



## RESEARCH PAPER

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## Post-germination changes in hormones, enzymatic activities and biochemical compounds of different domesticated almond varieties in response to stratification

Hossein Mohammadi<sup>1\*</sup>, Ali Imani<sup>2</sup>, Ali Ebadi<sup>3</sup>

<sup>1</sup>Islamic Azad University, Science and Research Branch, Tehran, Iran

<sup>2</sup>Faculty Member of Seed and Plant Improvement Institute, Karaj, Iran

<sup>3</sup>Department of Horticulture, University of Tehran, Karaj, Iran

**Key words:** Abscisic acid, amylase activity, fructose, gibberellic acid, glucose.

<http://dx.doi.org/10.12692/ijb/4.10.298-308>

Article published on May 26, 2014

### Abstract

Little biochemical information is available regarding the responses of domesticated almond varieties to stratification during the stages of seed germination. Therefore, this study carried out between seeds of Marcona, Rabie, Supernova, Perlis, D99, K-66 domesticated almond varieties to evaluate their hormonal [abscisic acid (ABA) and gibberellic acid (GA<sub>3</sub>)], enzymatic activities (amylase and peroxidase) and biochemical compounds (glucose, fructose, protein and proline) changes following stratification at 7 °C for 8 weeks. The results showed that after stratification and following germination ABA concentration significantly decreased, whereas GA<sub>3</sub> concentration increased. Stratification significantly affected amylase and peroxidase activities, sugars content (glucose and fructose) and proline content as the highest amounts were obtained after germination. Moreover, change in protein content depends on varieties as protein content significantly increased in Rabie, Supernova, D-99 and K-66 varieties, while Marcona and Perlis showed significantly decreasing in protein content. Overall, Rabie, Supernova and K-66 varieties showed the highest hormones, enzymatic activities biochemical compounds content after stratification and following germination.

\* **Corresponding Author:** Hossein Mohammadi ✉ [hossein8143@gmail.com](mailto:hossein8143@gmail.com)

## Introduction

Almond (*Prunus dulcis* (Mill.) D. A. Webb, syn. *P. amygdalus* Batsch, and *P. communis* (L.)) is an extensively grown nut crop of regions with a Mediterranean climate (Zacheo *et al.*, 2000). In *Prunus* species, seed dormancy is an adaptation mechanism in various species to delay germination after the seed has been shed from the tree until the appropriate time for germination (García-Gusano *et al.*, 2004).

Seeds displaying intermediate physiological dormancy usually require chilling stratification to release seeds from dormancy (Hartman *et al.*, 2002) and to reduce levels of growth inhibitor. Stratification, the standard method used to overcome seed dormancy involves storing seeds with an equal volume of moist medium for a period of time at a cold temperature (Bewley and Black, 1994).

Seed germination is a complex process involving numerous cellular events and wide variety of different enzymes. During germination, some of the seed reserve materials are degraded and used for respiration and synthesis of new cell constituents of the developing embryo, therefore causing significant changes in the biochemical and macromolecules (López-Amorós *et al.*, 2006). The plant hormones gibberellins (GAs) and abscisic acid (ABA) are involved in seed dormancy and germination. ABA is involved primarily in regulating seed germination, inducing storage, protein synthesis and in a potent germination inhibitor (Hopkins and Hüner, 2004; Al-Imam and Qrunfleh, 2007). Additionally, an increase in GA concentration during cold stratification or an increase to GA sensitivity may induce seed germination in many dormant species (Bewley and Black 1994; Hu *et al.*, 2012).

There is little or no reliable information on the changes in hormones, enzymatic activities and biochemical compounds during the germination of almond. Therefore, the main aim of this study was to evaluate the changes in hormones (GA and ABA), enzymatic activities (amylase and peroxidase) and

biochemical compounds (glucose, fructose, protein and proline) in seed germination of six almond varieties before and following stratification.

## Materials and methods

### *Seed materials and handling*

Studies were carried out in Seed and Plant Improvement Institute (SPII), Karaj, Iran in 2012. About 200 mature seeds of Marcona, Rabie, Supernova, Perlis, D99, K-66 domesticated almond varieties were collected at random from the Iranian national collection. Seeds were surface sterilized with sodium hypochlorite and then seeds were washed twice with sterile distilled water. To ensure that the seeds used for the experiments were viable and maintained their viability after treatments, seed viability was determined by the ability to reduce 2, 3, 5-triphenyltetrazolium chloride to red colored formation (ISTA, 1985). Thus, seeds were reserved before and after the treatments, which were tested by the ability to reduce 2, 3, 5-triphenyltetrazolium chloride to red colored formation. Seeds were washed again with sterile distilled water and allowed to dry at room temperature (at 25 °C and 60% relative humidity).

Then, seeds divided into two sub-samples. One sub-sample was immediately frozen in liquid nitrogen and immediately used for the biochemical analysis. The second sub-sample was used in stratification experiment. These seeds were stored in germination boxes and sealed. Then germination boxes placed in darkness and cooled incubator (7 °C) for 8 weeks. Seed germination was evaluated daily, and seeds were considered to have germinated when its radicle extended at least 0.5 mm out of the seed. When no germination was observed in all treatments at least in five consecutive days, the germination was considered completed, as recommended by Ranal and Santana (2006). Finally germinated seeds used for biochemical analysis.

### *Hormones analysis*

ABA and GA concentration were determined using high-performance liquid chromatography (HPLC), as

described by Yokota *et al.* (1994) and Pearce *et al.* (1994) with three replications. Briefly, two grams of the ground seeds from each treatment were homogenized in a 40 ml of 80% methanol (MeOH). This reaction solution was shaken in a shaker and kept in dark for 16 h at 4 °C. Aqueous part of the samples were centrifuged and filtered. The residues in the filtered were diluted with phosphate buffer. Filtered sample was injected to the HPLC (Waters) equipped with a UV-visible detector C18 column set at 257 nm. The mobile phase was a mixture of solvent 50:50 (v/v) methanol 80%:acetic acid 0.1 N. Samples and the column were thermostated and the eluent flow was at 0.8 ml min<sup>-1</sup>. The ABA and GA<sub>3</sub> standards were purchased from Sigma Chemical Company. The ABA and GA<sub>3</sub> concentrations were expressed in μmol g<sup>-1</sup>.

#### *Enzymes assay*

The Amylase activity was measured according to a modification of Bergmeyer *et al.* (1983). Samples were incubated with sodium phosphate (10 mM) and sodium chloride (3 mM) at pH= 5.5 and 25 °C. Aqueous was added to 3, 5-dinitrosalicylic acid (10 mg ml<sup>-1</sup>), sodium hydroxide (4 mM) and potassium sodium tartrate (30 mg ml<sup>-1</sup>) and kept in boiled water. The absorbance of samples was measured at 546 nm in UV/Visible spectrophotometer. Amylase activity was expressed as μmol gFW<sup>-1</sup> min<sup>-1</sup>.

POD activity was determined according to Hemeda and Kelin (1990). Enzyme extract of POD was prepared by first freezing a weighed amount of seed tissue (0.5 g) in liquid nitrogen followed by grinding with 1 ml extraction buffer [50 mM potassium phosphate buffer (pH=7), containing 0.5 mM Ethylenediaminetetraacetic acid (EDTA) and 2% Polyvinyl Polypyrrolidone (PVPP)]. Homogenate was centrifuged for 10 min at 15,000 g and the supernatant used to determine enzymes activity. POD activity was assayed by measuring spectrophotometrically the formation of guaiacol in 1 mL reaction mixture of 495 μL guaiacol, 495 μL H<sub>2</sub>O<sub>2</sub> and 10 μL crude enzymes. The increase in absorbance was recorded by the addition of guaiacol at 470 nm for 2

minutes (e, 26.6 μM<sup>-1</sup> cm<sup>-1</sup>). POD activity was expressed as μmol gFW<sup>-1</sup> min<sup>-1</sup>.

#### *Biochemical compounds assay*

Soluble sugars (glucose and fructose) were measured following the method of Irigoyen *et al.* (1992), with appropriate adaptations being made for our plant material. A sample of 0.5 g fresh seed matter was homogenized twice with 95% ethanol (v/v) and washed with 70% ethanol (v/v), followed by centrifuging at 3,500 ×g at 4 °C for 10 min. Glucose and fructose were determined in the resulting supernatant by spectrophotometry at 650 nm, using the colourimetric assay with anthrone reagent. Glucose and fructose concentrations were expressed as mg g<sup>-1</sup>.

Total protein content of samples was estimated based on Bradford (1976) method at 595 nm. Bovine serum albumin was used as standard and total protein content was expressed as mg g<sup>-1</sup>.

Proline was determined according to the method described by Bates *et al.* (1973). Approximately 0.5g of fresh or frozen seed material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman's No. 2 filter paper. Two ml of filtrate was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm. Appropriate proline standards were included for calculation of proline in the sample.

#### *Statistical analysis*

A two factorial experimental design with three replications was used where varieties and sampling times were the factors. Data were analyzed as a combined experiment model by SAS software (Ver. 9.1 2002–2003, SAS Institute, Cary, NC, USA). Before analysis of variance, data were tested for normality and homoscedasticity using the Kolmogorov–Smirnov and Cochran tests,

respectively. Turkey's test at  $P \leq 0.01$  was calculated to compare differences between means when F values were significant.

## Results and discussion

### Changes in ABA and GA<sub>3</sub> concentration

The results showed that the simple effect of stratification and varieties was significant on ABA concentration but their interaction has no

significantly affect (figure 1). ABA concentration significantly decreased from 53.61 to 39.89  $\mu\text{mol g}^{-1}$  after germination (figure 1a), also K-66 and Rabie varieties had the highest ABA concentration than others (figure 1b). Following stratification and germination GA<sub>3</sub> concentration significantly increased (figure 2). After germination Rabie variety had the highest GA<sub>3</sub> concentration (74.57  $\mu\text{mol g}^{-1}$ ).

**Table 1.** Main effects  $P$ -values and means for amylase and POD activities, glucose, fructose, protein and proline content in seed of six domesticated almond varieties in response to stratification.

		Amylase ( $\mu\text{mol gFW}^{-1} \text{min}^{-1}$ )	POD ( $\mu\text{mol gFW}^{-1} \text{min}^{-1}$ )	Glucose ( $\text{mg g}^{-1}$ )	Fructose ( $\text{mg g}^{-1}$ )	Protein ( $\text{mg g}^{-1}$ )	Proline ( $\text{mg g}^{-1}$ )
Time (T)		***†	***	***	***	***	***
Cultivars (C)		***	***	***	***	***	***
T × C		***	***	***	***	***	***
Before germination	Marcona	5.5 ± 0.43 e	2.7 ± 0.34 d	10.2 ± 1.19 e	3.7 ± 0.38 e	6.7 ± 0.29 cd	11.3 ± 1.05 g
	Rabie	8.1 ± 0.40 cd	4.4 ± 0.35 c	16.8 ± 0.81 e	5.9 ± 0.33 de	5.2 ± 0.17 def	21.1 ± 1.53 ef
	Supernova	8.2 ± 0.40 cd	4.1 ± 0.29 cd	17.4 ± 0.83 e	6.0 ± 0.32 de	6.6 ± 0.28 cd	18.8 ± 1.67 efg
	Perlis	6.8 ± 0.29 de	3.4 ± 0.26 cd	12.1 ± 0.97 e	3.9 ± 0.35 e	5.3 ± 0.20 def	15.0 ± 1.46 fg
	D-99	6.7 ± 0.32 de	3.5 ± 0.23 cd	12.3 ± 0.94 e	4.2 ± 0.26 de	6.9 ± 0.18 cde	17.0 ± 1.62 fg
	K-66	7.5 ± 0.36 cd	3.8 ± 0.29 cd	14.6 ± 0.86 e	4.7 ± 0.21 de	6.1 ± 0.28 cde	17.8 ± 1.70 fg
After germination	Marcona	5.6 ± 0.41 e	3.0 ± 0.38 cd	17.4 ± 1.76 e	10.3 ± 0.68 d	3.3 ± 0.32 f	17.5 ± 1.78 fg
	Rabie	10.9 ± 0.35 b	9.5 ± 0.43 a	51.6 ± 2.89 c	34.4 ± 1.60 b	18.2 ± 0.90 a	65.2 ± 2.79 b
	Supernova	13.6 ± 0.46 a	7.5 ± 0.47 b	84.6 ± 5.46 a	48.7 ± 2.75 a	11.3 ± 0.92 b	49.3 ± 1.19 c
	Perlis	5.7 ± 0.40 e	4.2 ± 0.30 c	18.2 ± 2.12 e	10.2 ± 0.65 d	4.3 ± 0.32 ef	34.8 ± 2.51 d
	D-99	8.7 ± 0.56 c	6.5 ± 0.51 b	37.5 ± 2.37 d	24.1 ± 2.08 c	7.6 ± 0.52 c	25.6 ± 3.01 e
	K-66	12.1 ± 0.18 ab	7.6 ± 0.49 b	65.5 ± 3.57 b	46.7 ± 3.03 a	11.6 ± 0.92 b	74.8 ± 2.92 a

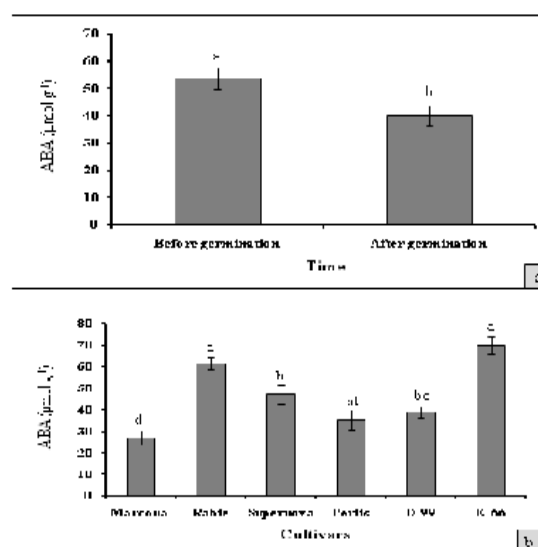
† NS, \*, \*\*, or \*\*\* indicates non-significant or significant at  $P \leq 0.05$ , 0.01, or 0.001, respectively.

‡ Means within each column followed by the same letter are not different at  $P \leq 0.01$  based on LSD test.

Similar results were obtained with other plants such as *Arabidopsis thaliana* (Ali-Rachedi *et al.*, 2004), *Fraxinus excelsior* (Blake *et al.*, 2002) and *Pseudotsuga menziesii* (Corbineau *et al.*, 2002). Germination is a very complex physiological process that is controlled by a range of developmental and external cues. Genetic and physiological studies have shown the important role played by plant hormones in regulating seed germination (Jacobsen *et al.*, 2002; Koornneef *et al.*, 2002).

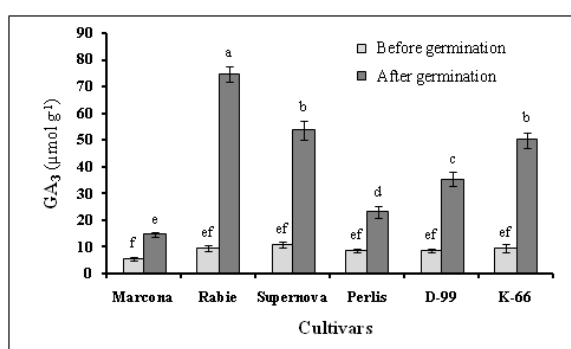
Many studies have shown that breaking dormancy by after-ripening, stratification and dark is strongly correlated with hormone-balance, a decrease of ABA and increase of GAs in seeds (Gubler *et al.*, 2005). ABA is a positive regulator of dormancy induction and most likely also maintenance, while it is a negative regulator of germination. GA releases

dormancy, promotes germination and counteracts ABA effects (Kucera *et al.*, 2005).



**Fig. 1.** Changes in ABA concentration following stratification and germination (a) in seed of six domesticated almond varieties (b).

Stratification commonly used to promote and synchronize seed germination, caused GA3ox1 mRNA expression in the entire radicle and in the aleurone layer, and an increase in bioactive GA content (Yamauchi *et al.*, 2004). The stimulatory role of GAs on seed germination was explained by at least two different mechanisms. First, GAs induce certain hydrolytic enzymes to overcome the mechanical resistance imposed by the endosperm and seed coat (Debeaujon and Koornneef, 2000). Second, GAs increase the growth potential of the embryo, as indicated in Arabidopsis (Debeaujon and Koornneef, 2000; Zhang *et al.*, 2010).



**Fig. 2.** Changes in GA<sub>3</sub> concentration in seed of six domesticated almond varieties following stratification and germination.

#### Enzymes activity

As the table 1 illustrates, amylase and POD activities affected by stratification as their activities significantly increase after germination. It was found that Supernova and K-66 had the highest amylase activity (13.6 and 12.1 µmol gFW<sup>-1</sup> min<sup>-1</sup> respectively), whereas the highest POD activity was observed in Rabie (9.5 µmol gFW<sup>-1</sup> min<sup>-1</sup>) as compared with other varieties.

Many reports have been on the changes in the enzymes activities during and following the dicot seed germination and the relationships between these enzymes and germination (Yan-fang *et al.*, 2007). Amylases are one of the important hydrolytic enzymes which are found in plants. During seed germination, amylase in the aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoots (Kaneko *et al.*, 2002).

Previous physiological and biochemical studies have revealed that amylase expression in the aleurone layer occurs as follows. First, active GA biosynthesis commences in the embryo, and the GAs are transported from the embryo to the aleurone layer (Fincher, 1989). Active GAs trigger the expression of amylase at the transcriptional level through the induction of a positive trans activating factor for amylase transcription. Then, amylase is secreted from the aleurone layer into the endosperm to catalyze the hydrating reaction of stored starch (Gubler *et al.*, 1995). On the contrary, ABA blocks the production of α-amylases and suppresses seed germination (Xie *et al.*, 2007).

The accumulation of reactive oxygen species (ROS), such as O<sup>2-</sup> and ·OH (Schopfer, 2001; Müller *et al.*, 2009) occurs during seed germination. The ROS production by germinating seeds has often been considered as a negative effect that might affect the germination process. For this reason, anti-oxidative mechanisms have been regarded as being of particular importance for the success of germination (Tommasi *et al.*, 2001; Barba-Espin *et al.*, 2010).

Antioxidative enzymes such as superoxide dismutase (SOD), POD, and catalase (CAT) are considered to be the main protective enzymes engaged in the removal of free radicals and activated oxygen species (Devi and Prasad, 2005). Peroxidase activity has also been shown to increase during germination and early seedling growth in many plant species (Prodanović *et al.*, 2007).

#### Changes in soluble sugars

Changes in glucose and fructose content displayed similar patterns (table 1). It was found that following stratification and germination soluble sugars content significantly increased. Among varieties, Supernova had the highest glucose and fructose content (84.6 and 48.7 mg g<sup>-1</sup> respectively) after germination.

Similar results were reported by Satyanarayana *et al.* (2011) and Tian *et al.* (2010). Carbohydrates, especially starch represent the major reserve substance in most seeds (Bewley and black, 1994).

During germination, mobilization of storage carbohydrates occurs, especially after radicle emergence. Once the high molecular weight carbohydrates are mobilized, they are converted to soluble forms such as glucose, fructose and sucrose, which are readily transportable to sites where they are required for growth. The soluble carbohydrates also seem to play an important role in osmotic regulation of cell during germination (Prado *et al.*, 2000).

#### *Changes in protein and proline content*

Germination, varieties and their interaction ( $P \leq 0.001$ ) had significantly effect on protein content (table 1). It was found that after stratification and following germination, protein content significantly increased in Rabie, Supernova, D-99 and K-66 varieties, whereas Marcona and Perlis showed significantly decreasing in protein content.

The increase in protein content after germination was also found by Mubarak (2005) and Rumiya *et al.* (2012). Plant growth regulators (PGR) such as ABA and GA play a vital and deciding role in seed development and germination. Proteins involved in the regulation of different PGRs exhibit differential expression during seed germination. In *Podophyllum* increase in the levels of GA biosynthetic proteins and decrease in the levels of ABA induced proteins in germinating seeds confirmed that the seeds germinate when GA levels exceed ABA levels (Dogra *et al.*, 2013).

Moreover, decrease in protein content during seed germination is in accordance with previous observations of Giami *et al.* (1999) and Satyanarayana *et al.* (2011). The loss of proteins from the cotyledons could be due to the transport of amino acids to the growing axes or to respiratory loss, or it might result in the accumulation of free amino acids in the cotyledons (Satyanarayana *et al.*, 2011).

Proline content significantly ( $P \leq 0.001$ ) affected by Germination, varieties and Germination  $\times$  varieties interaction (table 1). Proline content significantly increased after germination as the highest amounts

were obtained in K-66 and Rabie varieties (74.8 and 65.2 mg g<sup>-1</sup>).

As mentioned above, ROS content (as main abiotic stress agents) significantly increase during seed germination. It has previously emphasized the apparent importance of proline synthesis from glutamate in the modulation of plant cellular redox potential under conditions of abiotic stress and during cell division and differentiation (Hare *et al.*, 2001). Though a minor constituent of the amino acid pool, proline as an osmolyte act as cellular protector in several plant species in response to abiotic stress and scavenge ROS (Ashraf *et al.*, 2007). Accumulation of cellular proline may be due to increased synthesis and decreased degradation under a variety of stress conditions (Kavi-Kishor *et al.*, 2005). Increase in proline content may be due to the stimulation of Delta-1, Pyroline 5-carboxylate synthase (P5CS) responsible for proline biosynthesis under stressed conditions (Siripornadulsil *et al.*, 2002).

#### *Conclusion*

Our results revealed that stratification may be necessary to reduced ABA concentration and induce accumulation of GAs, and/or to increase the sensitivity of seeds to GAs, thus resulting in removal of dormancy. After stratification and following germination, amylase and peroxidase activities, glucose, fructose and proline content significantly increased, while change in protein content depends on domesticated almond varieties. Moreover, it was found that Rabie, Supernova and K-66 varieties showed the highest hormones, enzymatic activities biochemical compounds content after stratification and following germination.

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