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ORIGINAL PAPER

Arginase, glutamine synthetase and glutamate dehydrogenase activities in moist chilled and warm-incubated walnut kernels

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Abstract The activities of arginase, glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were studied in both moist chilled (5°C) and warm (27°C) incubated walnut (*Juglans regia*. L) kernels to asses whether the non-germinability of dormant kernels is associated with failure in amino acid metabolism. Warm-incubated kernels showed low germination (25%), whereas cold-stratified kernels displayed germination up to 61%. Arginase activity increased about twofold in imbibed kernels. It remained at a high level in cold-stratified kernels from mid-period of incubation onwards; however, in warm-incubated kernels the activity declined after an initial increase so that by 20 days, it was negligible. No significant differences in GS activity occurred between cold-stratified and warm-incubated kernels, but the activity of GDH was significantly more in kernels incubated at warm conditions. Thin-layer chromatographic separation of polyamines revealed greater ammonia, spermidine and an unknown polyamine accumulation in warm-incubated kernels. Thus, the declined rate of walnut kernel germination under warm conditions is mainly correlated with rapid inactivation of arginase, greater levels of ammonia and alterations in kernel polyamine composition. The enhanced activity of GDH in warm-incubated kernels implies that catabolic deamination of amino acids and their subsequent respiration is the favored pathway ongoing under warm conditions. This situation compromises germination-specific metabolism of amino acids which likely to operate better at lower temperatures during cold stratification of kernels.

Keywords Arginase · Cold stratification · Glutamate dehydrogenase · Glutamine synthetase · *Juglans regia*

Abbreviations

GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylene diamine tetraacetic acid
PVPP	Polyvinylpolypyrrolidone

Introduction

Seeds from many temperate tree species are dormant at maturity (Derkx 2000). To germinate they usually need periods varying from few to several weeks of moist chilling or cold stratification which weaken embryo dormancy and allows germination. According to metabolic inhibition theory, the germination failure of dormant embryos is due to blocks in reserve mobilization, which are removed following embryo moist chilling (Ross 1984; Lewak et al. 2000). Although no reserve mobilization occurs in warm-incubated dormant seeds, the mobilization of food reserves such as storage lipids, proteins and phytate has been reported during moist chilling of dormant seeds from both

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gymnosperms and angiosperms arboraceous species (Dawidowicz-Grzegorzewska 1989; Li and Ross 1990; Wang and Berjak 2000; Forward et al. 2001; Andriotis et al. 2004). This effect of cold is brought about by activation of various hydrolytic enzymes such as lipase (Li and Ross 1990; Zarska-Maciejewska 1992), protease (Zarska-Maciejewska and Lewak 1983; Forward et al. 2001) and phytase (Andriotis et al. 2004).

The subsequent metabolism of metabolites produced from reserve mobilization might also be a target of cold stimulus in the stratification treatment. In walnut (*Juglans regia* L.) kernels, for example, lipid mobilization proceeds equally under both cold and warm conditions. However, gluconeogenesis of lipid reserves would occur only during cold stratification (Nezamdoost et al. 2009). In addition, cold stratification may exert its beneficial effects by activating cellular repair mechanisms (Wang and Berjak 2000), and preventing deteriorative aging-related processes (Nezamdoost et al. 2009).

Persian walnut is an important nut tree from temperate regions. Cold stratification of walnut kernels promotes their germination (Kaur et al. 2006; Einali and Sadeghipour 2007). Storage proteins constitute about 17% of the walnut kernel food reserves (Sze-Tao and Sathe 2000). They are mobilized in both cold-stratified and warm-incubated walnut kernels; however, significant amino acid accumulation occurs only in kernels incubated at warm conditions, possibly due to failure in amino acid metabolism (Einali and Sadeghipour 2007). Because arginine is a major residue of walnut kernel proteins and constitutes a great proportion of the embryo-free amino acids before and during germination (Mapelli et al. 2001), comparison of its metabolism between cold-stratified and warm-incubated kernels might indicate the general failure in amino acid metabolism under warm conditions. This amino acid is also a major constituent of free amino acid pool in the nutritive tissues of other woody (Desmaison and Tixier 1986; King and Gifford 1997; Cantón et al. 2005) and herbaceous (Splittstoesser 1969; Capdevila and Dure 1977) species during protein mobilization. Being a rich source of nitrogen (C/N ratio of 2:1), arginine utilization and transport of the arginine-derived nitrogen following protein mobilization by the embryo require proper functioning of amino acid metabolizing enzymes, such as arginase, glutamine synthetase (GS) and glutamate dehydrogenase (GDH). Sequential activity of arginase and urease on their respective substrates, i.e., arginine and urea liberate ammonia, the latter is being used for glutamine synthesis by GS (King and Gifford 1997; Miflin and Habash 2002; Suarez et al. 2002). On the other hand, the extent of GDH activity might determine the flux of amino acids to either transport pathways or respiration (Miflin and Habash 2002). When considering that citrulline is the major transportable amino acid in germinating walnut kernels (Mapelli et al. 2001), the importance of these metabolic interconversions is further underlined. So far, there is no report on the activities of amino acid metabolizing enzymes during moist chilling of dormant seeds. Accordingly in the present work the activities of arginase, GS and GDH have been studied in imbibed dormant walnut kernels during cold stratification and warm conditions to find out whether the metabolism of amino acids is affected by cold stimulus in the stratification treatment.

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 2006 and 2007. 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 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 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. After isolating kernels from both cold-stratified and warm-incubated nuts, axes and cotyledons were excised with a razor blade from those which did not show any visible sign of germination and used for subsequent biochemical analyses.

Extraction and assay of arginase activity

Cotyledonary or axial tissues were ground and homogenized in cold homogenization buffer, consisting of 0.1 M Tris–0.5 M maleate buffer pH 8.0, 1 mM EDTA, 0.1 mM PMSF and 1% (v/v) 2-mercaptoethanol. The ratio of homogenization buffer to tissue was 2.5:1. 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 aliquots from the clear 10,000*g* supernatant was used for assaying arginase (EC 3.5.3.1)

activity (Goldraij and Polacco 1999). Before assay, arginase was activated by incubating enzymic crude extract with 100 mM Mn^{2+} at 35°C as described by Greenberg (1955).

Arginase activity was determined essentially by the method described by Roubelakis and Kliewer (1978). Arginase reaction mixture in a final volume of 2.5 ml consisted of 170 mM arginine pH 9.5, 2 mM $MnSO_4$ and aliquots from the enzyme crude extract. The reaction was carried out at 35°C and started by the addition of enzyme. It was allowed to proceed for 20 min and at every 5-min intervals, 0.5-ml aliquots were taken from the reaction mixture and the liberated ornithine due to arginase action determined spectrophotometrically (Chinard 1952). As control, enzymic extracts pretreated for 30 min at 95°C, i.e., heat-inactivated arginase was added to a similar reaction mixture and the reaction was followed accordingly. Arginase activity was expressed as nanomole ornithine produced per minute per gram fresh weight of tissue (nmol min^{-1} g^{-1} fw).

Extraction and assay of GS activity

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 PMSF, 0.1% (v/v) 2-mercaptoethanol and 0.6% (w/v) PVPP. The ratio of homogenization buffer to tissue was 3:1. The homogenate was filtered through five layers of muslin cloth and centrifuged at 10,000*g* for 30 min at 4°C. The 10,000*g* supernatant was used for the measurement of GS (EC 6.3.1.2) activity according to the method described by Montanini et al. (2003). Reaction mixture (pH 7.2) in a final volume of 2.0 ml consisted of 90 mM glutamine, 100 mM imidazole, 120 mM hydroxylamine, 2.4 mM $MnSO_4$, 0.5 mM ADP, 2 mM DTT, 50 mM Na_2HAsO_4 and aliquots from the enzyme extract. In the control assay mixtures, arsenate was excluded. The reaction was carried out for 20 min at 35°C. At every 5-min intervals, aliquots (450 µl) were taken from the reaction mixture and mixed with iron (III) chloride reagent (150 µl) to stop the enzymatic reaction. After centrifugation at 13,000*g*, the absorbance at 500 nm of the γ-glutamyl hydroxamate present in the clear supernatant was recorded. Assuming an extinction coefficient (ε_{500}) of 1.08 $(M\ cm)^{-1}$, GS activity was expressed as millimole of glutamine consumed per minute per gram fresh weight of tissue (mmol min^{-1} g^{-1} fw).

Extraction and assay of GDH activity

GDH (EC 1.4.1.2) activity was extracted and assayed as described by Restivo (2004). The tissue (1 g) was ground with 2.5-ml cold homogenization buffer consisting of 100 mM Tris buffer pH 8.0, 10 mM $MgSO_4$, 40 mM $CaCl_2$, 1 mM EDTA, 1 mM PMSF, 0.6% PVPP, 60 mM 2-mercaptoethanol and 0.05% Triton X-100. The homogenate was incubated at 5°C for 30 min, centrifuged at 15,000*g* for 15 min at 5°C and the clear 15,000*g* supernatant was used for assaying the enzyme activity. The assay mixture in a final volume of 1.3 ml contained 100 mM Tris and 120 mM glutamate adjusted to pH 8.0, 1 mM NAD^+ and aliquots from the enzyme extract. The reaction started by the addition of Tris–glutamate buffer and the increase in absorbance at 340 nm due to NADH formation was recorded for 5 min. As control, assay mixtures without glutamate were constructed to assess non-glutamate-dependent dehydrogenase activity of the enzyme extract. The enzyme activity was expressed as nanomole glutamate oxidized per min per gram tissue fresh weight (nmol min^{-1} g^{-1} fw) assuming an extinction coefficient (ε_{340}) of 6,220 $(M\ cm)^{-1}$ for the NADH product (Tian et al. 2005).

Other analytical methods

Extraction of free polyamines was carried out as described by Flores and Galston (1982). The extracted polyamines were dansylated subsequently by the same method with minor modifications. These include reducing the dansylation time to 1 h and performing at 45°C. Another modification was the use of proline rather than glycine to remove excess dansyl chloride from the dansylation mixture before partitioning of the dansylated polyamines into the benzene phase (Lapa-Guimaraes and Pickova 2004). Thin-layer chromatography of dansylated polyamines was carried out by the method of Lapa-Guimaraes and Pickova (2004) using Silica gel G-60-coated aluminum plates. Plates were developed in a mobile phase consisting of chloroform: diethyl ether: triethyl amine (4:1:4; v/v/v) and then photographed in a UV box.

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, NC, USA).

Results and discussion

Cold stratification of walnut kernels for 30 days increased their germination percentage significantly. Although non-stratified warm-incubated kernels showed germination of about 25%, the figure reached to 61% in cold-stratified

ones (data not shown). Our former studies have also shown that 30–40-day cold stratification of walnut kernels is sufficient for maximum germination (Einali and Sadeghipour 2007; Nezamdoost et al. 2009). Seed dormancy in walnut kernels is an innate one which originates from the embryo. Thus, germination is stimulated by both cold and GA_3 in either excised zygotic (Kaur et al. 2006) or somatic (Tang et al. 2000; San and Dumanoglu 2007) embryos. Because no germination occurred in kernels during 60 days of stratification at 5°C, all the biochemical changes reported here are attributed to dormancy release.

Non-stratified kernels, i.e., warm-incubated ones germinated after 10 days of incubation at 27°C and after 20 days of incubation most of them (about 75%) turned rancid (data not shown, Nezamdoost et al. 2009). Accordingly, samples were taken from these kernels only up to 20 days 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. Because cotyledons rather than axes appear to be the cold-perceiving organ in walnut kernels (Einali and Sadeghipour 2007; Nezamdoost et al. 2009), the present discussion is mainly based on the changes in this organ.

Mobilization of storage proteins is an important event during seed germination because it provides amino acids for de novo synthesis of germination-specific proteins (Rajjou et al. 2004). To be utilized by the growing radicle, cotyledonary-free amino acids initially must be converted into transportable forms (Canovas et al. 2007) by appropriate and consecutive activity of amino acid metabolizing enzymes such as arginase (King and Gifford 1997) and GS (Teixeira et al. 2005). This conversion might also be affected by cold stimulus in stratification treatment, especially in walnut kernels where protein mobilization commences in non-germinated kernels during release from dormancy (Einali and Sadeghipour 2007). Accordingly, the activity of arginase was compared between cold-stratified and warm-incubated kernels (Fig. 1). In cold-stratified kernels, arginase activity increased more than twofold within 40 days and, thereafter, it remained unaltered. Under warm conditions, a two to threefold increase in arginase activity occurred in 12-day-old kernels; however, it declined significantly in 20-day old kernels. Being a mitochondrial enzyme (Goldraij and Polacco 2000), dramatic decline in arginase activity might be due to oxidative stress observed by warm-incubated kernels (Nezamdoost et al. 2009). Because arginine is a major constituent of both storage proteins and free amino acid pool of walnut kernels (Sze-Tao and Sathe 2000; Mapelli et al. 2001), decline in arginase activity under warm conditions might compromise the amino acid metabolism and affects germination. Further comparative analysis of amino acids between cold-stratified and warm-incubated kernels is necessary to assess this point.

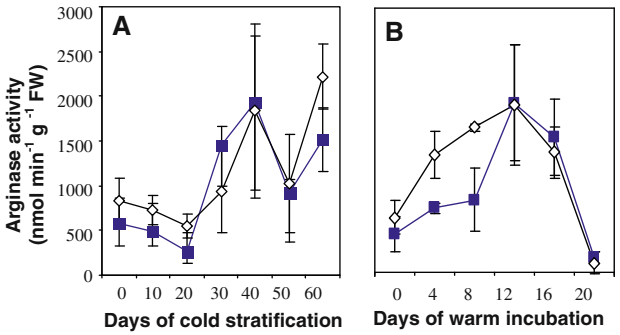

Fig. 1 Time course of changes in arginase activity in cotyledons (*filled square*) and axes (*diamond*) from cold-stratified (**a**) and warm-incubated (**b**) walnut kernels. Values are mean ± SE obtained from three separate extractions. Statistical significance for all data was calculated at $P < 0.05$

GS which is an active metabolic sink for the assimilation of ammonia (Miflin and Habash 2002; Suarez et al. 2002), is known to be affected by various environmental and internal stimuli (Oliveira et al. 2002; Pageau et al. 2006; Rana et al. 2008). The expression/activity of this enzyme might also be affected by cold stimulus either directly or indirectly, e.g. through phytohormones in stratifying walnut kernels. GS activity declined during cold stratification of kernels, but these changes were not significant (Fig. 2a). In warm-incubated kernels, the enzyme activity remained unaltered especially in cotyledons (Fig. 2b). Furthermore, means of GS activity were not significantly different between warm-incubated and cold-stratified kernels, as they were evaluated by nested design analysis for the whole period of incubation (data not shown). Although tissue GS activity and its protein level might not be directly correlated (Ishiyama et al. 2004), it appears that both cold-stratified and warm-incubated

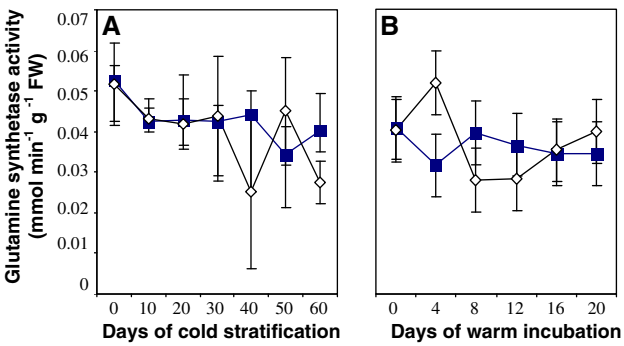

Fig. 2 Time course of changes in glutamine synthetase activity in cotyledons (*filled square*) and axes (*diamond*) from cold-stratified (**a**) and warm-incubated (**b**) walnut kernels. Values are mean ± SE obtained from three separate extractions. Statistical significance for all data was calculated at $P < 0.05$

walnut kernels have a continuous demand for GS activity. This idea may be justified by fairly arginase activity in cold-stratified kernels (Fig. 1a), and the accumulation of ammonia in warm-incubated kernels (to be discussed next), both of which are accompanied with significant mobilization of storage proteins following kernel imbibition (Einali and Sadeghipour 2007). The GS activity of walnut kernels is likely to be a cytosolic one, since in seeds and other non-photosynthesizing tissues; it is the predominant form (Gallardo et al. 2003). The enzyme activity is necessary during seed germination (Limami et al. 2002) and tissue death (Brugiere et al. 2000; Kichey et al. 2005; Pageau et al. 2006), corresponding to the situations of cold-stratified and warm-incubated walnut kernels, respectively. The NADH-glutamate 2-oxoglutarate amino transferase (NADH–GOGAT) activity was not measured in this study, since this activity is either in concert with GS (Glevarec et al. 2004), or insignificant (Cantón et al. 2005) in developing and germinating seeds.

Warm incubation leads to build-up of oxidative stress, declined sugar content (Nezamdoost et al. 2009), and amino acid accumulation (Einali and Sadeghipour 2007) in walnut kernels. Because GDH activity increases in sugar starved tissues (Miyashita and Good 2008), and is sensitive to sucrose, amino acids (Masclaux-Daubresse et al. 2005) and reactive oxygen species (Skopelitis et al. 2006), it could be used as a suitable marker of altered carbon/nitrogen nutritional statues of walnut kernels incubated at different temperatures. The activity of GDH showed fluctuations under both conditions (Fig. 3a, b). However, mean of the enzyme activity for the whole period of incubation was significantly greater in warm-incubated kernels as compared to cold-stratified ones (Fig. 3c, d). As many studies favor a de-aminating role for GDH in vivo (Stewart et al. 1995; Aubert et al. 2001; Purnell and Botella 2006; Skopelitis et al. 2007), the oxidative de-amination of glutamate is expected to be greater in warm-incubated walnut kernels. This conclusion is further supported by increased oxidative stress and declined levels of carbohydrates in warm-incubated walnut kernels (Nezamdoost et al. 2009), which favors amino acid respiration (Miyashita and Good 2008). Reduced levels of $NADP^+$–isocitrate dehydrogenase activity which is implied in amino acid biosynthesis (Hodges et al. 2003), in warm-incubated walnut kernels (Nezamdoost et al. 2009), along with ammonia accumulation in the latter (Fig. 4b), show that the balance of amino acid metabolism is shifted toward respiration rather than transport and/or biosynthesis.

Dramatic decline in arginase activity in kernels from mid-period of their incubation at warm conditions (Fig. 1b) is expected to provide more arginine for other metabolic pathways which utilize this amino acid. Arginine is known as a major substrate for polyamine and nitric oxide biosynthesis in cold-stratifying seeds and other plant tissues (Guoyao and Morris 1998; Santanen and Simola 1999; Crozier et al. 2000; Urano et al. 2005). Free polyamine profiles of 30–40-day cold-stratified walnut kernels showed significant differences to that from 20-day warm-incubated

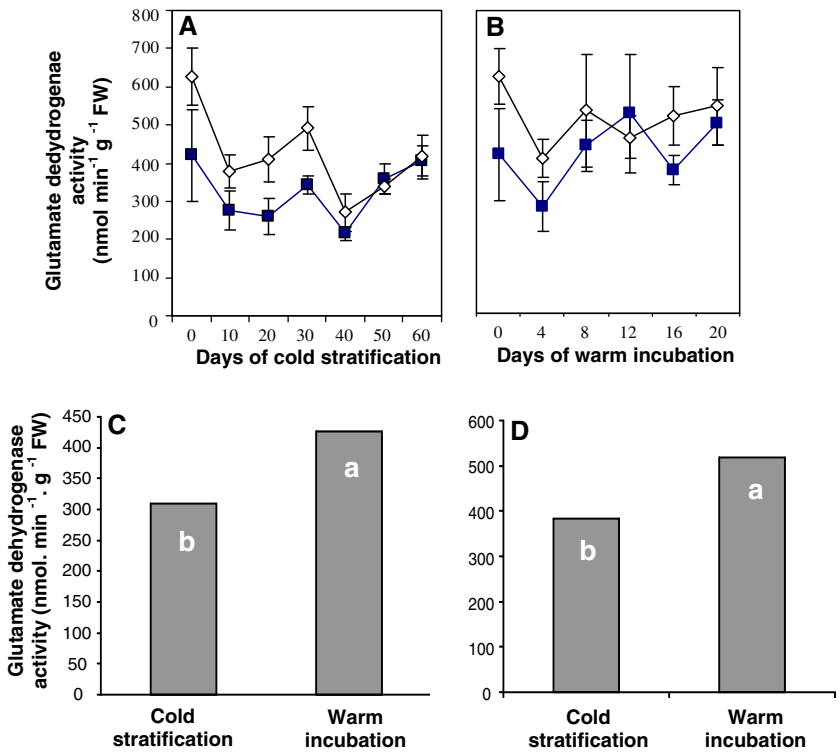

Fig. 3 Time course of changes in glutamate dehydrogenase activity in cotyledons (*filled square*) and axes (*diamond*) from cold-stratified (**a**) and warm-incubated (**b**) walnut kernels. Values are mean ± SE obtained from three separate extractions. Statistical significance for all data was calculated at $P < 0.05$, **c** and **d** represent, respectively, the mean of glutamate dehydrogenase activity in cotyledons and embryo axes for the whole period of incubation obtained after doing the nested design analysis. The *small letters a* and *b* on histograms represent statistically significant differences at $P < 0.05$

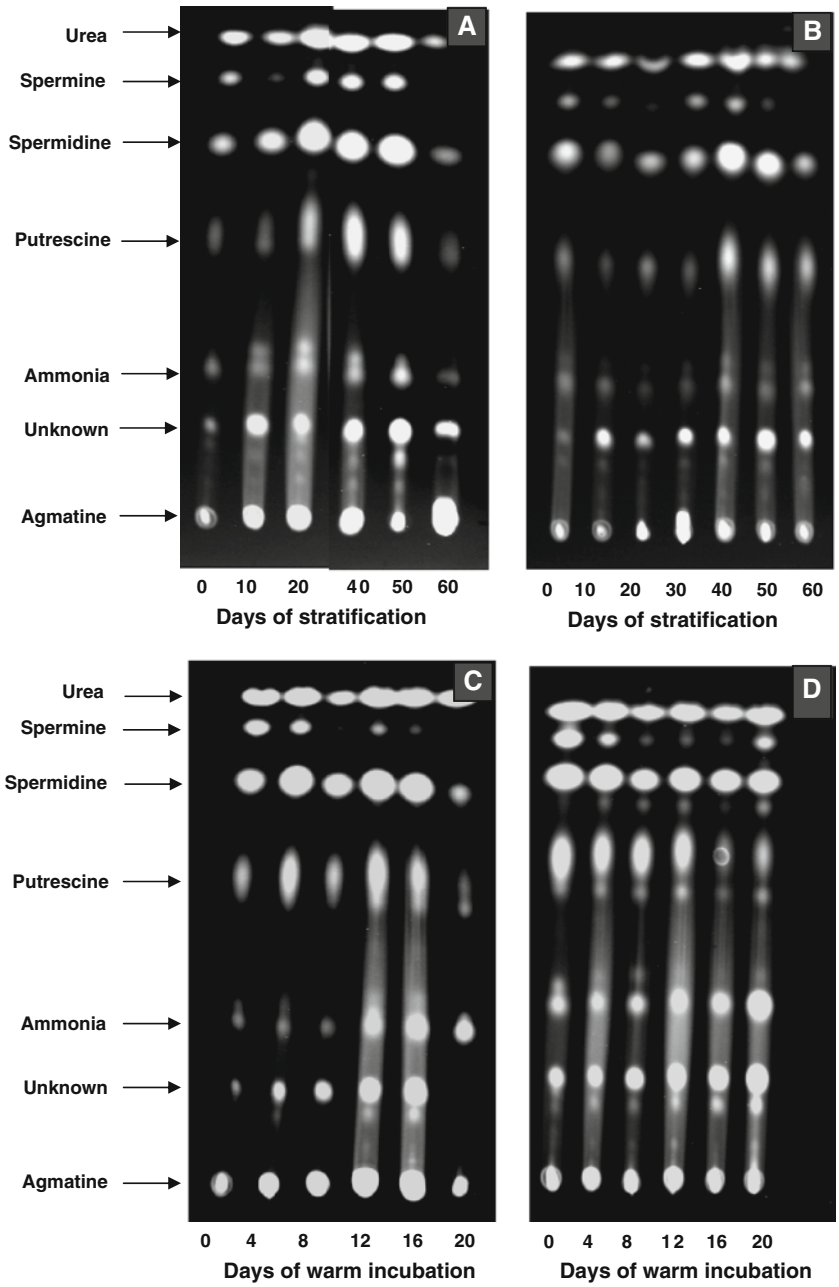

Fig. 4 Thin-layer chromatographic (TLC) separation of polyamines from the cotyledons (**a**, **c**) and axes (**b**, **d**) of cold-stratified (**a**, **b**) and warm-incubated (**c**, **d**) walnut kernels. Following extraction from kernels of different ages, polyamines were dansylated and resolved on Silica gel-coated TLC plates using a mobile phase consisting of chloroform: diethyl ether: triethyl amine (4:1:4; v/v/v). The dansylated polyamines and some other dansylated amino compounds were made visible after UV irradiation. Aliquots from the benzene phase corresponding to the polyamine contents present in 10-mg tissue fresh weight were loaded on each lane. Polyamine composition of 30-day-stratified cotyledons is not shown in **a** due to spoiled samples in all three replicates

kernels (Fig. 4). Major walnut kernel polyamines consist of agmatine, putrescine, spermidine, spermine, and an unknown one with an R_f of 0.16. Notably, the relative amount of spermidine increased in cold-stratified kernels, whereas it remained more or less constant in warm-incubated kernels. Warm-incubated kernels; however, showed the ever-increasing amount of an unknown polyamine with R_f of 0.16 and ammonium accumulation. Alteration in amino acid metabolism might have a direct bearing on tissue polyamine composition and contents (Gallardo et al. 2003). Polyamine profiles of apple (*Mallus domestica*) seed (Sinska and Lewandowska 1991) and linden (*Tilia cordata*) resting buds (Santanen and Simola 2007) also changed during dormancy release. Mention should be made that lower arginase activity under warm conditions should not necessarily correspond to greater polyamine content in walnut kernels. The extent of the activities of polyamine oxidases (Kusano et al. 2008) and other processes which affect polyamines such as conjugation processes (Santanen and Simola 2007; Dufeu et al. 2003) need to be known in walnut kernels. Polyamine profiles of walnut kernels also showed ammonia accumulation under warm conditions (Fig. 4). It might have been resulted from increased oxidative de-amination catalyzed by GDH as mentioned earlier and perhaps indicates the metabolic failure of kernels at warm conditions for assimilating ammonia into organic

metabolites that finally would compromise the kernel viability and germination.

Trends of changes in the activities of arginase, GS and GDH of the embryonic axes were nearly similar to cotyledons under the correspondingly either cold or warm conditions (Figs. 1, 2, 3a, b); however, the absolute activities of these enzymes remained significantly greater in axes than in cotyledons during 4–8 days of warm incubation which was also corresponded to greater ammonia accumulation (Fig. 4d). GDH was also the only enzyme that had significantly greater activity in axes than in cotyledons for the first 30 days of cold stratification (Fig. 3a). The significance of these observations requires further investigations. Having nearly similar trends of changes in enzyme activities in both cotyledons and axes (present study; Einali and Sadeghipour 2007; Nezamdoost et al. 2009), and considering that the mobilization of both storage proteins and lipids are more pronounced in walnut cotyledons rather than axes (Einali and Sadeghipour 2007; Nezamdoost et al. 2009), justify why data interpretation in our study was mainly based on the results obtained from the former tissue.

Recent proteomic studies in *Arabidopsis* point to the metabolic failure of the dormant seeds which is subsequently attenuated during dormancy removal and germination (Chibani et al. 2006). Studies like this, however, do not deal with enzymic activities in tissues. In conclusion, the present work is the first report on the activities of three major amino acid metabolizing enzymes during seed dormancy removal by cold stratification. The results established that during moist chilling arginine metabolism by arginase can proceeds to fulfill demands of kernels for nitrogen, whereas impairment of this conversion due to arginase downregulation under warm conditions, along with the enhancement of amino acid catabolism may account for compromising kernel viability and germination. Whether these differences in the activities of arginase and GDH are the direct effects of cold or associated with dormancy removal from walnut kernels require further study.

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