OPP, oxidative pentose phosphate; PDH, pyruvate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PMSF, phenylmethyl sulfonyl fluoride; PVPP, polyvinylpolypyrrolidone; SDH, succinate dehydrogenase; TPP, thiamine pyrophosphate.

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2011) states that dormant seeds are unable to mobilize their own food reserves and cold conditions make the embryo competent for the utilization of its reserves, thus allowing germination to proceed. Accordingly, increased lipase (Zarska-Maciejewska and Lewak, 1976; Li and Ross, 1990; Zarska-Maciejewska, 1992), protease (Zarska-Maciejewska and Lewak, 1983; Ranjan and Lewak, 1995; Forward et al., 2001) and phytase (Andriotis et al., 2004) activities and the respective mobilization of storage oils, proteins and phytate during cold stratification have been reported accompanying the increased capacities of embryos for germination in a few number of tree seeds. Cold conditions might also be beneficial for seed germination through activation of repair mechanisms and/or attenuation of aging-related deteriorative processes (Wang and Berjak, 2000; Nezamdoost et al., 2009).

Aging related processes in seeds is characterized by the build-up of oxidative stress, increased lipoxygenase activity, lipid peroxidation and volatilization of toxic aldehydes (Akimoto et al., 2004; McDonald, 2004; Terskikh et al., 2008). The latter as the final degradation products of lipid hydroperoxides (Taylor et al., 2004), can affect the mitochondrial dehydrogenases and other proteins involved in cell respiratory metabolism (Winger et al., 2005, 2007). Seed aging is furthermore associated with the declined activity of other dehydrogenases like glucose-6-phosphate dehydrogenase (G6PDH) (Bettey and Finch-Savage, 1996; Xin et al., 2011).

Mature walnut (*Juglans regia* L.) kernels possess physiological dormancy which can be overcome by cold stratification (Nezamdoost et al., 2009). In contrast to other studied tree seeds, there is no block to the hydrolysis of either storage oil (Nezamdoost et al., 2009) or protein (Einali and Sadeghipour, 2007; Shahmoradi et al., 2013) in dormant walnut kernels. They however, display metabolic failure in the gluconeogenesis of storage oil (Nezamdoost et al., 2009) and amino acid metabolism (Zarei-Ghadikolaee et al., 2010) under warm conditions. Warm incubated dormant walnut kernels undergo the process of aging, as evidenced by greater amounts of both hydrogen peroxide and malondealdehyde (MDA) i.e. an aldehyde derived from lipid peroxidation (Nezamdoost et al., 2009). The evolution of toxic aldehydes like hexanal has also been reported from aged walnut kernels (Martinez and Maestri, 2008). The aging process in warm incubated walnut kernels is possibly associated with the non-gluconeogenic operation of the glyoxylate cycle and increased respiration (Nezamdoost et al., 2009).

While former studies have shown the activation of some extra-mitochondrial glycolytic enzymes like pyruvate kinase and phosphofructokinase during seed moist chilling (Lewak, 2011), to the authors knowledge nothing is known on the impact of moist chilling on the mitochondrial respiratory metabolism of dormant seeds. Considering that alteration of mitochondrial respiration can affect the gluconeogenic competence of growing embryos (Falk et al., 1998), in the present study the activities of three major mitochondrial dehydrogenases i.e. pyruvate dehydrogenase (PDH), NAD^+-isocitrate dehydrogenase (NAD^+-IDH) and succinate dehydrogenase (SDH) were compared between cold-stratified and warm incubated walnut kernels. These were then correlated with the kernel G6PDH and phosphoenolpyruvate carboxykinase (PEPCK) activities as markers of oxidative pentose phosphate (OPP) metabolism and gluconeogenesis (Rylott et al., 2003a), respectively. Furthermore the extent of kernel aging-related hydroperoxidative events was assessed by analyzing lipid hydroperoxide contents and lipoxygenase activity. To the authors knowledge this is the first report on the activities of major mitochondrial dehydrogenases during the processes of seed dormancy release and furthermore indicates activation of a glyoxysomal succinate oxidase activity formerly reported in monocotyledons (Igamberdiev et al., 1995), and PEPCK can allow post-glyoxylate cycle gluconeogenesis to occur in cold stratifying walnut kernels.

2. Materials and methods

2.1. Plant material, stratification protocol and germination studies

Freshly harvested seeds of Persian walnut (*J. regia* L.) were procured from the Gorgan Office of Natural Resources during October of 2009. Kernels not older than eight 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 four times washing in distilled water. To stratify kernels, every 10 days lots of 75 nuts (in triplicates of 25) were wrapped in four layers of moistened cheesecloth covered with polytene bags and incubated at 5 °C in a refrigerator for up to 60 days. The experiment was carried out in a completely randomized design. 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 days in 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. Axes and cotyledons were excised with a razor blade from cold-stratified and warm-incubated kernels which did not show any visible sign of germination and used for subsequent biochemical analyses. Three separate kernel extractions and assays were made for each treatment and each biochemical data point represents the mean value of three separate extractions ± SE.

2.2. Extraction and assay of mitochondrial dehydrogenases

For extraction of PDH activity, cotyledonary tissues were ground and homogenized in cold homogenization buffer. The composition of homogenization buffer was essentially adopted from Millar et al. (1998). To this Triton X-100 was also added to make enzyme assay possible in the crude extract (Hinman and Blass, 1981). It was consisting of 0.05 M phosphate buffer pH 7.5, 0.3 M sorbitol, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol and 0.6% (w/v) polyvinylpolypyrrolidone (PVPP). The ratio of homogenization buffer to tissue was 3.5:1. The homogenate was filtered through five layers of muslin cloth. The filtrate was frozen and thawed three times during 24 h and then centrifuged at 13,000 × *g* for 15 min at 4 °C. The oil body layer (top layer of the homogenate after centrifugation) was collected with a spatula and aliquots from the clear 13,000 *g* supernatant was used for assaying PDH activity. PDH activity was determined essentially by the method described by Hinman and Blasss (1981), except that DTT was excluded from the reaction mixture (Schwab et al., 2005). The reaction mixture in a final volume of 1.0 ml consisted of 50 mM KH_2PO_4 buffer pH 7.5, 1 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 0.1% Bovine serum albumin, 0.6 mM iodonitrotetrazolium chloride (INT), 0.2 mM thiamine pyrophosphate, 0.1 mM acetyl-coenzyme A, 2.5 mM NAD^+, 6.5 μM phenazine methosulfate, 5 mM Pyruvate and aliquots from the enzyme crude extract. The reaction was started by the addition of Pyruvate and the increase in absorbance at 500 nm was recorded for 3 min using a Shimadzu UV-160A Spectrophotometer. As control, assay mixtures without Pyruvate were constructed to assess non-pyruvate dependent dehydrogenase activity of the enzyme extract. The enzyme activity was expressed as nmole pyruvate oxidized per min per g tissue fresh weight (nmol min^{-1} g^{-1} FW) assuming an extinction coefficient (ε_{500}) of 12.4 $(M\,cm)^{-1}$ for the reduced INT (Schwab et al., 2005).

Extraction and assay of NAD^+-IDH (EC 1.1.1.41) activity were carried out essentially as described by Behal and Oliver (1998). Cotyledonary tissues (1.5 g) were ground and homogenized in cold homogenization buffer (4.5 ml), consisting of 20 mM KH_2PO_4 (pH 7.1), 0.3 M sorbitol, 0.1% Bovine serum albumin, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1% (v/v) 2-mercaptoethanol and 0.6% (w/v) PVPP. The homogenate was filtered through two layers of muslin cloth and centrifuged at $1500 \times g$ for 5 min at 4 °C. The obtained $1500 \times g$ supernatant was re-centrifuged at $10{,}000 \times g$ for 15 min at 4 °C to precipitate mitochondria. The $10{,}000 \times g$ pellet containing mitochondria was re-suspended in suspension buffer consisting of 20 mM KH_2PO_4 (pH 7.1), 0.4 M sorbitol, 15% glycerol (w/w) and 0.1% (v/v) 2-mercaptoethanol. The mitochondrial suspension was centrifuged at $1500 \times g$ for 5 min at 4 °C. The obtained $1500 \times g$ supernatant was re-centrifuged at $10{,}000 \times g$ for 15 min at 4 °C to precipitate mitochondria. The mitochondrial $10{,}000 \times g$ pellet was re-suspended in the suspension buffer (450 μl) and frozen and thawed two times during 24 h to disrupt mitochondria. The preparation was then used as the enzyme source. The reaction mixture in a final volume of 1.0 ml consisted of 20 mM HEPES-KOH (pH 7.5), 1 mM $MgSO_4$, 1 mM NAD^+, 10 mM DL-isocitrate and aliquots from the enzyme source. The reaction was started by the addition of DL-isocitrate and the increase in absorbance at 340 nm was recorded for 2 min using a Shimadzu UV-160A Spectrophotometer. As control, assay mixtures without DL-isocitrate were constructed to assess DL-isocitrate independent dehydrogenase activity of the enzyme extract. The enzyme activity was expressed as nmole DL-isocitrate oxidized per min per g tissue fresh weight (nmol min^{-1} g^{-1} FW) assuming an extinction coefficient (ε_{340}) of 6220 $(M\,cm)^{-1}$ for the reduced NAD^+ (Tian et al., 2005).

SDH (EC 1.3.99.1) activity was extracted as described by Sarkar et al. (2009). The tissue (1 g) was ground with 3.0 ml of cold homogenization buffer consisting of 100 mM KH_2PO_4 buffer pH 8.0, 3 mM EDTA and 0.6% PVPP (w/w). The homogenate was centrifuged at $13{,}500 \times g$ for 10 min at 4 °C and the resulting clear supernatant was used for assaying the enzyme activity. SDH activity was determined by the method described by Munujos et al. (1993). The assay mixture in a final volume of 1.0 ml contained 100 mM triethanolamine (pH 8.3), 0.5 mM EDTA, 2 mM KCN, 0.42% (w/v) Cremophor EL, 2 mM INT, 20 mM succinate and aliquots from the enzyme extract. The reaction was started by the addition of succinate and the increase in absorbance at 500 nm was recorded for 2 min. The enzyme activity was expressed as nmole succinate oxidized per min per g tissue fresh weight (nmol min^{-1} g^{-1} FW) assuming an extinction coefficient (ε_{500}) of 19,300 $(M\,cm)^{-1}$ for the INT-formazan product.

2.3. Extraction and assay of glyoxysomal succinate oxidase activity

The homogenization medium for the extraction of glyoxysomal succinate oxidase consisted of 50 mM HEPES-KOH buffer pH 7.5, 3 mM EDTA and 0.6% PVPP (w/v). The tissue (1 g) was ground with 6.0 ml of cold homogenization buffer and the homogenate was centrifuged at $13{,}500 \times g$ for 10 min at 4 °C. The resulting clear supernatant was used for assaying succinate oxidase activity as described by Igamberdiev et al. (1995). The assay mixture in a final volume of 1.0 ml contained 50 mM HEPES-KOH (pH 7.8), 0.6 mM NAD^+, 40 mM succinate, 15.0 U malate dehydrogenase (from porcine heart) and aliquots up to 100 μl from the enzyme extract. The reaction was started by the addition of succinate and malate production was recorded for 60 s by the increase in absorbance at 340 nm due to the production of reduced NAD^+ from malate oxidation. The enzyme activity was expressed as μmole succinate oxidized per min per g tissue fresh weight (μmol min^{-1} g^{-1} FW) assuming an extinction coefficient (ε_{340}) of 6220 $(M\,cm)^{-1}$ for the reduced NAD^+ (Tian et al., 2005).

2.4. Extraction and assay of phosphoenolpyruvate carboxykinase (PEPCK)

For the extraction of PEPCK (EC 4.1.1.49) the tissue (1 g) was ground with 8.0 ml of cold homogenization medium consisting of 10 mM HEPES-KOH pH 7.0, 0.5 mM PMSF, 0.1% (v/v) 2-mercaptoethanol and 0.6% (w/v) PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at $13{,}500 \times g$ for 10 min at 4 °C. The clear supernatant was used for the assay of enzyme activity. The enzyme activity was determined in the carboxylation direction as described by Chen et al. (2004). It resulted in oxaloacetate production which was quantified after conversion to malate by malate dehydrogenase. The accompanying decrease in the absorbance at 340 nm due to oxidation of NADH coupled to oxaloacetate reduction was taken as a measure of enzyme activity. The assay mixture in a final volume of 1.0 ml contained 100 mM HEPES-KOH (pH 7.0), 90 mM $NaHCO_3$, 6.0 mM $MgSO_4$, 0.1% (v/v) 2-mercaptoethanol, 0.3 mM NADH, 2.0 mM ADP, 6.0 mM PEP, 15.0 U malate dehydrogenase and aliquots (10 μl) from the enzyme extract. The reaction was started by the addition of PEP and malate production was recorded for 2 min by decrease in absorbance due to NADH oxidation at 340 nm against a similar reaction mixture lacking ADP (Martin et al., 2007). The enzyme activity was expressed as μmole phosphoenol pyruvate consumed per min per g tissue fresh weight (μmol min^{-1} g^{-1} FW) assuming an extinction coefficient (ε_{340}) of 6220 $(M\,cm)^{-1}$ for the reduced NAD^+ (Tian et al., 2005).

2.5. Other analytical methods

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was assayed essentially as described by Deutsch (1983). The extraction and assay of lipoxygenase (EC 1.13.11.12) activity was essentially carried out as described by Axelrod et al. (1981). Lipid hydroperoxides were extracted according to Griffiths et al. (2000) and quantified spectrophotometrically by the Ferrous-Xylenol Orange (FOX) reagent as described by Delong et al. (2001).

2.6. Statistical analyses

Statistically significant differences at the 5% level were determined by Nested Design Analysis and the Duncan's multiple range test method (SAS software 2010, SAS Institute Inc., Cary, North Carolina, USA). The multiple fitted frequency curves were derived based on multiple histograms each depicting the relative frequency of kernels with a defined quantity of the studied biochemical parameter.

3. Results

3.1. Effects of cold stratification on germination rate of walnut kernels

Stratification of dormant walnut kernels at cold for 30 days had greatly improved their germination rate when compared to non-stratified warm incubated ones (Fig. 1). The rate of germination as measured by the time required for seeds to reach to 50% of their final germination was 0.15 $days^{-1}$ for 30 days cold stratified kernels whereas the corresponding figure for the non-stratified warm incubated ones was 0.06 $days^{-1}$.

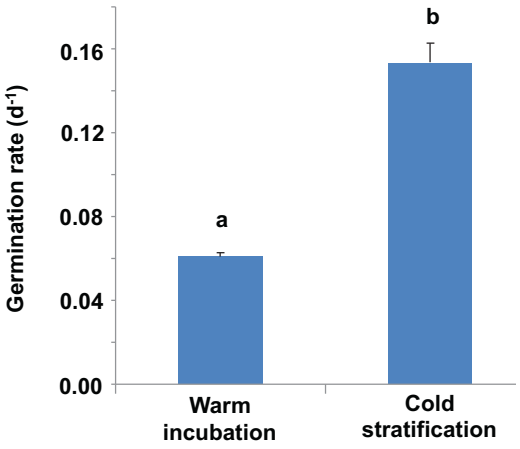

Fig. 1. Effect of stratification on the germination rate of dormant walnut kernels. Walnut kernels were cold stratified for 30 days at 5 °C, sown in the sand medium at 27 °C and their germination were compared with non-stratified warm incubated kernels incubated under the same conditions. Lots of 75 nuts (in triplicates of 25) were incubated under either cold or warm conditions. Each point represents the mean value of triplicate experiments, each consisting of 25 seeds ± SE. Statistically significant ($P < 0.05$) differences are shown by different small letters.

3.2. Lipoxygenase activity in warm incubated and cold stratified walnut kernels

The imbibed dormant walnut kernels initially had lipoxygenase activity of about 26 nmol min^{-1} g^{-1} FW. It increased gradually and reached to the greatest amount of about 46 nmol min^{-1} g^{-1} FW within four days under warm conditions (Fig. 2A). Significant decrease in enzyme activity occurred till 16 days of kernel incubation at warmth. Lipoxygenase activity however, remained relatively unchanged for about 60 days in cold stratified kernels (Fig. 2B). The distribution of lipoxygenase activities of warm incubated kernels compared to that of the cold stratified ones by Nested Design showed significantly ($P < 0.05$) greater mean of the enzyme activity in the former group (Fig. 2C).

3.3. Walnut kernel lipid hydroperoxide (LHP) contents under warm and cold conditions

Kernel LHP content was remained unaltered at the level of about 30 nmol g^{-1} FW till eight days under warm conditions (Fig. 3A). Significant increase in LHP content occurred from this time thereafter and reached to the greatest amount of 267 nmol g^{-1} FW after 16 days. From this time onwards, kernels were deteriorated so that the quantification of LHPs was not possible anymore. In cold stratified kernels however, LHPs were declined from an initial amount of 26 nmol g^{-1} FW in the imbibed seeds and reached to a non-detectable level in kernels that were cold stratified for 10 days and beyond (Fig. 3B).

3.4. The activities of walnut kernel mitochondrial dehydrogenases under warm and cold conditions

The activity of PDH was about 28 nmol min^{-1} g^{-1} FW in imbibed kernels. It increased under warm conditions and reached to the greatest amount of about 43 nmol min^{-1} g^{-1} FW after 12 days (Fig. 4A). The enzyme activity declined thereafter so that in kernels warm incubated for 20 days the lowest amount of 11 nmol min^{-1} g^{-1} FW was recorded. The activity of PDH however, declined following kernel moist chilling so that it remained undetectable from 10 days of cold stratification onward (Fig. 4B).

The activity of SDH increased under warm conditions from 21 to 35 nmol min^{-1} g^{-1} FW within 12 days of kernel incubation. It was then declined so that in warm incubated kernels for 16 days and beyond the figure had an average of about 20 nmol min^{-1} g^{-1} FW (Fig. 5A). The activity of SDH declined following kernel moist chilling so that it remained undetectable from 10 days of cold stratification onward (Fig. 5B).

The activity of another mitochondrial dehydrogenase i.e. NAD^+-IDH, showed some fluctuations in warm incubated kernels (Fig. 6A). The enzyme activity was significantly greater in 8 and 16 days warm incubated kernels (about 30 nmol min^{-1} g^{-1} FW) when compared to the figure (20 nmol min^{-1} g^{-1} FW) in imbibed kernels. The lowest enzyme activity of about 10 nmol min^{-1} g^{-1} FW occurred in 20 days old warm incubated kernels. The NAD^+-IDH activity was relatively unchanged at the level of about 20 nmol min^{-1} g^{-1} FW during 50 days of kernel moist chilling however, it declined significantly in 60 days old moist chilled kernels and reached to 11 nmol min^{-1} g^{-1} FW (Fig. 6B). The distribution of NAD^+-IDH activities of warm incubated kernels when compared to that of the cold stratified ones by Nested Design showed no significant difference between both groups (Fig. 6C).

3.5. Succinate oxidase and PEPCK activities in warm incubated and cold stratified walnut kernels

Succinate oxidase reportedly to be a glyoxysomal enzyme in germinating seeds of some gramineae oxidizes succinate into malate with the accompanying release of hydrogen peroxide (Igamberdiev et al., 1995). This activity has been implied for oxidizing excess succinate uncoupled with ATP production. The succinate oxidase activity of walnut kernels was determined and compared after incubating them for 20 days under either warm

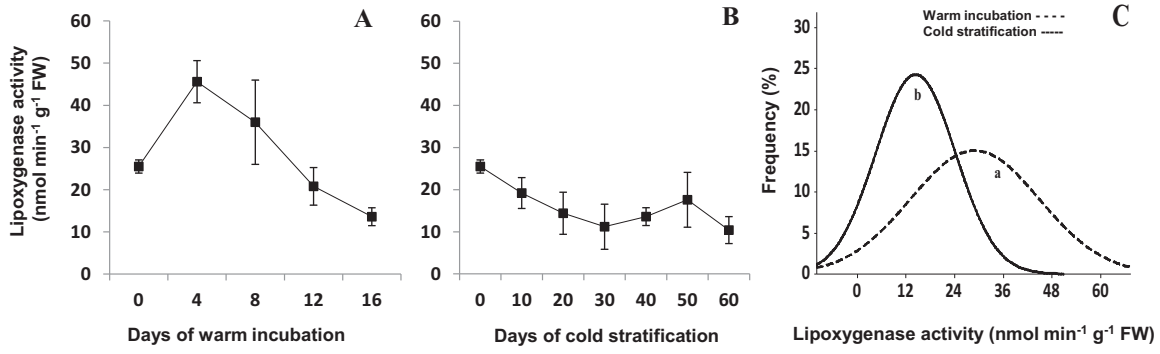

Fig. 2. Time course of changes in lipoxygenase activity in cotyledons of warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions. The relative frequencies of lipoxygenase activities of warm-incubated (dashed line) and cold-stratified (solid line) walnut kernels as obtained by Nested Design analysis are depicted in C. Statistically significant ($P < 0.05$) differences are shown by different small letters.

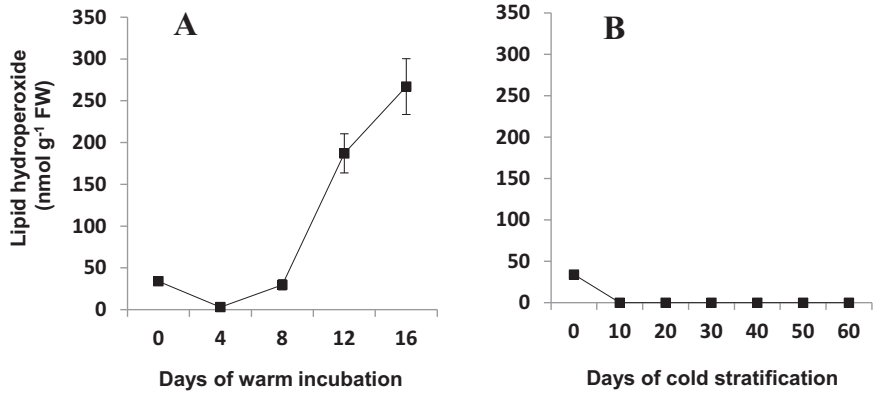

Fig. 3. Time course of changes in lipid hydroperoxide (LHP) contents of cotyledons from warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions.

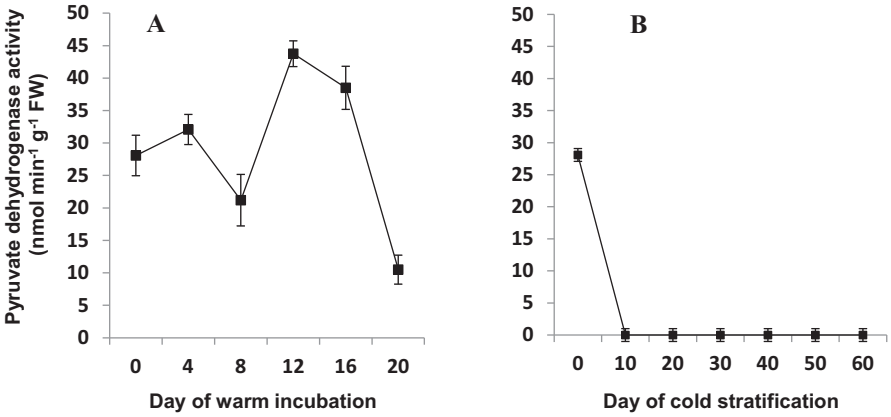

Fig. 4. Time course of changes in pyruvate dehydrogenase (PDH) activity of cotyledons from warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions.

or cold conditions (Fig. 7A and B). The enzyme activity was significantly greater in cotyledons (by 62%) and axes (by 109%) of 20 days cold stratified kernels compared to the corresponding figures obtained for 20 days warm incubated ones (Fig. 7A and B). Thus, the recorded enzyme activities in 20 days cold stratified kernels were1.144 ± 0.109 μmol min^{-1} g^{-1} FW for axes and 1.334 ± 0.030 μmol min^{-1} g^{-1} FW for cotyledons. In 20 days warm incubated kernels the measured enzyme activities for axes and cotyledons were 0.548 ± 0.077 and 0.821 ± 0.071 μmol min^{-1} g^{-1} FW, respectively.

The activity of PEPCK increased significantly following cold stratification of walnut kernels (Fig. 8A and B). Cold stratification of walnut kernels for 20 days increased the enzyme activity by about 4.0 and 2.5 folds in axes and cotyledons respectively, compared to 20 days walnut kernels incubated under warm conditions. The recorded enzyme activity in 20 days cold stratified axes was 237.7 ± 40.1 μmol min^{-1} g^{-1} FW, and in the case of cold stratified cotyledons it amounted to 346.6 ± 32.0 μmol min^{-1} g^{-1} FW. In 20 days warm incubated kernels the measured enzyme activities of axis and cotyledon

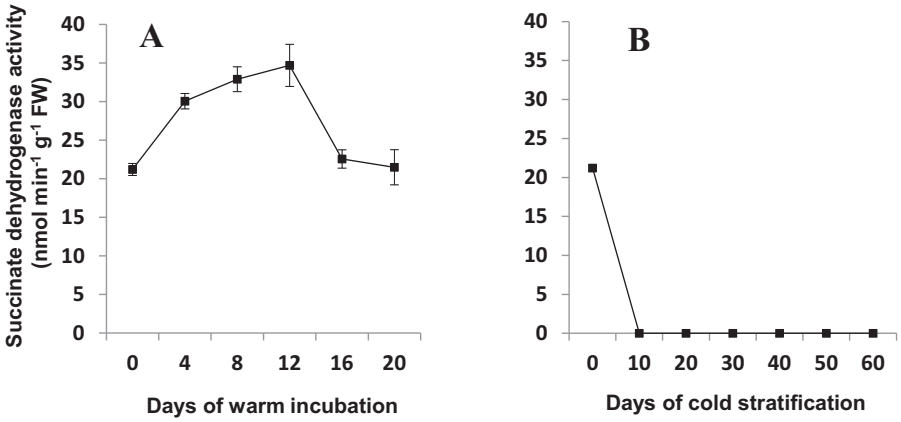

Fig. 5. Time course of changes in succinate dehydrogenase (SDH) activity of cotyledons from warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions.

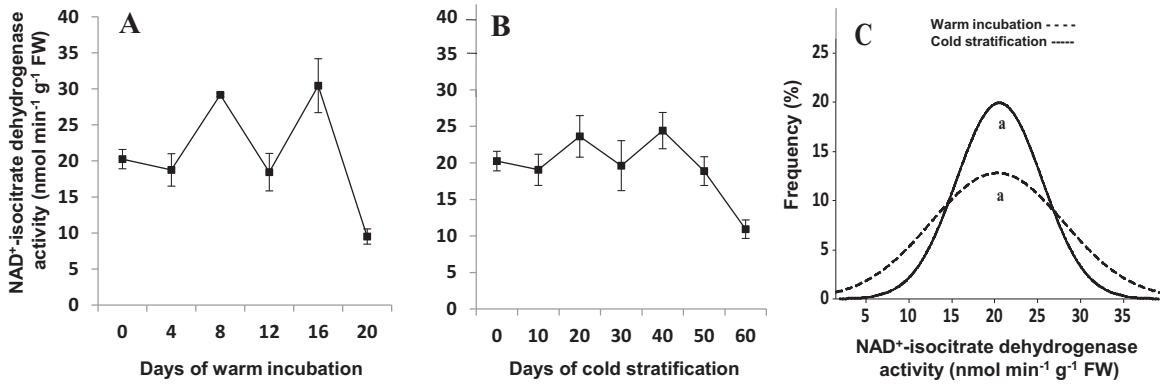

Fig. 6. Time course of changes in NAD$^+$-isocitrate dehydrogenase (NAD$^+$-IDH) activity in cotyledons of warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions. The relative frequencies of NAD$^+$-IDH activities of warm-incubated (dashed line) and cold-stratified (solid line) walnut kernels as obtained by Nested Design analysis are depicted in C. Statistically significant ($P < 0.05$) differences are shown by different small letters.

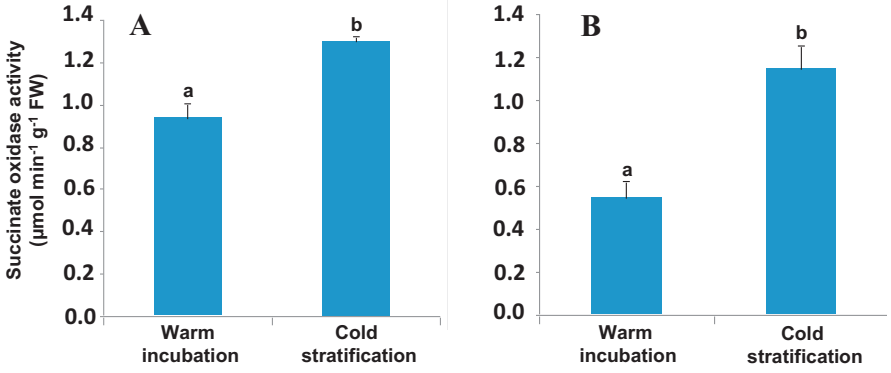

Fig. 7. Succinate oxidase activity in cold stratified and warm incubated walnut cotyledons (A) and axes (B). Tissue homogenates were prepared from 20 days cold stratified or warm incubated walnut kernels and used to assay succinate oxidase activity. Each point represents the mean value of three separate extractions ± SE. Statistically significant ($P < 0.05$) differences are shown by different small letters.

were 59.7 ± 21.0 and 139.3 ± 13.2 µmol min^{-1} g^{-1} FW, respectively.

3.6. Glucose-6-phosphate dehydrogenase activity in warm incubated and cold stratified walnut kernels

Under warm conditions, G6PDH activity gradually increased up to 16 days and remained unchanged till 20 days of kernel incubation (Fig. 9A). Under moist chilling conditions, the kernel enzyme activity increased significantly by about 90% within 10 days (Fig. 9B). It reached from 281.0 nmol min^{-1} g^{-1} FW in imbibed kernels to 538.0 nmol min^{-1} g^{-1} FW in those moist chilled for 10 days and thereafter it remained more or less constant. The mean enzyme activity of kernels (Fig. 9C) as analyzed by Nested Design was significantly lower (about 340 nmol min^{-1} g^{-1} FW) under warm conditions compared to that under the moist chilling conditions (435.71 nmol min^{-1} g^{-1} FW).

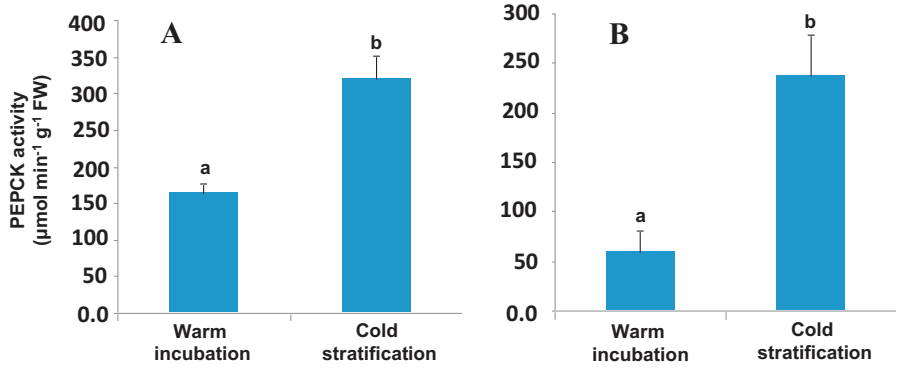

Fig. 8. Phosphoenolpyruvate carboxykinase (PEPCK) activity in cold stratified and warm incubated walnut cotyledons (A) and axes (B). Tissue homogenates were prepared from 20 days cold stratified or warm incubated walnut kernels and used to assay PEPCK activity. Each point represents the mean value of three separate extractions ± SE. Statistically significant ($P < 0.05$) differences are shown by different small letters.

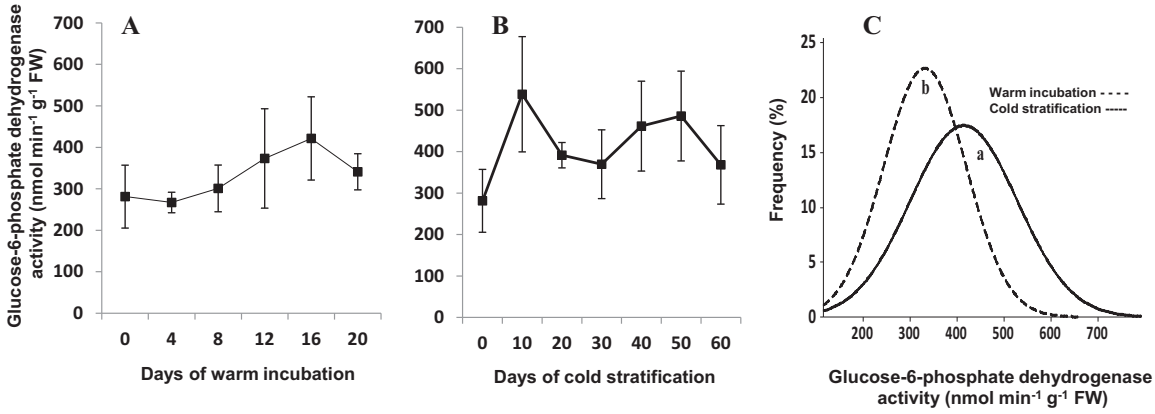

Fig. 9. Time course of changes in the activity of glucose-6-phosphate dehydrogenase (G6PDH) in cotyledons of warm-incubated (A) and cold-stratified (B) walnut kernels. Values are mean ± SE obtained from three separate extractions. The relative frequencies of G6PDH activities of warm-incubated (dashed line) and cold-stratified (solid line) walnut kernels as obtained by Nested Design analysis are depicted in C. Statistically significant ($P < 0.05$) differences are shown by different small letters.

4. Discussion

The impacts of moist chilling on walnut kernel metabolism are more pronounced and uniformly expressed in cotyledons rather than axes (Einali and Sadeghipour, 2007; Nezamdoost et al., 2009; Zarei-Ghadikolaee et al., 2010), thus mostly data from the former organ were used for making discussion. Detailed germination behavior of cold stratified and warm incubated walnut kernels have been described earlier (Nezamdoost et al., 2009). Cold stratification of walnut kernels for 30 days significantly improved their germination rate (Fig. 1), indicating it is an effective method for breaking kernel dormancy. There are also other commercially available stratification protocols for breaking walnut kernel dormancy (Vahdati and Hoseini, 2006). These methods though might be more efficient in breaking kernel dormancy, do not appear to act through a different mechanism. Radicle protrusion did not occur either at 5 °C for up to 60 days or 27 °C for up to 20 days, in all moist chilled and most of the warm incubated kernels, respectively. Beyond 20 days under warm conditions, most of the incubated kernels were deteriorated. Accordingly, all the experiments on warm incubated kernels were carried out up to 20 days and those on the cold stratified ones up to 60 days, though 30 to 40 days of cold stratification was sufficient to achieve maximum germination of kernels following their transfer to appropriate conditions i.e. 27 °C. The experiment was planned according to Nested Design to obtain and compare the distribution of the absolute values of the measured biochemical parameters between cold stratified and warm incubated kernels. This statistical analysis was found efficient when comparisons of the parameters were made between the two temperature treatments despite differences in incubation times and sampling intervals.

The lipoxygenase mediated peroxidation of lipids is the major cause of deterioration in moistened seeds (McDonald, 2004). Mature walnut kernels posses lipoxygenase activity (Buranasompob et al., 2007), thus the deterioration of dormant walnut kernels under warm conditions (Nezamdoost et al., 2009) might have been resulted from the increased lipoxygenase activity (Fig. 2) as it is reported in almond kernels during accelerated aging conditions (Zacheo et al., 1998). However, as data on the involvement of lipoxygenases in seed aging are not consistent (Kalpana and Rao, 1993; Suzuki et al., 1999), correlation between lipoxygenase activity and the in vivo changes of its immediate and final products on lipids i.e. LHPs and MDA, respectively (Porta and Rocha-Sosa, 2002; Bou et al., 2008), might be instructive for revealing the role of this enzyme in walnut kernel aging under warm conditions. The greater MDA content of warm incubated walnut kernels (Nezamdoost et al., 2009) might not be a good measure of bulk lipid peroxidation as it is produced only from unsaturated fatty acids having three or more double bonds (Halliwel and Gutteridge, 1989), whereas walnut kernel lipids mostly contain linoleic acid with two double bonds (Savage, 2001). Thus, the greater LHP content of warm incubated walnut kernels (Fig. 3), suggests lipid peroxidation events are predominant under these conditions. As the maximum lipoxygenase activity was not coincided to the greatest amount of LHPs under warm conditions (Figs. 2A and 3), its role in kernel aging and deterioration remains unresolved. The profile of walnut kernel bulk lipid peroxidation appears to depend on the tissue hydrogen peroxide content rather than lipoxygenase activity as it mostly followed the profile of kernel hydrogen peroxide contents (Nezamdoost et al., 2009). Negative correlations were also reported between beech seed germination potential with tissue lipid hydroperoxides and hydrogen peroxide contents (Pukacka and Ratajczak, 2005). Thus the beneficial effects of cold stratification in promoting walnut kernel germination, appears to be prevention of aging related peroxidative events.

The activity of a major mitochondrial dehydrogenase i.e. PDH increased during warm incubation of walnut kernels, while it declined following kernel moist chilling and mostly remained undetectable (Fig. 4). This activity is critically important, for it determines the flux of carbon into Krebs cycle and overall respiration rate (Juszczuk et al., 2007). The enzyme activity is considerably high in heterotrophic tissues whereas under situations favoring gluconeogenesis it declines to a significant extent (Gemel and Randall, 1992; Tovar-Mendez et al., 2003). The suppressed PDH activity in cold stratified walnut kernels (Fig. 4B) might be justified as they are active in gluconeogenesis (Nezamdoost et al., 2009). It guaranties lipid-derived C_4 carboxylic acids (mainly malate and its immediate oxidation product oxaloacetate) to be metabolized in the gluconeogenic direction. Furthermore, as lipids are the major food reserves of walnut kernels (Sze-Tao and Sathe, 2000), there would be little chance for pyruvate production and thus its gluconeogenesis. Pyruvate however, might be produced form amino acids like alanine, serine and cysteine and subsequently gluconeogenized via pyruvate orthophosphate dikinase in senescing organs engaged in nitrogen remobilization (Taylor et al., 2010). This situation might also exist in imbibed walnut kernels as they are engaged in storage protein mobilization (Einali and Sadeghipour, 2007; Shahmoradi et al., 2013).

The activity of PEPCK in 20 days cold stratified kernels was more than that of the warm incubated ones of the same age (Fig. 8). The 20 days cold stratified kernels were supposed to be active in gluconeogenesis as they lacked PDH activity. The greater kernel PEPCK

activity under cold conditions indicates the prevalence of gluconeogenic metabolism. This enzyme has been shown to be essential for gluconeogenesis of lipid mobilization products in germinating seeds (Rylott et al., 2003a). Furthermore, it is in congruence with the idea denoting that activation of lipid gluconeogenesis in cold stratified walnut kernels occurs beyond the glyoxylate cycle (Nezamdoost et al., 2009). The latter contention was drawn as no sugar was accumulated in warm incubated kernels but they had isocitrate lyase (EC 4.1.3.1) activity not significantly different from cold stratified ones. Malate synthase activity i.e. another key enzyme of the glyoxylate cycle was not recorded in walnut kernels as its expression is coordinated to that of isocitrate lyase in germinating oilseeds (Rylott et al., 2003b). Thus, in contrast to cold stratified walnut kernels, the operation of glyoxylate cycle in warm incubated kernels is not gluconeogenic (Nezamdoost et al., 2009). The non gluconeogenic operation of the glyoxylate cycle has also been pointed out by other investigators (Eastmond and Graham, 2001).

The activity of SDH was detected only in crude homogenates of warm incubated walnut kernels (Fig. 5), and assumed to be mitochondrial as this enzyme is localized in mitochondria (Millar et al., 2004). In the soluble non-mitochondrial fractions however, this enzyme remains inactive and for succinate oxidation to occur enzyme partitioning into mitochondria is necessary (Nakayama et al., 1980). As the continued operation of the glyoxylate cycle and gluconeogenesis of lipid reserves require mitochondrial participation of SDH activity (Cooper and Beevers, 1969), the absence of this activity in gluconeogenically active cold stratified walnut kernels was unexpected and needs to be justified. Thus other routes for succinate oxidation such as glyoxysomal succinate oxidase (Igamberdiev et al., 1995) might be operative in cold stratified walnut kernels. Glyoxysomal succinate oxidase converts succinate directly into malate, hence compensating the lack of SDH activity. As 20 days old cold stratified kernels lacked SDH activity, they were investigated for succinate oxidase activity. Substantial levels of succinate oxidase activity were found in walnut kernels however, cold stratification stimulated this activity significantly (Fig. 7). The activity was insensitive to malonic acid, i.e. an inhibitor of SDH (data not shown). The existence of succinate oxidase activity in cold stratified walnut kernels can allow the continued operation of the glyoxylate cycle without the need for SDH activity. In actively respiring warm incubated walnut kernels with non-gluconeogenic operation of the glyoxylate cycle (Nezamdoost et al., 2009) this activity however, might allow overflow succinate oxidation uncoupled from ATP synthesis (Igamberdiev et al., 1995). It is noteworthy that walnut is the first reported dicotyledonous species with succinate oxidase activity. So far this enzyme has only been reported in germinating seeds of some gramineae such as *Zea mays* L. and *Triticum aestivum* L. (Igamberdiev et al., 1995). The SDH supression of cold stratified walnut kernels might be at either transcriptional or translational levels. To reveal this issue further studies are necessary.

The activity of NAD^+-IDH i.e. another mitochondrial dehydrogenase remained relatively unchanged and indifferent between kernels incubated either under cold or warm conditions (Fig. 6). This enzyme regulates the flux of lipid mobilization products to either pathways of gluconeogenesis or respiration via the Krebs cycle in some germinating oilseeds (Falk et al., 1998). However, in imbibed walnut kernels as discussed above this regulation is possibly achieved by PDH and SDH rather than NAD^+-IDH. The greater activities of both PDH and SDH in warm incubated walnut kernels versus the cold stratified ones might suggest the prevalence of mitochondrial respiratory metabolism operating in the former. This view is supported by both declined carbohydrate levels (Nezamdoost et al., 2009) and enhanced amino acid catabolism (Zarei-Ghadikolaee et al., 2010) in kernels under warm conditions. Thus, the accumulation of hydrogen peroxide in warm incubated walnut kernels (Nezamdoost et al., 2009), might have been resulted from the increased respiratory metabolism (Figs. 4 and 5). Recent finding on the SDH mediation of mitochondrial reactive oxygen production further supports this idea (Gleason et al., 2011). The possible in vivo inhibitory role of toxic aldehydes on mitochondrial dehydrogenases (Millar and Leaver, 2000; Winger et al., 2007) in warm incubated walnut kernels needs further investigations as they had significantly greater activities of both PDH and SDH compared to cold stratified ones. This inhibition however, might only be observed in walnut kernels which are incubated for sufficiently long time e.g. 20 days under warm conditions. These kernels displayed significant decline in the activities PDH, SDH and NAD^+-IDH which were corresponded to the stage of greatest LHP accumulation (Fig. 3).

While the mechanism is not yet known, declined functioning of OPP pathway is associated with the decreased germination potential of dormant seeds (Bethke et al., 2007). In congruence, the activity of G6PDH i.e. the marker enzyme of OPP pathway was lower in walnut kernels under warm versus to the moist chilling conditions (Fig. 9). The G6PDH activity is important for counteracting oxidative stress as it provides reducing equivalents (Kruger and Von Schaewen, 2003; Scharte et al., 2009). The declined G6PDH activity in warm incubated walnut kernels might represent the progression of seed aging process (Bettey and Finch-Savage, 1996; Xin et al., 2011). Greater enzyme activity under moist chilled conditions however, implies increased kernel competence for counteracting oxidative stress and nucleotide biosynthesis. The activity of another extra mitochondrial enzyme i.e. $NADP^+$-isocitrate dehydrogenase ($NADP^+$-IDH) of walnut kernels was also declined from mid period of kernel incubation under warm conditions (Nezamdoost et al., 2009). This possibly points to the compromised kernel competence for both amino acid and reducing equivalent biosynthesis (Hodges et al., 2003; Zarei-Ghadikolaee et al., 2010).

5. Conclusions

The present study for the first time has explored the important role played by mitochondrial dehydrogenases in metabolic regulation of seeds during cold-induced dormancy release. Former studies however, have mainly dealt with investigating extra-mitochondrial metabolic respiratory pathways like OPP, glycolysis and β-oxidation during this stage (Penfield et al., 2007; Lewak, 2011). It appears that in cold stratifying walnut kernels, the mitochondrial respiration of either pyruvate or succinate is restricted through down regulation of PDH and SDH activities, respectively. Meanwhile the cold induced stimulation of glyoxysomal succinate oxidase and PEPCK activities may show the post-glyoxylate cycle activation of gluconeogenesis. Despite PDH suppression, Krebs cycle and hence ATP generation can proceed in cold stratified walnut kernels. This can be achieved following transfer and subsequent oxidation of acetyl moieties of either glyoxysomal citrate or isocitrate (derived from β-oxidation of lipid reserves) into mitochondria (Pracharoenwattana et al., 2005). The energetic efficiency for acetyl-CoA oxidation in cold stratified walnut kernels however, is expected to be less as succinate oxidation occurs via succinate oxidase rather than SDH. The oxidation of cytosolically generated reducing equivalents by external NAD(P)H-dehydrogenases of inner mitochondrial membranes (Millar et al., 2011), might also contribute to ATP production of kernels under moist chilled conditions. The present study further suggests that the beneficial effects of moist chilling on seed dormancy release might be exerted through limiting the respiration-induced oxidative burst and the associated deleterious lipid peroxidative events. These possibly lead to the activation of some germination-specific metabolism such as the OPP pathway known to be activated during

seed dormancy release (Bettey and Finch-Savage, 1996; Nonogaki et al., 2010).

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