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ORIGINAL ARTICLE

Storage reserve mobilization, gluconeogenesis, and oxidative pattern in dormant pistachio (*Pistacia vera* L.) seeds during cold stratification

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Abstract
Key message **Cold treatment promotes germination of pistachio kernels and prevents deteriorative processes through operating cellular repair mechanisms and activating gluconeogenic processes.**
Abstract Effects of cold stratification on enhancing germination capacity of pistachio (*Pistacia vera* L.) kernels was investigated through the study of storage reserve mobilization, gluconeogenesis, and enzymatic antioxidant defense system in both cotyledon and embryonic axis organs. Every week, imbibed pistachio seeds were cold stratified at 5 °C for increasing periods up to 42 days. Stratifying seeds for 42 days significantly increased their germination potential. Lipid hydrolysis in both organs during cold stratification was accompanied by increased isocitrate lyase activity and the accumulation of starch and soluble sugars. Total protein mobilization occurred in cotyledons rather than embryonic axes but was accompanied by high proteolytic activity and accumulation of amino acids in both organs. However, the pattern of soluble proteins changes in both tissues was nearly identical. The activity of a typical protease at a particular pH was not detected in stratifying pistachio kernels, but rather different proteases might be activated depend on cold treatment period. Cold stratification also led to increased H_2O_2-scavenging enzymes activity and consequently decreased hydrogen peroxide and lipid peroxidation in pistachio kernels. These data suggest that beneficial effects of cold treatment in germination induction of pistachio kernels arise from prevention of processes causing seed deterioration by turning on cellular repair mechanisms and activating gluconeogenic processes, which subsequently enhance seed capability for germination.

Keywords Cold stratification · Gluconeogenesis · Isocitrate lyase · *Pistacia vera* · Protease · Reserve mobilization

Introduction

Dormancy refers to a developmental block period in many mature seeds in which they are not able to germinate despite favorable conditions. Alleviation of dormancy in many seeds of temperate tree species can be achieved by incubating them in moist and cold (5 °C) conditions, a process known as cold stratification. Many biochemical changes involved in removal of dormancy, including changes in metabolism or perception of phytohormones (Corbineau et al. 2002; Jacobsen et al. 2002; Schmitz et al. 2002) and changes in enzymatic activities involved in seed reserve mobilization (Li and Ross 1990a, b; Bogatek et al. 2002; Andriotis et al. 2004; Nezamdoost et al. 2009), have been reported during cold stratification. Changes in seed storage reserves may also occur by cold stratification, and ultrastructural studies have displayed a gradual decrease in seed reserves during this period (Dawidowicz-Grzegorzewska 1989; Wang and Berjak 2000; Andriotis et al. 2004).

Storage proteins and lipid mobilization during cold stratification are accompanied by the increase in enzymatic activities related to proteolysis and gluconeogenesis, which

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facilitate accumulation of free amino acids and carbohydrates (Ranjan and Lewak 1995; Forward et al. 2001; Todd et al. 2001; Einali and Sadeghipour 2007; Nezamdoost et al. 2009). Free amino acid accumulation induced by cold stratification is an important process to germinate seeds due to de novo synthesis of germination-specific proteins (Rajjou et al. 2004). Likewise, carbohydrates accumulation due to lipid mobilization has an important role in seed dormancy breaking (Li and Ross 1990a, b; Zarska-Maciejewska 1992) and prepares the required carbon and energy of seeds for germination (Cooper and Beevers, 1969).

It has been shown that the breaking of seed dormancy by cold stratification may be associated with accumulation of reactive oxygen species (ROS) and oxidative stress (Debska et al. 2013; Shalimu et al. 2016). Enzymatic antioxidant defense system, including catalase (CAT), ascorbate peroxidase (APX), and other non-specific peroxidases, can scavenge excessive ROS and prevent lipid peroxidation, a process that known as primary reason of seed deterioration (McDonald 2004). Thus, cold treatment may play a pivotal role in the alleviation of seed dormancy and promoting germination by operating cellular repair mechanisms and preventing oxidative damage (Wang and Berjak 2000; Einali and Sadeghipour 2007; Nezamdoost et al. 2009).

Persian pistachio (*Pistacia vera* L.) is an important tree species, which received much attention because of commercially valuable edible nut. Storage lipids, proteins, and carbohydrates constitute 55, 22, and 14% of food reserves in the pistachio kernel, respectively (Clarke et al. 1976). This is a dioecious, deciduous, and drought tolerant species, which well adapted with hot and dry weather. It tolerates low winter temperature but is sensitive to spring frost and does not tolerate high humidity in the growing season (Khan et al. 1999). It was reported that all the *Pistacia* species might be used as rootstock in horticulture (Almehdi et al. 2002). Because of difficulties in propagation of pistachio rootstocks by rooted stem cuttings (Tilkat et al. 2009) or by tissue culture techniques (Almehdi et al. 2002), all pistachio rootstocks are presently propagated from seeds (Isfendiyaroglu and Ozeker 2001; Almehdi et al. 2002). However, germination of pistachio seeds is generally low under ordinary conditions. If the seeds germinate, they will produce very weak seedlings that will not be desirable for rootstocks breeding (Khan et al. 1999). Moist chilling treatment has been proposed as a practical technique to enhance seed germination and to produce uniform and functional rootstocks for pistachio cultivation (Khan et al. 1999; Isfendiyaroglu and Ozeker 2001). Most of our knowledge about biochemical aspects of seed stratification (e.g., reserve mobilization) comes from studies of gymnosperms and few species of arboraceous angiosperms, such as almond, apple, walnut, and hazelnut (Dawidowicz-Grzegorzewska 1989; Forward et al. 2001; Andriotis et al. 2004; Einali and Sadeghipour 2007). So far, there is no report on the biochemical aspects of moist chilling in Persian pistachio kernels. In the present work, pistachio was taken as a model system to assess the biochemical changes during moist chilling and its beneficial effects in the breaking of dormancy.

Materials and methods

Plant material and germination test

Freshly harvested fruits of Persian pistachio (*Pistacia vera* L.) were obtained from the Zahedan office of Agricultural and Natural Resources in September 2014 and 2015. Dormant seeds were isolated from the pericarp of fruit, dried at room temperature, and dry stored at 5 °C. Imbibed nuts in tap water for 24 h were surface sterilized with 0.5% (w/v) sodium hypochlorite solution for 10 min followed by four times washing with distilled water. Seeds stratification was performed according to Einali and Sadeghipour (2007). Every week, lots of 100 nuts (in quadruplicate) were enveloped in two layers of moistened cheesecloth, put in polyethylene bags and incubated at 5 °C up to 6 weeks (42 days). The stratified seeds were then transferred into well-irrigated cocopeat pots, incubated in culture room at 25 °C in darkness, and their germination was recorded for 25 days. Seeds were taken as germinated when the average radicle length was 10 mm and were obvious as protrusions on the cocopeat surface. Kernels were isolated from cold-stratified nuts that have no visible sign of germination. The cotyledons and embryonic axes were cutoff from kernels with a razor blade and used for succeeding biochemical analyses.

Total lipid determination

Extraction and estimation of total lipid were carried out gravimetrically according to the method of Hara and Radin (1978). Total lipids were extracted from cotyledonary or axial tissue (1 g) by grinding the tissue in a mortar with a pestle in 24 ml of n-hexan: isopropanol solvent (3:2; v/v). The homogenate was filtered through a Whatman No. 1 filter paper. The filtrate was washed with 24 ml of 6% sodium sulfate solution in a separatory funnel to pure filtrate from non-lipid substances. The organic phase was transferred into a pre-weighted flask, and total lipid contents were determined by re-weighing the flask after solvent evaporation.

Extraction and measurement of soluble sugars and starch

The defatted powder obtained from tissue total lipid extraction was used for extraction and determination of soluble sugars, including reducing and non-reducing sugars, and starch content. Soluble sugars were extracted from the defatted powder (40 mg) with 5 ml of 80% (v/v) ethanol at 70 °C for 10 min. After centrifugation at 2000*g* for 10 min, the supernatant was transferred into a flask and the pellet was re-extracted four times with the same volume of 80% ethanol as above. The ethanolic extracts were combined and concentrated to one-fifth of their initial volume by evaporation, and used for determination of soluble sugar content. Reducing sugars were determined by the dinitrosalicylic acid method of Miller (1959), and non-reducing sugars were quantified according to the method of Handel (1968). The pellet after final centrifugation was used for extraction and determination of starch content according to the method of McCready et al. (1950). Briefly, 0.2 ml of distilled water was added to the pellet in an ice bath and then mixed with 0.26 ml of 52% perchloric acid and incubated in ice for 15 min. The suspension was mixed with 0.4 ml of distilled water and centrifuged at 2000*g* for 10 min. The supernatant was saved in ice, and the pellet was re-extracted with 0.1 ml of distilled water and 0.13 ml of 52% perchloric acid. The extracts were pooled and reached final volume of 2 ml with distilled water. Total soluble sugar concentration in the mixture was quantified by the anthrone method of McCready et al. (1950) using glucose as the standard. Starch content was determined by multiplying the glucose content by the glucose equivalent of 0.9.

Total protein and free amino acid determination

Total protein was extracted from the defatted powder by the method of Stone and Gifford (1997) with some modifications. Briefly, 20 mg of defatted powder was extracted with 0.5 ml of sample buffer containing 60 mM Tris–HCl buffer (pH 6.8), 10% (v/v) glycerol, and 2% (w/v) SDS at 90 °C for 1 h. After centrifugation at 10,000*g* for 15 min, the supernatant was used for determination of total protein content. Because of interference by SDS in the Bradford (1976) protein assay, the Markwell et al. (1981) method was used to determine total protein content.

Free amino acid extraction was performed as described for soluble sugars except that 1 g of cotyledonary or axial tissue used rather than defatted powder. The concentrated ethanolic extract was mixed with chloroform (1:5; v/v) to separate lipids and pigments from the aqueous phase. Total free amino acid content in the aqueous phase was determined by the ninhydrin method of Yemm and Cocking (1955) using glycin as the standard.

Enzyme extraction and assays

Enzymes were extracted from 1 g of cotyledonary or axial tissue in a cold mortar with a pestle in 2 ml of extraction buffer containing 100 mM cold potassium phosphate buffer (pH 7.0), 0.4 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), 70 mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100, and 1% (w/v) Polyvinylpolypyrrolidone (PVPP). The extraction buffer also contained 5 mM ascorbic acid when extracting ascorbate peroxidase (APX). For extraction of kernel protease(s), PMSF and EDTA were excluded from the extraction buffer. The homogenate was filtered through four layers of cheesecloth and incubated at 4 °C for 1 h. The filtrate was then centrifuged (12,000*g* for 10 min at 4 °C), and the supernatant (soluble protein fraction) was used to assay enzymatic activities after total soluble protein (TSP) estimation by the method of Bradford (1976).

Catalase (CAT) activity was assayed according to the method of Luck (1965). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2, and enzyme extract. The H_2O_2 breakdown was monitored at 240 nm using the extinction coefficient of 0.0394 cm^2 μmol^{-1}.

APX activity was assayed as described by Chen and Asada (1992). The reaction mixture (1 ml) composed of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 1 mM H_2O_2, and enzyme extract. The rate of ascorbate oxidation was measured at 290 nm using the extinction coefficient of 2.8 cm^2 μmol^{-1}.

Pyrogallol peroxidase (PPX) activity was assayed according to the method of Nakano and Asada (1981). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2, 40 mM pyrogallol, and enzyme extract. The increase in the absorbance due to purpurogallin production was measured at 430 nm using the extinction coefficient of 2.47 cm^2 μmol^{-1}.

Isocitrate lyase (EC 4.1.3.1) activity was assayed as described by Cooper and Beevers (1969) with slight modifications. The reaction mixture (1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 5 mM $MgSO_4$, 20 mM phenyl hydrazinium hydrochloride, 50 mM 2-mercaptoethanol, 13 mM isocitrate, and enzyme extract. The increase in the absorbance due to production of glyoxylate–phenylhydrazone complex was measured at 324 nm using the extinction coefficient of 17 cm^2 μmol^{-1}.

The proteolytic activity in the cold-stratified kernels was assayed by estimating the rate of release of soluble amino nitrogen according to the method of Peoples and Dalling

(1978) with some modifications. The reaction mixture containing 0.1 ml of enzyme extract, 0.5 ml of substrate [1% Bovine Serum Albumin (BSA) in 50 mM phosphate buffer (pH 4.5, 7.5, 9)], and 0.1% (v/v) 2-mercaptoethanol was incubated for 2 h at 37 °C. The reaction was stopped by adding 0.7 ml of 15% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 5000*g* for 10 min, and the TCA-soluble nitrogen was quantified by the ninhydrin method of Yemm and Cocking (1955) using glycine as the standard.

Determination of hydrogen peroxide (H_2O_2) and lipid peroxidation

Tissue H_2O_2 concentration was determined according to the method of Alexieva et al. (2001). Cotyledonary or axial tissue (1 g) was homogenized with 3 ml of 0.1% (w/v) cold TCA in an ice bath and filtered through four layers of cheesecloth. The filtrate was then centrifuged at 12,000*g* for 15 min at 4 °C, and the resulting supernatant was used for estimation of H_2O_2 and lipid peroxidation content as well. The reaction mixture contained 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4), 0.5 ml of the supernatant, and 2 ml of 1 M potassium iodide. The mixture was incubated for 1 h at room temperature in dark. The absorbance was recorded at 390 nm. A calibration standard of hydrogen peroxide was used for calculating the concentration of H_2O_2.

Lipid peroxidation content in cold-stratified kernels was determined by measuring the concentration of malonyldialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction, as described by Heath and Packer (1968). The reaction mixture containing 1 ml of supernatant and 2 ml of reagent (0.5% (w/v) TBA in 20% TCA) was incubated for 30 min at 95 °C. The reaction was terminated by incubating the mixture in ice bath. The mixture was centrifuged at 10,000*g* for 10 min. The absorbance of the samples was recorded at 532 nm and subtracted from the non-specific absorbance at 600 nm. The concentration of MDA–TBA complex was calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical analysis

All results except the germination test were obtained from calculation of the mean ± standard deviations (SD) of at least three independent experiments. Germination test data were presented as the mean ± SD of four separate experiments. Statistically, significant differences were determined at $P < 0.05$ using analysis of variance (ANOVA) with a Duncan multiple comparisons post hoc test. The relationship between measured variables in cotyledons and embryonic axes was determined by calculating correlation coefficients between these variables separately for each organ.

Results

Effect of moist chilling on germination of pistachio kernels

Control seeds (non-stratified seeds), which were imbibed for 25 days at 25 °C, displayed germination percentage and rate up to 45% and 0.13 day^{-1}, respectively. Moist chilling heightened both percentage and rate of pistachio seed germination compared to non-stratified seeds (Fig. 1a). The highest germination percentage (94%) and highest rate of germination (0.55 day^{-1}) were found in cold-stratified seeds for 42 days (Fig. 1a). Control seeds achieved the maximum germination percentage after 15 days culture at 25 °C, while cold-stratified seeds for 42 days attained a greatly higher maximum percentage after a shorter period (4 days) (Fig. 1b).

Changes in total lipid content of cotyledonary and axial tissues of cold-stratified pistachio kernels

A significant decrease in total lipid content was found in both cotyledons and embryonic axis tissues of pistachio kernels following cold stratification (Fig. 2). No statistically significant change was observed in lipid mobilization of cotyledonary tissues between different cold stratification periods, whereas the lipid mobilization of axial tissues was significant after 35 days of moist chilling with reference to other periods (Fig. 2).

Changes in soluble sugars and starch contents of cotyledonary and axial tissues of cold-stratified pistachio kernels

Reducing sugar content of both cotyledonary and axial tissues was statistically unchanged during the first 28 days of cold stratification but increased significantly after 35 days, so that the reducing sugar concentration was much higher in axis compared with cotyledon tissues (Fig. 3a). The amount of non-reducing sugars of both cotyledonary and axial tissues gradually decreased concomitantly with increase of cold stratification period. A decrease of 43 and 16% was found for cotyledons and axes of cold-stratified pistachio kernels for 42 days relative to control, respectively (Fig. 3b). Starch concentration of cotyledons increased concomitantly with stratification period and the highest amount was found in cold-stratified kernels for 35 days. The starch content of axes increased significantly after 14 days of moist chilling compared with control, but

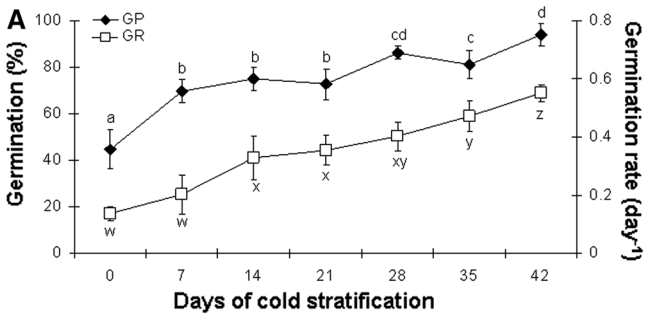

Fig. 1 **a** Effect of moist chilling on germination percentage (GP) and germination rate (GR) of pistachio seeds. **b** Time course of germination at 25 °C of non-stratified (control) and cold-stratified pistachio seeds. Every 7 days for a period of 42 days, seeds were imbibed for 24 h, surface sterilized, incubated at 5 °C, and then placed in a well-irrigated cocopeat medium at 25 °C. Germination was recorded for a period of 25 days. Each value represents the mean ± SD of four independent experiments, each consisting of 100 seeds. Different letters (*a–d* for GP and *w–z* for GR) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

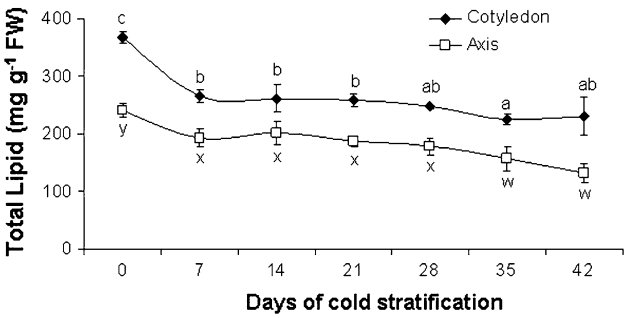

Fig. 2 Changes in total lipid content of cotyledons and embryonic axes of cold-stratified pistachio kernels. The values are mean ± SD of three independent experiments. Different letters (*a–c* for cotyledon and *w–y* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

it was with fluctuations and the highest amount was observed in cold-stratified kernels for 42 days (Fig. 3c).

Changes in isocitrate lyase activity of cotyledonary and axial tissues of cold-stratified pistachio kernels

Isocitrate lyase activity of both tissues was positively affected by cold stratification. The enzyme activity increased significantly in cotyledons after 21 days of moist chilling and then increased slightly but not significantly during the remaining of cold stratification periods (Fig. 4). The isocitrate lyase activity in the embryonic axes of cold-stratified pistachio kernels increased gradually, but a significant increase occurred after 35 days of moist chilling.

The activity of H_2O_2-decomposing enzymes in cotyledonary and axial tissues of cold-stratified pistachio kernels

CAT activity in both tissues showed a rise and fall during different cold stratification periods. The activity increased significantly in cotyledons after 14–21 days of cold treatment but reverted thereafter (Fig. 5a). CAT activity in the axes highly increased following cold stratification. However, the activity came back to control or higher level in the remaining of cold periods. APX activity of cotyledonary tissues remained unchanged until 14 days of stratification, but a significant increase in the enzyme activity observed in cotyledons of stratified kernels for 21 and 28 days, before returning to initial level (Fig. 5b). APX activity in the axes showed a similar pattern to CAT activity with a pronounced increase in the kernels that had been cold stratified for 7 days. PPX activity of cotyledons decreased concomitantly with cold periods and reached minimum level in the stratified kernels for 42 days (Fig. 5c). Such pattern of PPX activity was also found for the embryonic axes with the difference that the enzymatic activity increased unexpectedly after 42 days of cold stratification.

Hydrogen peroxide and MDA contents of cotyledonary and axial tissues of cold-stratified pistachio kernels

Hydrogen peroxide content of cotyledons decreased significantly following exposure the kernels to cold treatment and a nearly continuous decrease was found throughout the cold period (Fig. 6a). In the embryonic axes, hydrogen peroxide content increased after 7 days but decreased in cold-stratified kernels for 14 days and then remained constant until the end of cold period.

MDA concentration of cotyledons decreased significantly subsequent cold stratification and then continued without change (Fig. 6b). In contrast, the axial tissues showed a pattern of MDA content with fluctuations, so that MDA concentration increased after 14 days of cold treatment, but highly decreased after 21 days, then increased

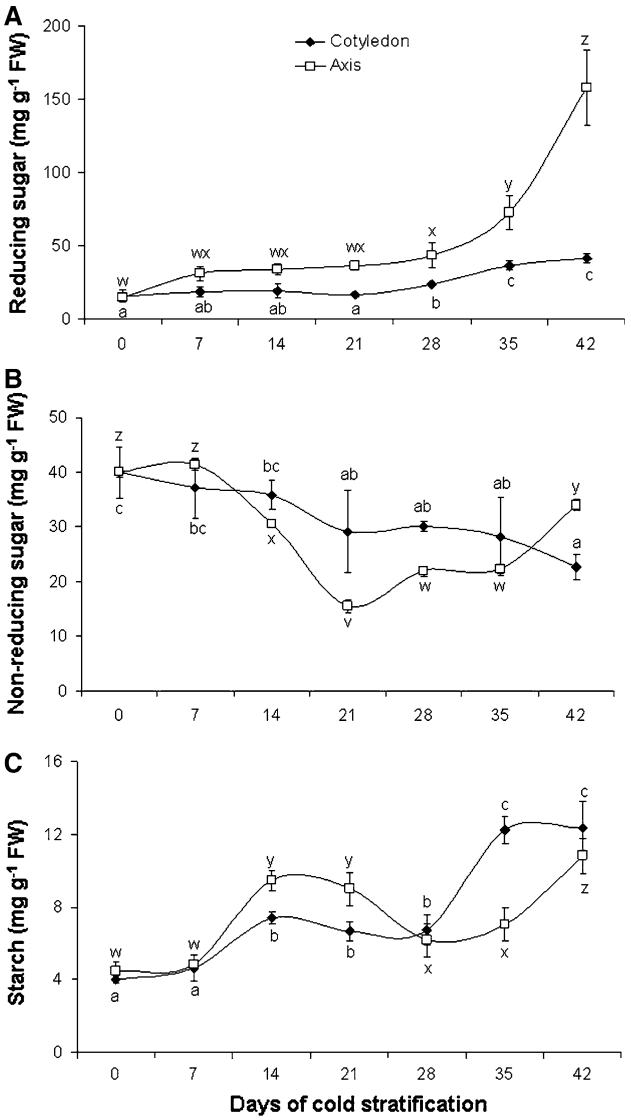

Fig. 3 Changes in reducing sugars (**a**), non-reducing sugars (**b**), and starch (**c**) of cotyledonary and axial tissues of cold-stratified pistachio kernels. Results are mean ± SD of three separate experiments. Different letters (*a–c* for cotyledon and *w–z* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

significantly after 28 days and came back again to the control level.

Changes in soluble protein, total protein, and amino acid contents of cotyledonary and axial tissues of cold-stratified pistachio kernels

Total soluble protein (i.e., proteins soluble in extraction buffer without SDS and TSP) content in both cotyledonary and axial tissues was unchanged up to 14 days of moist chilling but significantly decreased after 21 and 28 days, before returning to initial level on the 35th day (Fig. 7a).

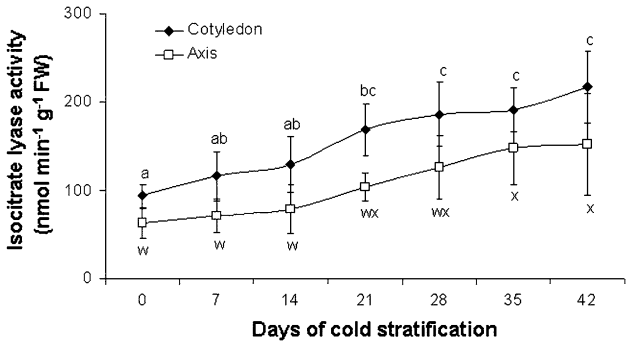

Fig. 4 Changes in isocitrate lyase activity of cotyledonary and axial tissues of cold-stratified pistachio kernel. The values are mean ± SD of three independent experiments. Different letters (*a–c* for cotyledon and *w–x* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

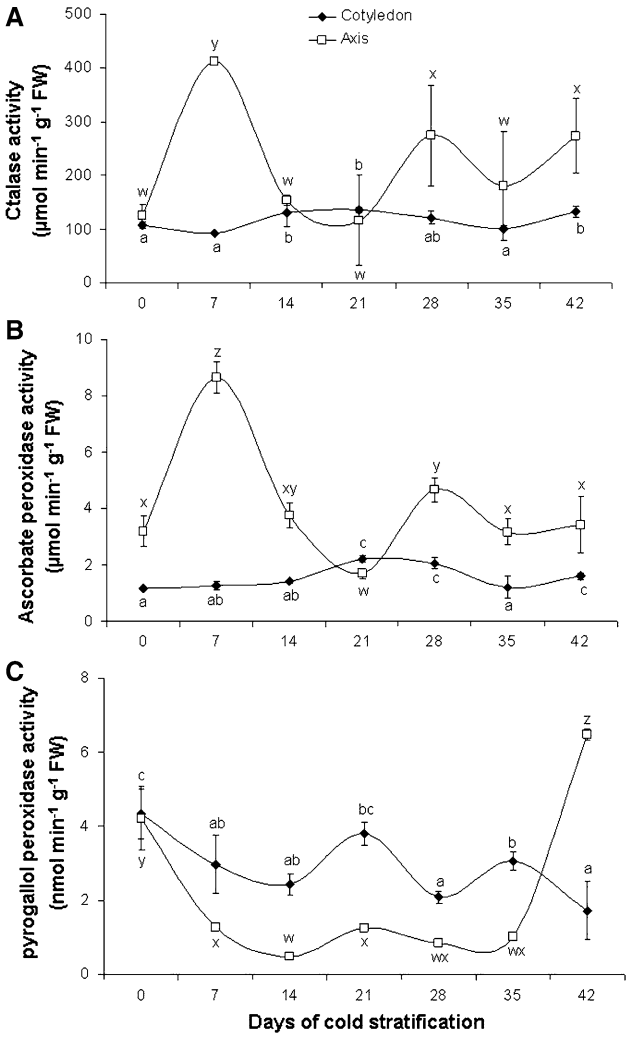

Fig. 5 Changes in activity of catalase (**a**), ascorbate peroxidase (**b**), and Pyrogallol peroxidase (**c**) in cotyledonary and axial tissues of cold-stratified pistachio kernel. Each value represents the mean ± SD of three independent experiments. Different letters (*a–c* for cotyledon and *w–z* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

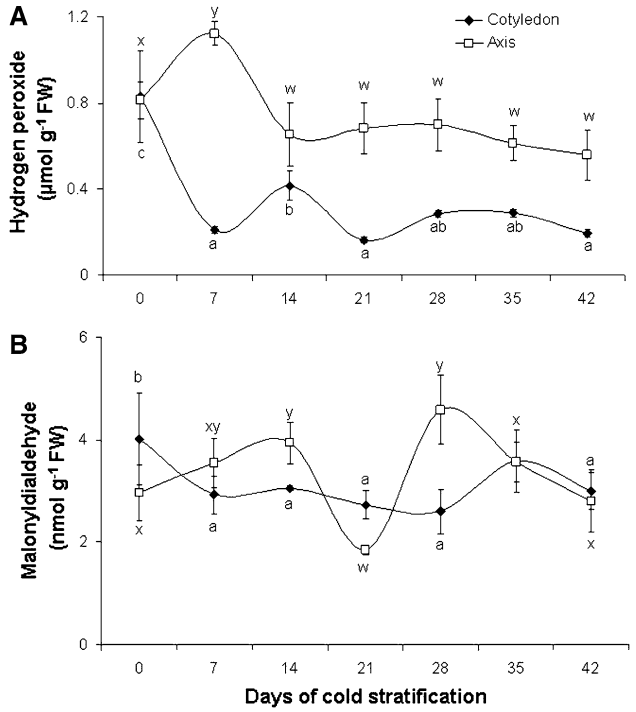

Fig. 6 Changes in hydrogen peroxide (**a**) and MDA content of cotyledonary and axial tissues of cold-stratified pistachio kernels. The values are mean ± SD of three separate experiments. Different letters (*a–c* for cotyledon and *w–y* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test

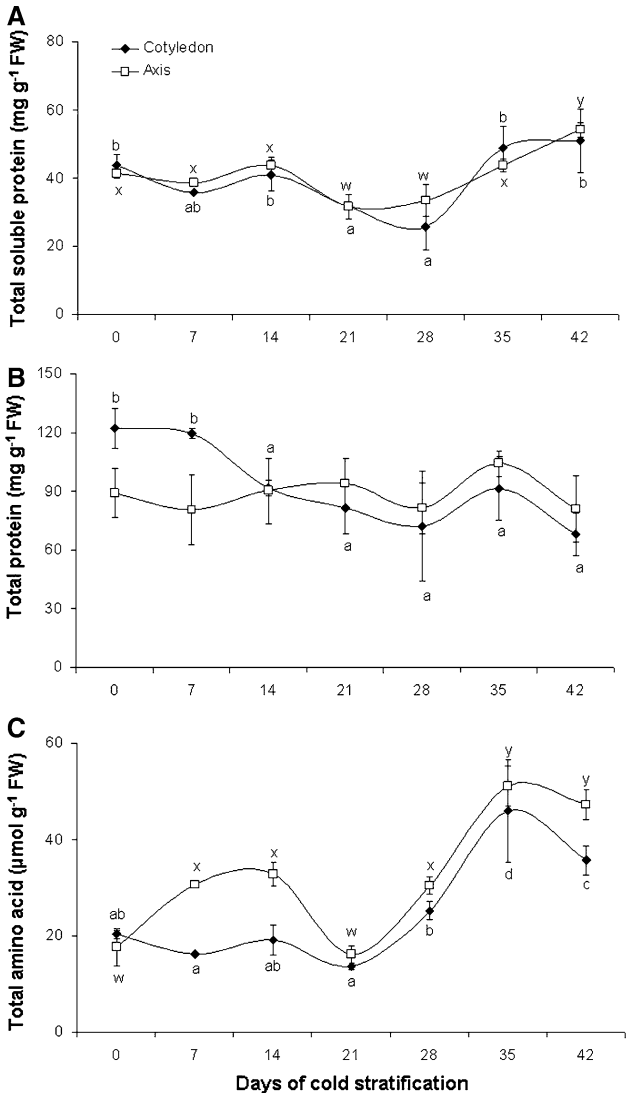

Fig. 7 Changes in total soluble protein (**a**), total protein (**b**), and amino acid (**c**) contents in cotyledonary and axial tissues during cold stratification of pistachio kernels. Results are mean ± SD of three separate experiments. Different letters (*a–d* for cotyledon and *w–y* for axis) indicate significant differences between the various treatments at $P < 0.05$ according to the Duncan test. No significant differences were observed in total protein concentration between the various treatments of axial tissues

Total protein is the proteins extracted with sample buffer in the presence of SDS. Although SDS does not extract all proteins (Einali and Sadeghipour 2007), the amount of pistachio proteins extracted with sample buffer was more than twofold higher from that extracted with extraction buffer (Fig. 7a, b). Total protein content of cotyledonary tissues decreased by about 25% after 14 days of cold stratification and remained statistically unchanged during the remaining of moist chilling periods (Fig. 7b). In contrast, no significant change was found in total protein content of axial tissue of pistachio kernels during cold stratification. Total free amino acid content of cotyledonary tissue did not change significantly up to 28 days after moist chilling, but drastically increased in cold-stratified tissues for higher periods (Fig. 7c). Total amino acid concentration in axes increased significantly following cold stratification and reached about 190 and 170% of control in axes that had been stratified for 35 and 42 days, respectively.

Proteolytic activity in cotyledonary and axial tissues of cold-stratified pistachio kernels

Proteolytic activity at different pHs showed a similar pattern in both tissues of cold-stratified pistachio kernels (Fig. 8). Proteolytic activity did not change in both organs at acid pH until 21 days of cold stratification, but there was a significant increase in proteolytic activity of stratified kernels for 28–42 days. Cold stratification caused a significant increase in the proteolytic activity of both tissues at neutral and alkaline pHs, particularly in kernels that had been stratified for 28–42 days. However, the activity at acid pH surpassed other pHs in both organs of stratified kernels for 28–42 days (Fig. 8).

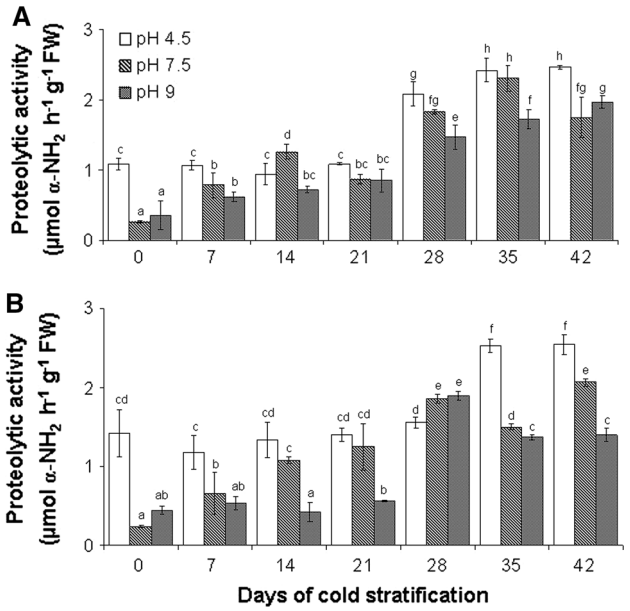

Fig. 8 Proteolytic activity in cotyledonary (**a**) and axial (**b**) tissues of cold-stratified pistachio kernels at different pHs. Each column represents the mean value of three separate samples ± SD. Different letters indicate significant differences between the various treatments and pHs at $P < 0.05$ according to the Duncan test

Correlation patterns of biochemical variables

The pattern of correlation between measured variables, including gluconeogenic markers and proteolytic activities during cold stratification, was investigated. This pattern was separately calculated as a correlation matrix for measured variables (14 × 14) for cotyledons and embryonic axes (Table 1). Lipid hydrolysis was negatively correlated with isocitrate lyase activity and germination variables. A positive correlation was found between isocitrate lyase activity with germination variables and carbohydrate reserves, including starch and reducing sugars, while this correlation was negative with non-reducing sugars in cotyledons. There was no correlation between total protein with total amino acids and proteolytic activities in both organs, except proteolytic activity of cotyledons at alkaline pH. However, there was a strong positive correlation between the total amino acid content and proteolytic activities of cotyledons at different pHs (Table 1).

Discussion

Non-stratified pistachio seeds (control seeds) exhibited a germination percentage up to 45%, showing that pistachio kernels are not deeply dormant. However, cold treatment significantly enhanced germination percentage of seeds to a maximum value of 94% for seeds that had been received cold stimulus for 42 days. Such stimulating effects of cold stratification have also been observed for other arboraceous seeds, including walnut (Einali and Sadeghipour 2007), hazelnut (Li and Ross 1990a; Andriotis et al. 2004), and pear seeds (Lin et al. 1994). The rate of germination also increased concomitantly with cold duration, which is consistent with previous report on walnut seeds (Einali and Sadeghipour 2007). It indicates that in contrast to apple seeds (Lewak 2011), dormancy of pistachio kernels may not rely on solely physiological status of the embryo. It means that other factors, such as the tissues surrounding the embryo, may also have a role in the germination process, as previously reported for walnut (Einali and Sadeghipour 2007) and yellow-cedar (Ren and Kermode 1999) seeds.

The pattern of lipid mobilization in both organs of cold-stratified pistachio kernels was well associated with an increase of isocitrate lyase activity, which indicates that cold treatment stimulates mobilization of storage lipids and provides the required substrate to operate glyoxylate cycle and gluconeogenesis for carbohydrate production. Increased isocitrate lyase activity along with accumulation of starch and reducing sugars and decreased level of non-reducing sugars during cold stratification of pistachio kernels is indicative of the gluconeogenesis process, which is well correlated with the percentage and rate of germination (Table 1). Decreased content of non-reducing sugars in cold-stratified pistachio kernels may be indicative of formation of starch from those produced during gluconeogenesis to avoid the increase of soluble sugars to suppressive levels. Enhanced isocitrate lyase activity and the accumulation of carbohydrates during moist chilling have also been previously reported as a sign of preparedness for germination in most stratification-requiring seeds (Noland and Murphy 1984; Dawidowicz-Grzegorzewska 1989; Li and Ross 1990a, b; Nezamdoost et al. 2009), which further support our results. However, alleviation of dormancy in walnut kernels by cyanide showed that despite enhanced competence for gluconeogenesis, the isocitrate lyase activity remained unchanged following cyanide treatment, although walnut kernel exposure to cyanide led to increased activities of glyoxysomal succinate oxidase and phospho*enol*pyruvate carboxykinase (Gerivani et al. 2016). Both activities are known as markers of gluconeogenesis activation in walnut kernels during moist chilling (keshavarzian et al. 2013). Thus, increased capacity for gluconeogenesis is a general response generated by any dormancy breaking agent in oil seeds (Gerivani et al. 2016). However, the pattern of gluconeogenesis activation is different and may be depend on dormancy breaking agent.

Glyoxysomes gluconeogenic activity in synthesis of carbohydrates from storage lipids is associated with

Table 1 Correlation coefficients between 14 analyzed variables

	ICL	Starch	RS	NRS	GP	GR	H_2O_2	MDA	Total protein	Total AA	Proteolytic (pH4.5)	Proteolytic (pH7.5)	Proteolytic (pH9)
Total lipid													
Cotyledon	−0.81*	−0.78*	ns	0.77*	−0.93*	−0.84*	0.89*	ns	ns	ns	ns	−0.83*	−0.77*
Axis	−0.91*	ns	−0.87*	ns	−0.93*	−0.93*	ns	ns	ns	−0.79*	−0.79*	−0.89*	ns
ICL													
Cotyledon		0.84*	0.81*	−0.98*	0.89*	0.97*	−0.67*	ns	−0.90*	ns	0.85*	0.83*	0.95*
Axis		ns	0.79*	ns	0.83*	0.95*	ns	ns	ns	ns	0.88*	0.91*	0.83*
Starch													
Cotyledon			0.94*	−0.84*	0.75*	0.92*	ns	ns	ns	0.86*	0.83*	0.84*	0.90*
Axis			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
RS													
Cotyledon				−0.80*	ns	0.86*	ns	ns	ns	0.90*	0.92*	0.80*	0.93*
Axis				ns	ns	0.83*	ns	ns	ns	ns	0.84*	ns	ns
NRS													
Cotyledon					−0.86*	−0.95*	ns	ns	0.87*	ns	−0.80*	ns	−0.91*
Axis					ns	ns	ns	ns	ns	ns	ns	ns	ns
GP													
Cotyledon						0.91*	−0.80*	ns	−0.86*	ns	ns	0.83*	0.87*
Axis						0.91*	ns	ns	ns	ns	ns	0.95*	ns
GR													
Cotyledon							ns	ns	−0.89*	ns	0.83*	0.87*	0.95*
Axis							−0.76*	ns	ns	ns	0.81*	0.95*	ns
H_2O_2													
Cotyledon								0.80*	ns	ns	ns	ns	ns
Axis								ns	ns	ns	ns	ns	ns
MDA													
Cotyledon									ns	ns	ns	ns	ns
Axis									ns	ns	ns	ns	ns
Total protein													
Cotyledon										ns	ns	ns	−0.78*
Axis										ns	ns	ns	ns
Total AA													
Cotyledon											0.89*	0.82*	0.82*
Axis											0.83*	ns	ns
Proteolytic (pH4.5)													

Table 1 continued

	ICL	Starch	RS	NRS	GP	GR	H_2O_2	MDA	Total protein	Total AA	Proteolytic (pH4.5)	Proteolytic (pH7.5)	Proteolytic (pH9)
Cotyledon												0.85*	0.96*
Axis												ns	ns
Proteolytic (pH7.5)													
Cotyledon													0.90*
Axis													0.82*

N 7 for cotyledons or embryonic axes

* Statistically significant correlation coefficients ($P < 0.05$)

ns non-significant, *ICL* Isocitrate lyase, *RS* Reducing sugars, *NRS* Non-Reducing sugars, *GP* Germination percentage, *GR* Germination rate, *Total AA* Total amino acid

generation of ROS (Beevers 1969; Lin et al. 1983; Bailly 2004; El-Maarouf-Bouteau and Bailly 2008). It has been demonstrated that ROS at proper concentrations have a positive role in seed dormancy removal (Bailly et al. 2008; Liu et al. 2010). Hydrogen peroxide is as an indicator of ROS, which is generated through different metabolic pathways, including gluconeogenesis (Huang et al. 1983; Li and Ross 1990a; Diaz-Vivancos et al. 2013), and can induce lipid peroxidation (Oracz et al. 2007; Ahmad et al. 2010; Shalimu et al. 2016). Thus, the activation of enzymatic defense systems against oxygen radicals is common during gluconeogenesis process (Huang et al. 1983; Li and Ross 1990a) to regulate the ROS level for the transition from dormancy phase to germination (De Gara et al. 1997; Tommasi et al. 2001). Increased CAT activity along with isocitrate lyase development and accumulation of starch during cold treatment implies that fully functional glyoxysomes develop simultaneously with the beginning of lipid hydrolysis (Li and Ross 1990a). Therefore, increased level of CAT and APX activity during cold stratification of pistachio kernels can be due to the need to detoxify the cells involved in lipid mobilization, as has been previously documented for many tree species seeds during stratification or germination (Li and Ross 1990a; Jordy et al. 2000; Bailly 2004; Einali and Sadeghipour 2007). However, the decrease of PPX activity during cold stratification of both tissues can be attributed to the specificity of hydrogen peroxide scavenging enzymes in detoxification of oxygen radicals generated during lipid hydrolysis.

Dynamic changes in H_2O_2 content during seed germination of many species has been documented before (Gidrol et al. 1994; Caliskan and Cuming 1998; Hite et al. 1999; Schopfer et al. 2001; Bailly et al. 2002; Morohashi 2002; Wojtyla et al. 2006). Increased H_2O_2 level in apple seeds and H_2O_2 with MDA contents in pomegranate seeds during cold stratification have also been reported (Debska et al. 2013; Shalimu et al. 2016). In contrast, H_2O_2 and MDA concentrations of cold-stratified pistachio kernels decreased significantly throughout the cold period (Fig. 6). It can be due to increased antioxidant enzymes activities, such as CAT and APX, during cold stratification of pistachio kernels, which scavenge H_2O_2 and prevent MDA production. In agreement with our results, it has been reported that increased CAT activity in germinating sunflower seeds before radicle protrusion was associated with a decrease in H_2O_2 and lipid peroxidation level (Bailly 2004). However, a transient increase in H_2O_2 content in embryonic axes of stratified pistachio kernels for 7 days may probably act as a signal molecule to induce antioxidant enzymes activities, as correlated with CAT and APX activity in this cold period (Fig. 5a, b). Hydrogen peroxide level of both cotyledons and embryonic axes during cold stratification of walnut kernels

remained unchanged, while MDA level significantly decreased (Nezamdoost et al. 2009).

Moist chilling of pistachio kernels caused protein mobilization in cotyledonary tissues without significant change in total protein content of embryonic axes. However, accumulation of amino acids occurred in both organs (Fig. 7a, b). Such a case was also reported for apple embryos, in which slight mobilization of total protein was associated with high proteolytic activity during cold stratification (Lewak 2011). The transport of amino acids from cotyledons to embryonic axes was reported for walnut kernels (Einali and Sadeghipour 2007). However, the increase in proteolytic activity accompanied by an increased amino acid content in both tissues suggests that amino acid transport is low or absent between cotyledons and embryonic axes. Thus, it can be postulated that amino acid metabolism and new protein synthesis in embryonic axes are more drastic than cotyledons. However, the pattern of protein solubility in both tissues was nearly identical (Fig. 7a). Because of increased proteins solubility prior to mobilization of storage proteins (Yano et al. 2001; Wong et al. 2004), unchanged or decreased level of soluble proteins can be attributed to the relative coordination or superiority of proteolytic activities compared to protein-solubilizing processes, which cause solubility of insoluble proteins and mobilization of soluble proteins and amino acid accumulation. It has been demonstrated that seed reserve mobilization during cold stratification takes place mainly in embryonic axes rather than cotyledons and the former organ acts as the receiver of cold stimulus (Dawidowicz-Grzegorzewska 1989; Bogatek et al. 1989; Li and Ross 1990a, b; Zarska-Maciejewska 1992; Andriotis et al. 2004). However, although protein mobilization did not occur in the embryonic axes of the stratifying pistachio kernels (Fig. 7b), but proteolytic activity and the extent of lipid hydrolysis were similar in both organs (Fig. 8). Thus, cold-sensing organ in moist chilled pistachio kernels could not be recognized.

Proteolytic activity of pistachio kernels showed that each protease had a role in a specific duration of cold treatment (Fig. 8). In both tissues of stratified pistachio kernels for lower than 21 days, proteases most active at neutral to alkaline rather than acidic pHs might be involved in protein mobilization. However, proteolytic activity of both organs was higher at acidic pHs compared to neutral, or alkaline in kernels that had been stratified for 28–42 days. It indicates that a typical protease solely is not involved in protein mobilization of stratifying pistachio kernels, but also different proteases may be activated depend on cold treatment period. Increased proteolytic activity at acidic to neutral pHs occurred for germinating-walnut kernels (Einali and Sadeghipour 2007). Cold-stratified Douglas-fir seeds showed protease activity at neutral to alkaline pHs (Forward et al. 2001). Increased proteolytic activity during cold stratification of apple seeds has been observed in embryonic axes rather than cotyledons (Zarska-Maciejewska and Lewak 1983), which is contrary to present results. However, enhanced proteolytic activity during cold stratification can be because the new proteases synthesis and inactivation of protease inhibitors that naturally exist in the kernels tissues (Salmia and Mikola 1980).

These results suggest that increased germination capacity of pistachio kernels by cold stratification can be ascribed to multiple factors, including direction of metabolites derived from lipid and protein catabolism to appropriate metabolic pathways. Lipid mobilization of cold-stratified pistachio kernels is accompanied by activation of gluconeogenic pathway. Moist chilling may also stimulate the protein mobilization by enhancing proteolytic activities and accumulating amino acid pool, which is accompanying by their subsequent direction to germination-inducing metabolic pathways. Increased certain amino acid metabolism, such as arginine during moist chilling, has been reported (King and Gifford 1997). Cold stratification induces the activity of H_2O_2-scavenging enzymes, such as CAT and APX, to regulate the level of ROS generated during gluconeogenesis or other metabolic pathways and decreases the level of hydrogen peroxide and MDA in pistachio kernels. It has been determined that accumulation of MDA caused seed deterioration and decrease in seed competence for germination (McDonald 2004; Nezamdoost et al. 2009). Therefore, as has been substantiated before (Wang and Berjak 2000; Einali and Sadeghipour 2007; Nezamdoost et al. 2009) and the present results confirm it, the beneficial effects of moist chilling in germination stimulation of pistachio kernels are because of prevention of seed deteriorative processes by operating cellular repair mechanisms and activating gluconeogenic processes, which subsequently enhance seed capability for germination.

Author contribution statement AE designed and carried out all the research and was the main author of this manuscript. JV provided all the technical support during the laboratory work and helped in conception and design of the experiment. Both authors have read and approved the submitted manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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