PLANTA PERSICA
An International Journal of Comparative Plant Physiology
Volume 1, Number 1, Autumn 2017

The promotion of walnut kernel germination by exogenous hydrogen peroxide is accompanied by enhanced gluconeogenesis metabolism

E. Kaviani[a], H.R. Sadeghipour[a,*], M. Aghdasi[a], M. Azimmohseni[b]

[a]*Department of Biology, Faculty of Science, Golestan University, Gorgan, Iran.*
[b]*Department of Statistics, Faculty of Science, Golestan University, Gorgan, Iran.*
*Corresponding author. E-mail: h.r.sadeghipour@gmail.com

Received 2017/01/22; Accepted after revision 2017/11/08

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; PMSF, phenylmethylsulfonyl fluoride; PVPP, polyvinylpolypyrrolidone; SDH, succinate dehydrogenase; TPP, thiamine pyrophosphate

Abstract
Seed dormancy in Walnut (*Juglans regia* L.) can be alleviated by moist chilling which increases kernel gluconeogenesis ability. Hydrogen peroxide also stimulates seed germination of both herbaceous and woody plants; however, its impact on biochemical events in dormant seeds is largely unknown. Accordingly, the effects of irrigating imbibed walnut kernels with exogenous hydrogen peroxide (10 mM) solution for 2, 4, 6 and 8 days on promoting germination and gluconeogenesis metabolism were investigated. Kernels that were irrigated for 6 days with hydrogen peroxide solution displayed the greatest germination percentage. Piercing walnut shells significantly increased kernel germination, and the 6-day irrigation with hydrogen peroxide was again the most efficient. Thus hydrogen peroxide affects both shell and embryo components of walnut seed dormancy. Changes in the amounts of lipids and carbohydrates were also studied in hydrogen peroxide-irrigated kernels. Hydrogen peroxide promoted starch accumulation whereas starch mobilization occurred in non-treated kernels. Total and non-reducing sugars increased in kernels and they were greater especially in the axes of hydrogen peroxide-treated kernels. Reducing sugars, though declined during the experiment, remained higher in hydrogen peroxide-treated kernels. Thus hydrogen peroxide like other chemical or physical agents that can break seed physiological dormancy enhances kernel lipid gluconeogenesis competence as a prerequisite to germination.

***Keywords*:** Seed dormancy, Germination, Gluconeogenesis, Hydrogen peroxide, *Juglans regia* L.

Introduction

In contrast to lower vascular plants, the embryo transition into seedling in seed plants is accompanied with a phase of growth cessation called seed dormancy. The embryo growth cessation in some species is due to inadequacy of suitable environmental conditions most importantly moisture and temperature. In some other species especially those growing in temperate regions, the embryonic axis growth (i.e., germination) cannot proceed despite availability of moisture and suitable temperature. Failure in the embryonic axis growth in this case is an innate feature of embryo known as physiological dormancy. Mechanical, physical and/or chemical characteristics of the embryo enclosing structures, such as seed coat, could also affect the growth competency of the embryonic axis (Bewley et al., 2013).

Naturally, embryos are released from the physiological dormancy after incubation under moist chilled or dry warm conditions which are available in the cold and warm seasons of temperate and tropical regions, respectively. The embryo physiological dormancy can also be overcome by treating

the seeds with some chemicals; such as nitric oxide (Zhang et al., 2003), nitrate and nitrite (Bethke et al., 2006), sodium nitroprusside (Liu et al., 2010a), hydrogen cyanide (Oracz et al., 2009), smoke derived compounds (Light et al., 2010) and hydrogen peroxide (Sarath et al., 2007, Oracz et al., 2009, Liu et al., 2010 b, Bahin et al., 2011).

In plants reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH°), are deleterious to cellular constituents at high concentrations causing oxidative damage to lipids, proteins and nucleic acids; however, at low concentrations they act as signaling molecules regulating many processes as diverse as growth and development, programmed cell death, hormone signaling and response to various stresses (Mittler et al., 2011). Reactive oxygen species stimulate the germination of seeds in many plants (El-Maarouf-Bouteau et al., 2007, Bailly et al., 2008). Thus, the exogenous application of hydrogen peroxide enhances the germination of seeds from woody gymnosperms (Riffle and Springfield 1968) and herbaceous plants like *Hordeum vulgar* (Bahin et al., 2011), *Oryza sativa* (Naredo et al., 1998), *Zinnia elegans* (Ogawa and Iwabuchi 2001), *Arabidopsis thaliana* (Liu et al., 2010b), *Helianthus annuus* (Oracz et al., 2009) to many warm season grasses (Sarath et al., 2007). The accumulation of ROS, e.g., hydrogen peroxide, as well as some other free radicals, e.g., nitric oxide, has been reported during the germination of many seeds (Bailly et al., 2008). This is essential for seed dormancy alleviation, since these compounds mediate the regulation of ABA catabolism and GA biosynthesis (Liu et al., 2010b). The various forms of ROS are furthermore downstream signaling components of hormones like GA in some germination related processes like α-amylase secretion (Ishibashi et al., 2012). There are evidences which support that the dormancy breakage of seeds by nitric oxide and cyanide is mediated by various forms of ROS (Oracz et al., 2009; Gniazdowska et al., 2010).

Embryo transition from dormant into non-dormant state following seed moist chilling is associated with increased competence for reserve mobilization (Lewak et al., 2011), especially gluconeogenesis of lipids (Li and Ross 1990a,b; Lewak 2011; Keshavarzian et al., 2013), as well as amino acid metabolism (Forward et al., 2001; Zarei-Ghadikolaee et al., 2010) and phosphate mobilization (Andriotis et al., 2004). While the impacts of some chemicals such as nitric oxide (Zhang et al., 2003 and 2005) and cyanide (Lewak et al., 2011; Gerivani et al., 2016) have been reported on some aspects of seed reserve mobilization, information on the effects of various forms of ROS such as hydrogen peroxide are scarce.

Persian walnut (*Juglans regia* L.) is a temperate woody species whose embryo, hereafter kernel, is dormant at maturity (Vahdati et al., 2012). The nuts are a rich source of triacylglycerols and proteins which constitute up to 70% and 20% of its dry weight, respectively (Sze-Tao and Sathe, 2000). The kernel physiological dormancy can be removed by moist chilling. This treatment increases the kernel competence for germination which is accompanied with both the activation of lipid gluconeogenesis (Nezamdoost et al., 2009; Keshavarzian et al., 2013) and organic nitrogen mobilization (Einali and Sadeghipour 2007, Zarei-Ghadikolaee et al., 2010, Shahmoradi et al., 2013) in favor of germination. Warm incubation of moistened kernels, however, favors lipid respiration (Keshavarzian et al., 2013) and is associated with perturbations in amino acid metabolism (Zarei-Ghadikolaee et al., 2010) resulting in compromised germination. So far, there is no report on carbon metabolism of seeds during hydrogen peroxide induced dormancy release. Taking walnut kernels as a representative seed of woody species, the extent to which hydrogen peroxide affects the release of seed dormancy was assessed through alterations in carbon metabolism. As dormancy release of walnut kernels by cold stratification is accompanied with the activation of lipid gluconeogenesis pathway (Keshavarzian et al., 2013), the ability of hydrogen peroxide in altering carbon metabolism of dormant kernels in favor of gluconeogenesis was investigated.

Materials and methods

Plant materials

Seeds of Persian walnut (*Juglans regia* L.) were freshly harvested during the October of 2011 and 2012. After soaking in tap water for 24 h, seeds were surface sterilized with 0.5% (w/v) sodium hypochlorite for 15 min, washed four times in distilled water and transferred into a sand medium. They were then kept in a temperature controlled culture room at 27˚C in darkness and irrigated daily with distilled water for up to 35 days. To evaluate the effect of hydrogen peroxide on germination of walnut kernels, they were irrigated daily with hydrogen peroxide solution (10 mM) for 2, 4, 6, and 8 days. In each of these four treatments, subsequent irrigations were carried out with distilled water till 35th day. Each treatment consisted of 72 nuts in triplicates of 24. The experiment was carried out in a completely randomized design. The germination of kernels was recorded daily for 35 days since transferring seeds into the sand medium. The average radicle length of 10 mm was considered as kernel germination. During irrigation of kernels with either hydrogen peroxide (up to 6 days) or water, axes and cotyledons were excised from kernels at 2, 4, 6, 8 and 10 days from the start of the experiment and used for biochemical analyses. There were no sign of germination *i.e*. radicle emergence up to 10 days.

Quantification of hydrogen peroxide

The hydrogen peroxide contents of kernels were determined according to Sergive et al., (1997). Samples (0.2 g) from cotyledons or axes of different ages were homogenized with 0.1% (w/v) cold trichloroacetic acid (TCA) on ice. The obtained extract was centrifuged at 15000 ×*g* for 15 min at 4˚C. To aliquots (0.5 mL) from the clear supernatant, 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1.0 mL freshly prepared Potassium iodide solution (1 M) in potassium phosphate buffer (10 mM, pH 7.0) were added and allowed for color development. The absorbance was taken at 390 nm using a Shimadzu UV-160A spectrophotometer and the H_2O_2 concentration was estimated based on a standard curve with hydrogen peroxide concentrations in the range of 10 to 100 µM.

Other analytical methods

Isocitratelyase (EC, 4.1.3.1) activity of walnut kernels was extracted and assayed as described earlier (Nezamdoost et al., 2009). Extraction and quantification of total lipids from walnut kernels were carried out according to the method of Hara and Radin (1978). The defatted powder obtained following tissue total lipid extraction was used for the extraction and measurement of starch, reducing and non-reducing sugars. Starch content was quantified according to the method of McCready et al., (1950). Reducing and non-reducing sugars were determined by the methods of Prado et al., (1998) and Handel (1968), respectively. D, L-Isocitrate trisodium salt obtained from Sigma Chemical Company. All other chemicals were analytical grade and obtained from Merck.

Statistical analyses

Statistically significant differences at the 5% level were determined by Nested Design Analysis and the Duncan method (SAS software 2010, SAS Institute Inc., Cary, North Carolina, USA).

Results

Effect of hydrogen peroxide on germination of walnut kernels

The control kernels displayed germination percentage up to 61%. Irrigation with hydrogen peroxide significantly increased the germination of walnut kernels (Figure 1A). The greatest germination percentage (87.4 %) occurred for those kernels irrigated with hydrogen peroxide for 6 days (Figure 1B). The germination percentages of kernels irrigated for 2, 4 and 8 days with hydrogen peroxide were more or less equal (about 77%). Although these were greater than the figure observed for the control ones, still lower than that of the 6 days hydrogen peroxide treated kernels.

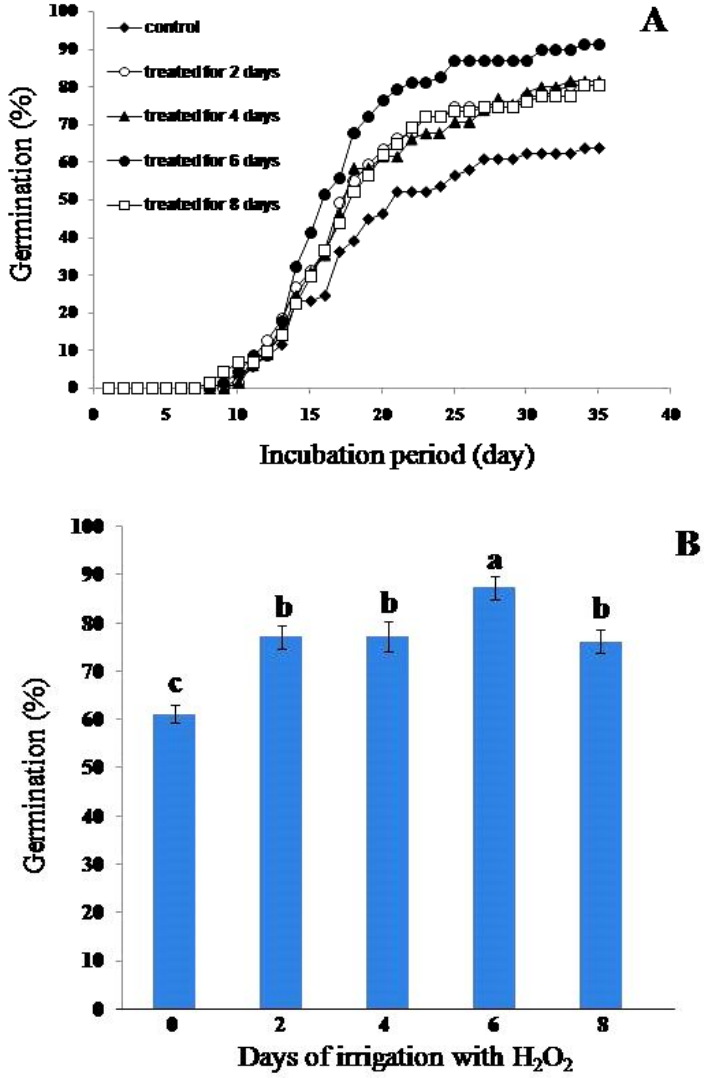

Figure 1. (A) Time course of germination of walnut kernels irrigated initially with 10 mM hydrogen peroxide solution for 2 (○), 4 (▲), 6 (●) and 8 (□) days and subsequently with distilled water up to 35 d. The water irrigated control kernels are shown by the symbol " ♦ ". (B) The germination percentage of walnut kernels as affected by duration of irrigation with 10 mM hydrogen peroxide solution. Different small letters indicate significant differences at $P < 0.05$. Nuts with pierced shells at peduncle were used in this experiment.

Effect of shell intactness on the enhanced germination of walnut kernels by hydrogen peroxide

Shells from walnut kernels are reported to exert an inhibitory effect on the germination of embryos (Vahdati et al.,, 2012). However, the nature of this inhibitory effect is not clearly defined. Shells from intact nuts were pierced at the peduncle region. Both intact and pierced nuts were irrigated with hydrogen peroxide solution for 6 days and their germination after 35 d were compared with the corresponding control ones i.e. those irrigated only with distilled water (Figure 2A). The maximum germination of kernels from intact seeds was about 41% (Figure 2B). Piercing shells significantly increased this value to about 49%. Irrigating kernels with hydrogen peroxide for 6 days had a significant effect on stimulating the germination of both intact and pierced seeds. The germination percentages of intact and pierced seeds in this case were 54 and 61% respectively.

All the subsequent biochemical analyses were carried out on nuts with pierced shells irrigated with both hydrogen peroxide for 6 days as treatment and water as control.

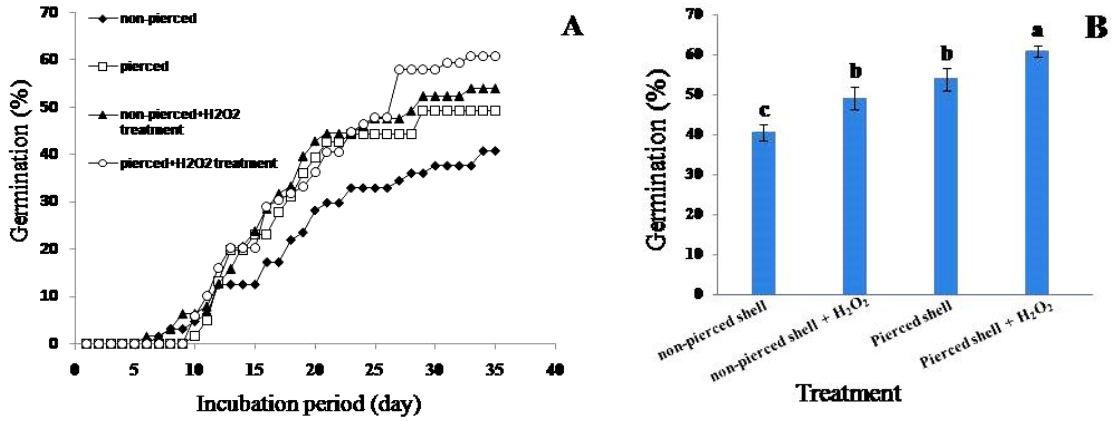

Figure 2. (A) Time course of germination of walnut kernels with either intact (non-pierced) or pierced shells after irrigation with 10 mM hydrogen peroxide solution for 6 days compared to those irrigated with water. The symbols "♦" and "□" stand for water irrigated intact and pierced nuts, respectively and the symbols "▲" and "○" represent hydrogen peroxide irrigated intact and pierced nuts. (B) The interaction of shell intactness and hydrogen peroxide irrigation on germination percentage of walnut kernels. Different small letters indicate significant differences at $P < 0.05$. Nuts with both intact and pierced shells were used in this experiment.

Changes in total lipid content of hydrogen peroxide treated kernels

Total lipid content of cotyledons from imbibed walnut kernels was initially 587 mg. g^{-1}FW which declined significantly in both water- and hydrogen peroxide-irrigated kernels after 4 and 2 days, respectively (Figure 3A) and continued to decline till 10th day. As with cotyledons, the lipid content of axes from both water- and hydrogen peroxide-irrigated kernels declined significantly during 10 days of incubation (Figure 3B). As revealed by Nested Design Analysis for the whole 10 days of kernel incubation, the mean lipid content of hydrogen peroxide-irrigated kernels (469 ± 51.7 mg. g^{-1}FW) was not significantly different from that of the water-irrigated ones (474.8±35.6 mg.g^{-1}FW).

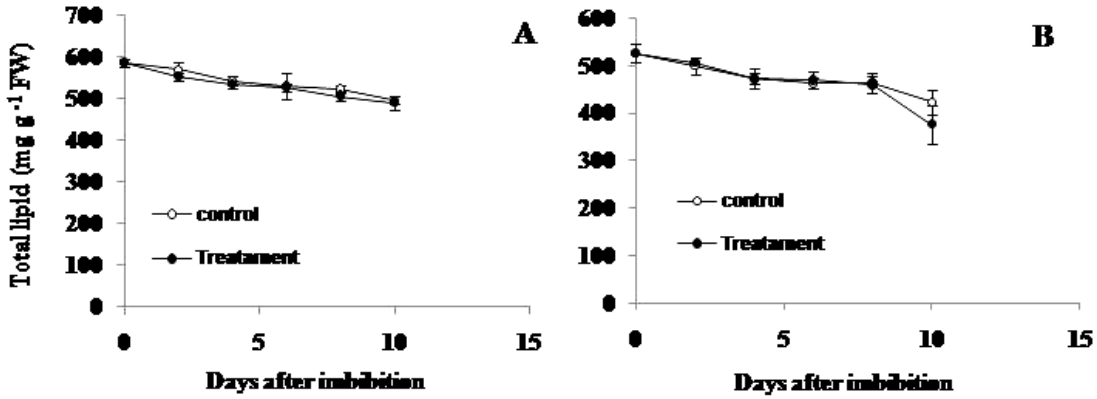

Figure 3. Changes in total lipid contents of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE.

Changes in carbohydrate contents of kernels after irrigation with hydrogen peroxide

The reducing sugar content of cotyledons decreased in both control and hydrogen peroxide treated kernels (Figure 4A). It decreased from an initial amount of 4.0 mg. g^{-1}FW in imbibed cotyledons to about 2.4 mg.g^{-1} FW in 4 d old water-irrigated control kernels. The decline in reducing sugar content of hydrogen peroxide treated-cotyledons was much slower and after 4 days it reached to about 3.4 mg.g^{-1} FW. The content of reducing sugar in water irrigated embryonic axes also decreased, however, in hydrogen peroxide treated axes it increased at day two and thereafter declined (Figure 4B). The means for reducing sugar contents of both cotyledons and axes of hydrogen peroxide treated-kernels were significantly greater than those of the control water irrigated-seeds, as revealed by Nested Design Analysis (Figure 4C and D).

In cotyledons of water irrigated-kernels, non-reducing sugars were accumulated from an initial amount of 3.8 mg.g^{-1} FW to 5.6 mg. g^{-1} FW after 10 days (Figure 5A). The figure in the 10-day old hydrogen peroxide irrigated-cotyledons corresponded to 6.2 mg. g^{-1} FW. The accumulation of non-reducing sugars in axes was greater than cotyledons (Figure 5B). Thus, the amount of non-reducing sugars in control axes increased from an initial amount of 4.7 mg. g^{-1} FW to 12.1 and 12.4 mg. g^{-1} FW in water- and hydrogen peroxide-irrigated kernels, respectively. Nested Design Analysis revealed that the non-reducing sugar contents of hydrogen peroxide irrigated cotyledons and axes were significantly greater than those in the control kernel (Figure 5C and D).

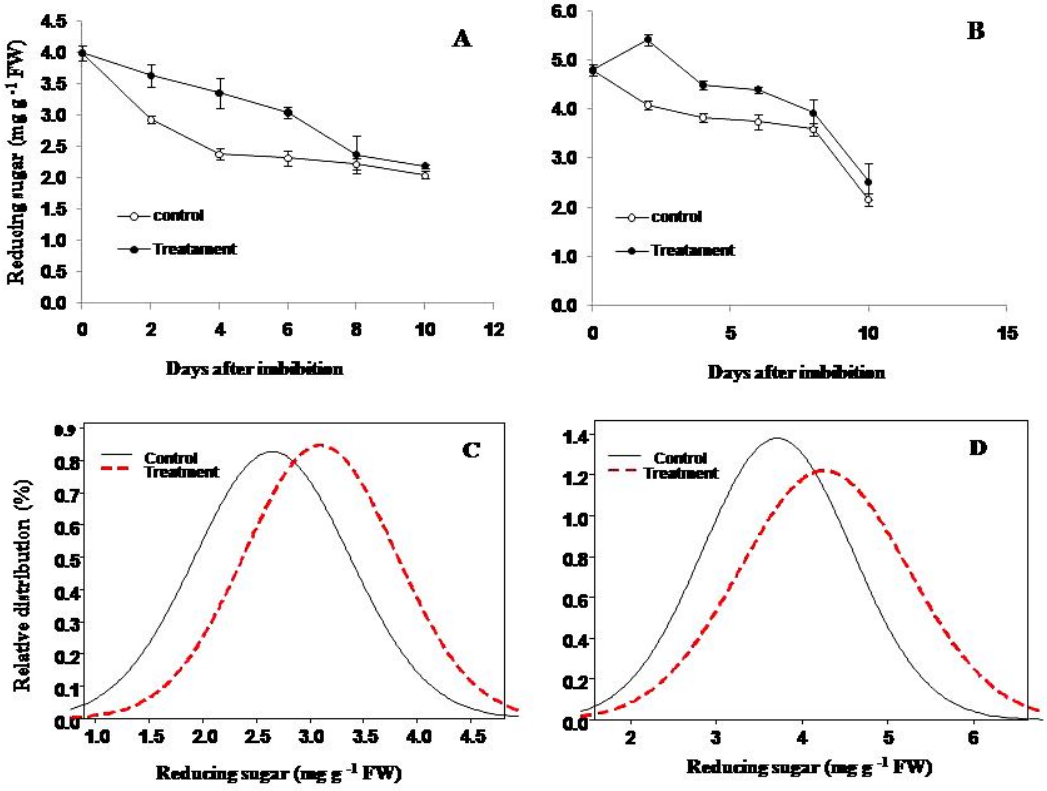

Figure 4. Changes in reducing sugar contents of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE. The relative frequencies of reducing sugar contents of cotyledons (C) and axes (D) from hydrogen peroxide treated (dashed line) and water irrigated (solid line) kernels were obtained by Nested Design Analysis

The total soluble sugar contents of cotyledon and axis increased during the 10 days of kernel incubation, and this increase was even more pronounced after irrigation by hydrogen peroxide (Figure 6A and B). Nested Design Analysis revealed that the total sugar contents of hydrogen peroxide irrigated cotyledons and axes were significantly greater than the corresponding figures in the control kernel (Figure 6 C and D). The hydrogen peroxide-stimulated increase in total soluble sugars was much greater in the embryonic axes than in cotyledons; thus the mean total soluble sugar content of hydrogen peroxide irrigated cotyledons was greater by only about 4% compared with the control ones during the 10 days of kernel incubation, whereas the corresponding figure for the axes rose to 13%.

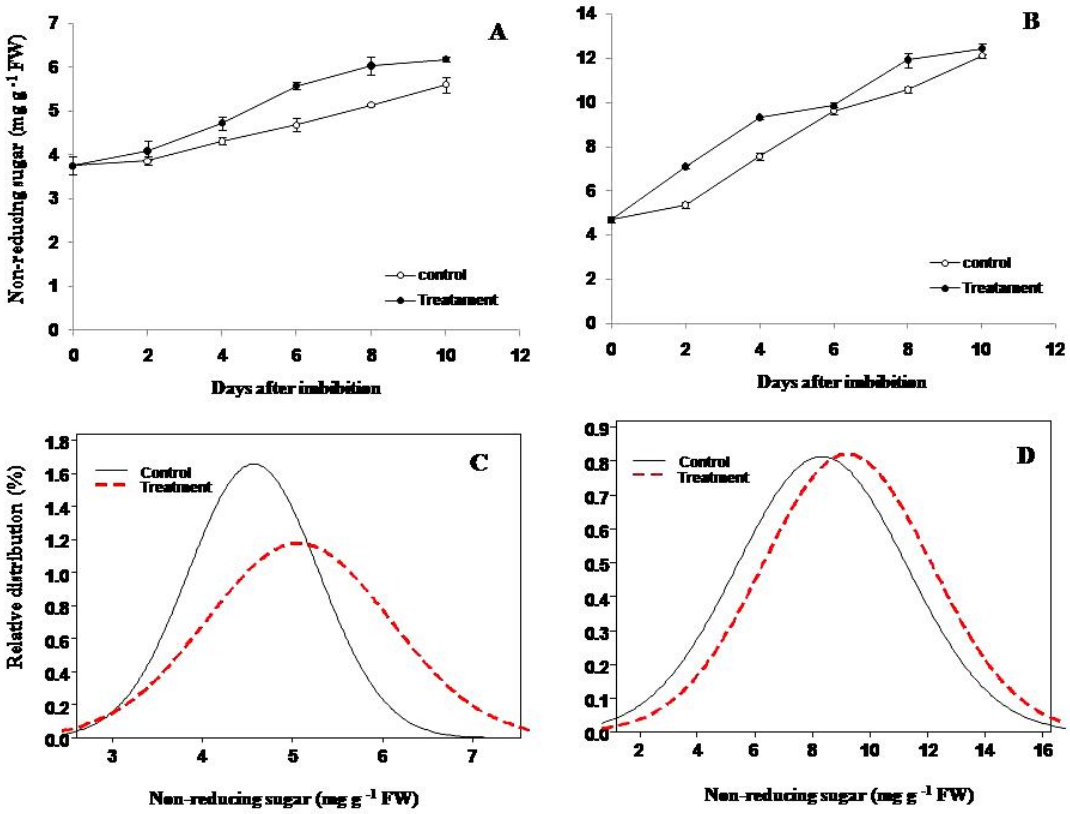

Figure 5. Changes in non-reducing sugar contents of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE. The relative frequencies of non-reducing sugar contents of cotyledons (C) and axes (D) from hydrogen peroxide treated (dashed line) and water irrigated (solid line) kernels were obtained by Nested Design Analysis.

The starch contents of both cotyledons and axes of water irrigated kernels declined from 3.0 to 2.4 mg. g^{-1} FW and from 3.5 to 2.7 mg. g^{-1} FW, respectively, after 10 days of kernel incubation (Figure 7A and B). In kernels irrigated with hydrogen peroxide, however, after an initial decline at day 2, the starch contents of both cotyledons and axes increased and reached to the greatest amount of 3.6 and 4.0 mg.g^{-1} FW, respectively. Nested Design Analysis revealed a significantly greater mean for the starch content of hydrogen peroxide irrigated-kernels compared with the water irrigated ones (Figure 7C and D).

Changes in isocitrate lyase activity of walnut kernels

Isocitrate lyase activity was not detectable initially in imbibed walnut kernels however it appeared after 2 to 4 days of incubation under both control and hydrogen peroxide

irrigation (Figure 8 A and B). There were no conspicuous trend in enzyme activity and mostly it showed fluctuations under the aforementioned conditions in both cotyledons and axes. Means for the enzyme activity in both cotyledons and axes of water irrigated kernels were slightly greater than those irrigated by hydrogen peroxide (data not shown).

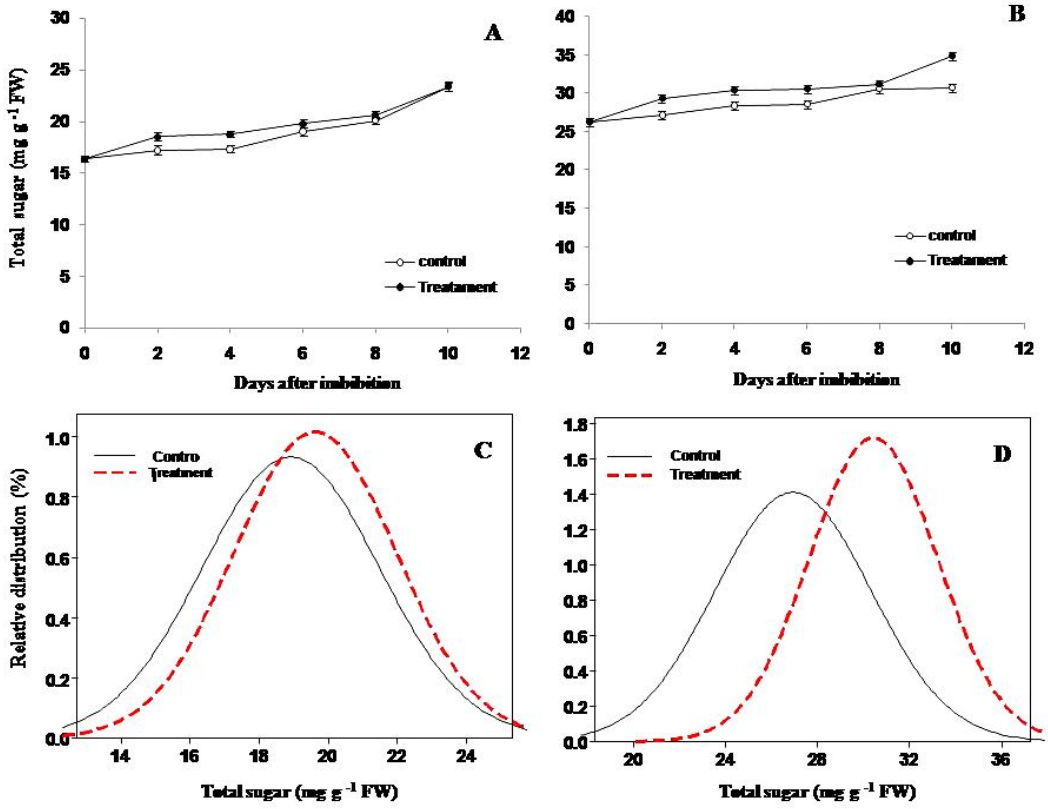

Figure 6. Changes in total sugar contents of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE. The relative frequencies of total sugar contents of cotyledons (C) and axes (D) from hydrogen peroxide treated (dashed line) and water irrigated (solid line) kernels were obtained by Nested Design Analysis.

Changes in endogenous hydrogen peroxide contents of walnut kernels

The initial hydrogen peroxide contents of cotyledons and axes were 0.26 and 0.13 µmol. g^{-1} FW, respectively. During the 4 days of kernel irrigation with water, it increased significantly and reached to the greatest level of 0.41 µmol. g^{-1} FW in both cotyledons and axes (Figure 9A and B). A rapid decline in kernel hydrogen peroxide content occurred especially in cotyledons from this time onwards so that it was least in 10 days old kernels. Changes in hydrogen peroxide content of hydrogen peroxide irrigated kernels were more or less similar to those of the water irrigated ones. However, the endogenous hydrogen peroxide content of axes (and not cotyledons) of kernels irrigated with hydrogen peroxide was lower than that of the water irrigated ones (data not shown).

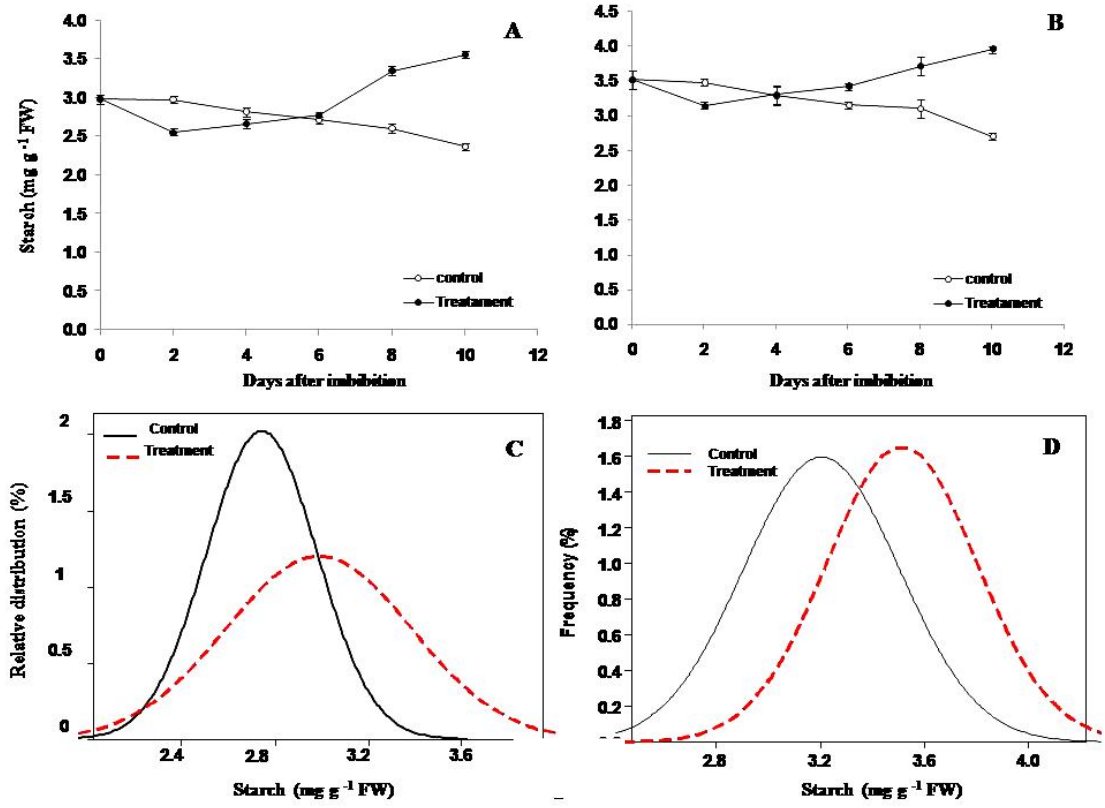

Figure 7. Changes in the starch contents of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE. The relative frequencies of starch contents of cotyledons (C) and axes (D) from hydrogen peroxide treated (dashed line) and water irrigated (solid line) kernels were obtained by Nested Design Analysis.

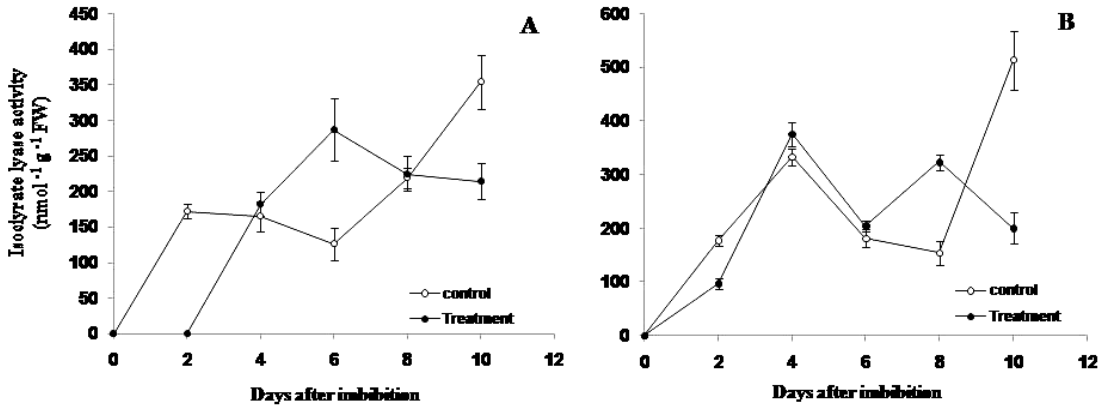

Figure 8. Changes in isocitrate lyase activity of cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE.

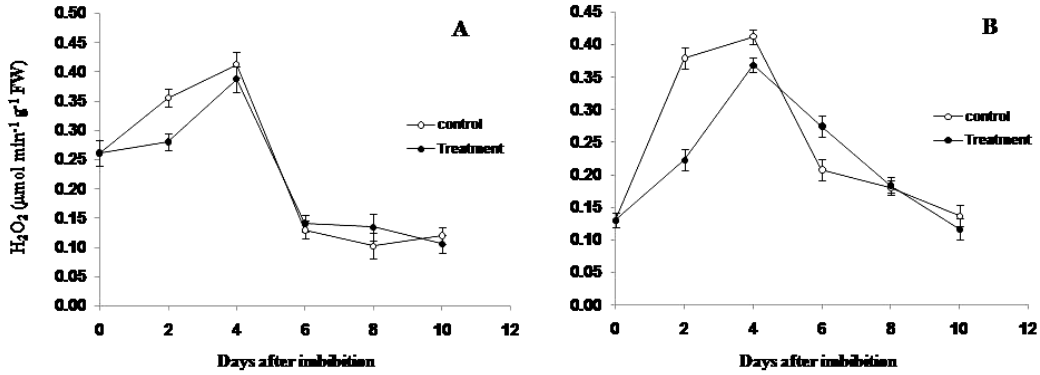

Figure 9. Changes in the level of hydrogen peroxide in cotyledons (A) and axes (B) of walnut kernels after irrigation with either hydrogen peroxide (●) or water (○). Each point represents the mean value of three separate extractions ± SE.

Discussion

Walnut kernels are not deeply dormant since its germination occurs in the absence of any treatment (Figure 1), in agreement with former reports (Einali and Sadeghipour, 2007). Irrigating kernels with hydrogen peroxide significantly enhanced their germination and the 6-day irrigation treatment was found to be more efficient. The enhancement of germination by exogenous hydrogen peroxide has been reported for seeds of many herbaceous (Sarath et al., 2007; Oracz et al., 2009; Liu et al., 2010a, b, Bahin et al., 2011) and woody species (Riffle and Springfield, 1968; Bogatek et al., 2003). Hydrogen peroxide might oxidize and inactivate germination inhibitory compounds present in walnut shell and/or kernel (Petriccione and Ciniglia, 2012), a situation that is reported for seeds of some herbaceous species such as *Zinnia elegans* (Ogawa and Iwabuchi, 2001). Alternatively, hydrogen peroxide may affect the embryo hormonal metabolism in favor of GA accumulation and/or ABA breakdown thus promoting dormancy release (Liu et al., 2010b). Furthermore, the beneficial effects of hydrogen peroxide on germination could be due to its ability to limit infestation of seeds by fungi (Szopinska, 2014).

Piercing the shells improved kernel germination (Figure 2), however, it does not appear to be related to the shell mechanical properties only (Vahdati et al., 2012) or impermeability of the shells to water. Accordingly both pierced and intact seeds displayed identical water absorption isotherms (data not shown). In hydrogen peroxide treated nuts, piercing might have resulted in more efficient penetration of hydrogen peroxide and thus restricting embryo-shell prohibiting effects on germination. The dormancy of walnut seeds can be regarded as a mix type or combinatorial dormancy as both shell and embryo contribute to dormancy.

The moist chilled-induced dormancy release of walnut kernels (Nezamdoost et al., 2009; Keshavarzian et al., 2013) and some other arboraceous seeds (Li and Ross, 1991a,b; Lewak, 2011) accompanies with the enhanced competence for gluconeogenesis of lipid reserves. As the exogenous hydrogen peroxide improved the germination of walnut kernels, its ability for promoting gluconeogenesis of lipid reserves was investigated during a 10-day period started from imbibition time. This period was adopted as there were no visual signs of radicle emergence and thus any possible biochemical changes in kernels could be attributed to the process of dormancy release or pre-germination events. Lipid mobilization occurred in both water and hydrogen peroxide treated kernels (Figure 3). The kernel lipid mobilization might be due to the activity of an alkaline lipase already present in the mature dry seeds (Yesiloglu and Demirkan, 2010). The irrigation of kernels with hydrogen peroxide, was accompanied with

the accumulation of starch, whereas in the water irrigated kernels starch was mobilized (Figure 7). Although non-reducing and total soluble sugars increased in both hydrogen peroxide- and water-irrigated kernels, their accumulations were greater in the hydrogen peroxide-treated ones (Figures 5 and 6). Several conclusions can be drawn from these observations; first of all, during the initial 10 days of kernel incubation, irrespective of the type of treatment, gluconeogenesis metabolism prevails (although at dissimilar rates). Thus, the predominance of respiratory metabolism leading to death in the water irrigated kernels must be beyond this period; as the estimation of the activities of major respiratory enzymes might imply this idea (Nezamdoost et al., 2009; Keshavarzian et al., 2013). Second, gluconeogenesis of lipid reserves in the control water-irrigated kernels is manifested by the accumulation of non-reducing sugars (mainly sucrose) whereas, in hydrogen peroxide-treated ones beside sucrose, starch was also accumulated. The significance of the latter observation might be understood by comparing the extent of non-reducing sugar accumulation between cotyledons and axes. While non-reducing sugars accumulated by only 50% in cotyledons, the figure approached to about 200% in the embryonic axes. This could partly be attributed to a greater rate of lipid gluconeogenesis and sugars production such as sucrose, in axes compared with cotyledons (Figure 5). However, a greater proportion of the sucrose is seemingly due to the translocation of gluconeogenically-derived sugars from cotyledons into axes. An enhanced sugar transport from cotyledons to axis due to cyanide exposure has recently been reported in walnut kernels (Gerivani et al., 2016). Application of hydrogen peroxide to pea seeds results in carbonylation of specific proteins notably those involved in glycolysis which in turn limits carbon flux into this respiratory pathway (Barba-Espin et al., 2011). Therefore, in the hydrogen peroxide-irrigated kernels the probable attenuation of carbon respiration favors starch accumulation as revealed by the greater levels of reducing sugars as well as sucrose.

Despite the differential competences for starch accumulation and thus gluconeogenesis in the hydrogen peroxide-treated kernels versus the water-irrigated ones, they showed almost comparable isocitrate lyase activities (Figure 8). As pointed out earlier, this implies the non-gluconeogenic operation of the glyoxylate cycle (Eastmond and Graham 2001). This is consistent with the recent findings that the activities of glyoxysomal succinate oxidase and phosoho*enol* pyruvate- carboxykinase rather than isocitrate lyase are the markers of gluconeogenesis metabolism in moist chilled walnut kernels (Keshavarzian et al., 2013). Starch accumulation and isocitrate lyase activities, however, have been reported in some other woody species in the course of seed dormancy release by moist chilling (Noland and Murphy, 1984; Dawidowicz-Grezegorezewka, 1989; Li and Ross, 1990b).

Hydrogen peroxide content increased for 4 days and then it decreased suddenly in both control- and hydrogen peroxide-treated kernels. Accumulation of H_2O_2 during germination has been reported for seeds of many herbaceous plants such as soybean (Gidrol et al., 1994), wheat (Caliskan and Cuming 1998), radish (Schopfer et al., 2001), sunflower (Oracz et al., 2009), tomato (Morohashi, 2002) and pea (Wojtyla et al., 2006). This could be due to the resumption of seed metabolic processes, such as respiration (Bailly et al., 2008). Exogenous application of hydrogen peroxide remarkably decreased kernel hydrogen peroxide content within two days (Figure 9). This early effect of external hydrogen peroxide was subsequently compensated, as beyond this time the hydrogen peroxide contents of these kernels were nearly equal to those found in the water-irrigated ones. This behavior could indicate the increased potential for ROS scavenging in kernels irrigated with hydrogen peroxide. The accumulation of hydrogen peroxide predominates during the first 4 days of kernel incubation, which suggests inefficient anti-oxidative mechanism during the aforementioned

period. This is consistent with gradual development of catalase activity in walnut kernels as incubation proceeds (Einali and Sadeghipour, 2007). Furthermore it has been shown that other dormancy breaking chemicals, e.g. nitric oxide and cyanide, regulate the activities of ROS-scavenging enzymes in germinating apple embryos (Krasuska and Gniazdowska, 2012). In contrast to walnut kernels, the application of hydrogen peroxide to pea seeds resulted in increased levels of endogenous hydrogen peroxide (Barba-Espin et al., 2011). The basis for this difference however, might reside in the non-dormant state of pea seeds used in that study.

In conclusion, using walnut kernels, this study showed the activation of gluconeogenesis during seed dormancy release by hydrogen peroxide. This activation might be mediated through changes in the levels of various endogenous ROS, reactive nitrogen species and/ or phytohormones (Oracz et al., 2009, Ishibashi et al., 2012), which finally affect some enzymes of primary metabolism as downstream signaling components. Thus, the application of hydrogen peroxide like other chemicals or physical agents, which can break seed physiological dormancy, leads to the development of kernel gluconeogenesis competence. Recent proteomic studies on Arabidopsis seed dormancy removal by either moist chilling or nitrate support this contention as both treatments resulted in similar functional proteomic changes accompanying seed dormancy release (Arc et al., 2012).

Acknowledgments

We thank the Golestan University Deputy of Research and Office of Higher Education for financial support to E. Kaviani in the form of grant for M.Sc. research project.

References

Andriotis, V.M.E., Smith, S.B., Ross, J.B., 2004. Phytic acid mobilization is an early response to chilling of the embryonic axes from dormant oilseed of hazel (*Corylusavellana*. L). J. Exp. Bot. 56, 537-545.

Arc, E., Chibani, K., Grappin, P., Jullien, M., Godin, B., Cueff, G., Valot, B., Balliau, T., Job, D., Rajjou, L., 2012. Cold stratification and exogenous nitrates entail similar functional proteome adjustment during Arabidopsis seed dormancy release. J. Proteome Res. 11, 5418-5432.

Bahin, E., Bailly, C., Sotta, B., Kranner, I., Corbineau, F., Leymarie, J., 2011. Crosstalk between reactive oxygen species and hormonal signaling pathway regulates grain dormancy in barley. Plant Cell Environ. 34, 980-993.

Bailly, C., El-Maarouf-Bouteau, H., Corbineau, F., 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. CR Boil. 331, 806-814.

Barba-Epsin, G., Diaz-Vivancos, P., Job, D., Belghazi, M., Job, C., Hernandez, J.A., 2011. Understanding the role of H_2O_2 during pea seed germination: a combined proteomic and hormone profiling approach. Plant Cell Environ. 34, 1907-1919.

Bethke, P.C., Libourel, I.G.L., Reinohl, V., Jones, R.L., 2006. Sodium nitroprusside, cyanide, nitrite, and nitrate break *Arabidopsis* seed dormancy in an oxide-dependent manner. Planta. 223, 805-812.

Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M., Nonogaki, H., 2013. Seeds: Physiology of development, germination and dormancy. 1st edition, Springer, New York.

Bogatek, R., Gawronska, H., Oracz, K., 2003. Involvement of oxidative stress and ABA in CN-mediated elimination of embryonic dormancy in apple. In: Nicolas G, Bradford KJ, Côme D, Pritchard HW, editors. The biology of seeds: recent research advances, 1st edition, City, Country, Publisher.pp. 211-216.

Caliskan, M., Cuming, A.C., 1998. Spatial specificity of H_2O_2-generating oxalate oxidase gene expression during wheat embryo germination. Plant J. 15, 165-171.

Dawidowicz-Grzegorzewska, A., 1989. Degradation of protein and lipid bodies during dormancy removal in apple seeds. J. Plant Physiol. 135, 43-51.

Eastmond, P.J., Graham, I.A., 2001. Re-examining the role of the glyoxylate cycle in oil seeds. Trends Plant Sci. 6, 72-77.

Einali, A.R., Sadeghipour, H.R., 2007. Alleviation of dormancy in walnut kernels by moist chilling is independent from storage protein mobilization. Tree physiol. 27, 519-525.

El-Maarouf-Bouteau, H., Job, C., Job, D., Corbineau, F., Bailly, C., 2007. ROS signaling

in seed dormancy alleviation. Plant Signal Behav. 2, 362-364.

Forward, B.S., Tranbarger, T.J., Misra, S., 2001. Characterization of proteinase activity in stratified Douglas-fir seeds. Tree Physiol. 21, 625-629.

Gerivani, Z., Vashaee, E., Sadeghipour, H.R., Aghdasi, M., Shobbar, Z.S., Azimmohseni, M., 2016. Short versus long term effects of cyanide on sugar metabolism and transport in dormant walnut kernels. Plant Sci. 252, 193-204.

Gidrol, X., Lin, W.S., Degousee, N., Yip, S.F., Kush, A., 1994. Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J. Biochem. 224, 21-28.

Gniazdowska, A., Krasuska, U., Czajkowska, K., Bogatek, R., 2010. Nitric oxide, hydrogen cyanide and ethylene are required in the control of germination and undisturbed development of young apple seedlings. Plant Growth Reg. 61, 75-84.

Handel, E.V., 1968. Direct microdetermination of sucrose. Anal. Biochem. 22, 280-283.

Hara, A., Radin, N.S., 1978. Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90, 420-426.

Ishibashi, Y., Tawaratsumida, T., Kondo, K., Kasa, S., Sakamoto, M., Aoki, N., Zheng, S.H., Yuasa, T, Iwaya-Inoue, M., 2012. Reactive oxygen species are involved in gibberellin/abscisic acid signalling in barley aleurone cells. Plant Physiol. 158, 1705-1714.

Keshavarzian, M., Garivani, Z., Sadeghipour, H.R., Aghdasi, M., Azimmohseni, M., 2013. 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. Sci. Hort. 161, 314-323.

Krasuska, U., Gniazdowska, A., 2012. Nitric oxide and hydrogen cyanide as regulating factors of enzymatic antioxidant system in germinating apple embryos. Acta. Physiol. Plant. 34, 683-692.

Lewak, S., 2011. Metabolic control of embryonic dormancy in apple seed: seven decades of research. Acta. Physiol. Plant. 33, 1-24.

Li, L., Ross, J.D., 1990a. Lipid mobilization during dormancy breakage in oilseed of Corylusavellana. Ann. Bot. 66, 501-505.

Li, L., Ross, J.D., 1990b. Starch synthesis during dormancy breakage in oilseed of Corylusavellana. Ann. Bot. 66, 507-512.

Light, M.E., Burger, B.V., Staerk, D., Kohout, L., Van Staden, J., 2010. Butenolides from plant-derived smoke: Natural plant growth regulators with antagonistic actions on seed germination. J. Nat. Prod. 73, 267-269.

Liu, X., Deng, Z., Cheng, H., He, X., Song, S., 2010a. Nitrite, sodium nitroprusside, potassium ferricyanide and hydrogen peroxide release dormancy of *Amaranthusretroflexus* seeds in a nitric oxide-dependent manner. Plant Growth Regul. 64, 155-161.

Liu, Y., Ye, N., Liu, R., Chen, M., Zhang, J., 2010b. H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. J. Exp. Bot. 11, 2979-2990.

McCready, R.M., Guggolz, J., Silviera, V., Owns, H.S., 1950. Determination of starch and amylose vegetables. Anal. Chem. 22, 1156-1158.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., and Vandepoele, K., 2011. ROS signalling: the new wave? Trends Plant Sci. 16, 300-309.

Morohashi, Y., 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. J. Exp. Bot. 53, 1643-1650.

Naredo, M.E.B., Juliano, A.B., Lu, B.R., de Gusman, F., Jackson, M.T., 1998. Responses to seed dormancy-breaking treatments in rice species (*Oryza* L.). Seed Sci. Technol. 26, 675-689.

Nezamdoost, T., Tamaskani, F., Abdolzadeh, A., Sadeghipour, H.R., 2009. Lipid mobilization, gluconeogenesis and aging-related processes in dormant walnut kernels during moist chilling and warm incubation. Seed Sci. Res. 19, 91-101.

Noland, T.L., Murphy, J.B., 1984. Changes in isocitratelyase activity and ATP content during stratification and germination of sugar pine seeds. Seed Sci. Technol. 12, 777-787.

Ogawa, K., Iwabuchi, M., 2001. A mechanism for promoting the germination of *Zinnia elegans* seeds by Hydrogen peroxide. Plant Cell Physiol. 42, 286-291.

Oracz, K., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F., Bailly, C., 2009. The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. Plant Physiol. 150, 494-505.

Petriccione, M., Ciniglia, C., 2012. Comet assay to assess the genotoxicity of Persian walnut (*Juglansregia* L.) husks with statistical evaluation. Bull. Environ. Toxicol. 89, 166-171.

Prado, D.E., Gonzalea, J.A., Boero, C., Sampietro,

A.R., 1998. A simple and sensitive method for determining reducing sugars in plant tissues. Application to quantify the sugar contant in Quinoa (*Chenopodium quinoa* Willd.) seedlings. Phytochem. Anal. 9, 58-63.

Riffle, J.W., Springfield, H.W., 1968. Hydrogen peroxide increases germination and reduces microflora on seed of several southwestern woody species. For. Sci. 14, 96-101.

Sarath, G., Hou, G., Baird, M.L., Mitchell, B.R., 2007. ABA, ROS and NO are key players during Switchgrass seed germination. Plant Signal. Behav. 6, 492-493.

Schopfer, P., Plachy, C., Frahry, G., 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin and abscisic acid. Plant Physiol. 125, 1591-1602.

Sergive, I., Alexieva, V., Karanov, E., 1997. Effect of spermine, atrazine and combination between them on endogeneus protective systems and stress markers in plants. Compt. Rend. Acad. Bulg. Sci.51, 121-124.

Shahmoradi, Z., Tamaskani, F., Sadeghipour, H.R., Abdolzadeh, A., 2013. Redox changes accompanying storage protein mobilization in moist chilled and warm incubated walnut kernels prior to germination. J. Plant Physiol. 170, 6-17.

Sze-Tao, K.W.C., Sathe, S.K., 2000. Walnut (*Juglans regia* L.): proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. J. Sci. Food Agric.80, 1393-1401.

Szopinska, D., 2014. Effect of hydrogen peroxide treatment on the germination, vigor and health of Zinnia elegans seeds. Folia Hort. 26, 19-29.

Vahdati, K., Aslani Aslamars, A., Rahemi, M., Hassani, D., Leslie, C., 2012. Mechanism of seed dormancy and its relationship to bud dormancy in Persian walnut. Environ. Exp. Bot. 75, 74-82.

Wojtyla, L., Garnczarska, M., Zalewski, T., Bednarski, W., Ratajczak, L., Jurga, S., 2006. A comparative study of water distribution, free radical production and activation of antioxidative metabolism in germinating pea seeds. J. Plant Physiol. 163, 1207-1220.

Yesiloglu, Y., Demirkan, B., 2010. Biocatalytic properties of lipase from walnut (*Juglansregia* L.)
seeds. J. Amer. Oil Chem. Soc. 87, 659-665.

Zarei-Ghadikolaee, M., Abdolzadeh, A., Sadeghipour, H.R., 2010. Arginase, glutamine synthetase and glutamate dehydrogenase activities in moist chilled and warm incubated walnut kernels. Trees. 24, 425-433.

Zhang, H., Shen, W.B., Xu, L.L., 2003. Effect of nitric oxide on the germination of wheat seeds and its reactive oxygen species metabolism under osmotic stress. Acta. Bot. Sin. 45, 901-905.

Zhang, H., Shen, W.B., Zhang, W., Xu, L.L., 2005. A rapid response of β-amylase to nitric oxide but not gibberellin in wheat seeds during the early stage of germination. Planta. 220, 708-716.