Scientia Horticulturae 161 (2013) 314–323

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

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

Maryam Keshavarzian[a], Zahra Gerivani[a], Hamid Reza Sadeghipour[a,*], Mahnaz Aghdasi[a], Majid Azimmohseni[b]

[a] Department of Biology, Faculty of Science, Golestan University, Gorgan, Iran
[b] Department of Statistics, Faculty of Science, Golestan University, Gorgan, Iran

ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form 22 July 2013
Accepted 24 July 2013

Keywords:
Juglans regia
Glyoxysomal succinate oxidase
Lipoxygenase
Phosphoenolpyruvate carboxykinase
Pyruvate dehydrogenase
Seed dormancy

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.

* Corresponding author. Tel.: +98 9111775149; fax: +98 1712245964.
E-mail addresses: h.r.sadeghipour@gmail.com, hamidrezasadegh@yahoo.com (H.R. Sadeghipour).

0304-4238/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scienta.2013.07.026

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).