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ORIGINAL ARTICLE

The effects of free amino acids profiles on seeds germination/dormancy and seedlings development of two genetically different cultivars of Yemeni Pomegranates

Alhadi Fatima A.[1], Adnan A.S. AL-Asbahi[2*], Arif S.A. Alhammadi[3], and Qais A.A. Abdullah[4]

[1] *Department of Biology (Plant Physiology-Ecology), Faculty of Science, Sana'a University Plant, Sana'a Republic of Yemen.*

[2*]*Department of Biology (Biotechnology and Molecular Genetics), Faculty of Sciences, Sana'a University, P. O Box 14686, Sana'a, Republic of Yemen, email:*

[3] *Department of Biology (Plant Genetics), Faculty of Science, Sana'a University Plant, Sana'a Republic of Yemen.*

[4] *Department of Biology (Fungal Microbiology), Faculty of Sciences, Sana'a University, Sana'a, Republic of Yemen.*

[*]*E mail: adnanasbahi@yahoo.com*

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Plant seeds used rely on a wide range of internal mechanisms and physio-chemical factors to ensure their germination under favorable environmental conditions. Most plant seeds have complex process of germination, including water, oxygen, temperature availability, genome-wide gene expression, signal transduction, hormones stimulations, inhibitors removal and catalytic protein synthesis. In addition, influences of seeds nutrient values such as, protein, lipids, sugars and free amino acids have a special importance. Regarding, seeds free amino acids. Discussion of these individual factors needs to be put in context of their role in germination processes. Regarding, free amino acids seed storage, there is limited information about their relevant functions in activation and/or deactivation of required metabolic mechanisms and interactive compounds involved in this process in commercial plant cultivars. Therefore, current study was aimed to determine the probable influence of free amino acid compositions of seeds on germination process of two different (*Punica granatum* L.) pomegranate cultivars including wild type Automi cultivar and edible Khazemi cultivar. In particular, we focused on the impact of amino acids contents variations on germination process and associated AAs compositional changes during various stages of germination and seedlings establishment. Amino acid analysis using HPLC detected all the essential and non-essential amino acids in the raw seeds of the studied cultivars, Automi and Khazemi along with AAs compositional changes occurred during different stages of seed germination. These AAs have been extensively analyzed in the context of their role in dormancy breaking capacities in plants species. Automi raw seeds are rich in Phe, that, is strongly related to ABA synthesis and hence might be responsible for the dormancy of Automi seeds, Khazemi raw seeds have sufficient levels of Arg, Glu and Met that have been reported to enhance seeds germination in plant, therefore Khazemi germination capacity was assumed to be regulated more or less by these AAs. In addition, changes in amino acid composition in the germinated Khazemi cultivar during various stages of seeds germination including imbibition, germination, and sprouts stages have been noticed to change in response with germination demands. This suggests that amino acids reserves in dry seeds are major determinant for germination capacity and germination behavior in the following steps of germination. The noticed particular AAs increase/decrease along the time course of Khazemi pomegranate germination till establishment of heterotrophic seedlings were used as cornerstones for elucidation and deduction of putative function and relevant biochemical pathways controlling initiation of seeds germination and seedlings developments. Based on publicly available databases of model plants and literatures surveys, we established correlations between prevailing AAs factors as biochemical parameters actively involved in seeds dormancy-breaking and germination process.

Key words: Amino acids (AAs), pomegranate seeds, germination, dormancy, arginine, HPLC

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Key words: Amino acids (AAs), pomegranate seeds, germination, dormancy, arginine, HPLC

Pomegranate (*Punica granatum* L.), is one of the first five cultivated fruit trees in the world (Ranade et al, 2009) belonging to punicaceae family (IBPGR, 1986). It grows in a wide range of geographical and climatic zones within dry, semi dry and moist areas worldwide, preferring area with warm temperatures and lots of sunlight (Morton, 1987 and Orwa et al, 2009). It has a high capacity for growth in a wide range of alkaline soils with a good drainage (Joy et al, 1998) and requires very little amount of water, nitrogen and other fertilizer (Shamseldin et al, 2010). Despite, it is grown mainly for its edible fruits, the other parts of the tree including bark, root bark, leaves, and leafy shoots are being used for various medicinal purposes (Langley, 2000; Singh et al, 2002; Kelawala et al, 2004; Fuhrman et al, 2005; Sumner et al, 2005 and Lansky and Newman, 2007). It is known as a native plant to the Middle East and South Asia (Narzary et al, 2009).

Since pomegranate cultivation is mostly rely on easily propagation by hardwood cuttings, seed propagation method is not being used in large-scale for pomegranate production (Olmez et al, 2007). Nevertheless, seeds are still required for the purposes of preservation and domestication of important plant species. Moreover, seeds propagation of wild types varieties is necessary for breeding programs and genetic studies for production of high competitive varieties with high fruit quality to be used for extension of cultivable lands (Jalikop and Sampath 1990). Therefore, wild types become more important for production of new bred varieties through artificial cross-pollination as pomegranate varietiesare mostly self-pollinating plants (Ghorbel et al, 1996). Generally, seeds of wild type species have some sort of deep dormancy preventing sprouting during dry and hot seasons (Koning, 1994) thereby increasing their survival from a single seed set (Baskin et al, 2001). Similarly, pomegranate wild types have shown dormancy problem, although seeds of commercial varieties commonly germinate very easily without the need for dormancy-breaking period (Rawat et al, 2010). Consequently, pomegranate wild types breeding programs are faced by seed dormancy barriers prohibiting breeders to use them as parents for establishment new high quality commercial varieties.

Generally seeds dormancy is attributed to a wide range of internal mechanisms, environmental factors and chemical stimulants including; a) dehydration of dry seeds due to water loose, b) state of food reserves as dense crystalline bodies (Berjak et al, 1996), c) physical barriers by impermeable seed coat to oxygen, and water (Barre, 1983), d) chemical inhibitors presented in the embryo (Al-Charchafchi et al, 1988), e) absence of growth promoters required for necessary metabolism of important organic compounds and/or f) less developed embryo (Baskin et al, 1992). In fact, seeds germination is a very complicated biological process not only restricted on the previous factors, but also involving many other biochemical and physiological reactions toward activation of metabolic enzymes and utilization or synthesis of wide range of chemical compounds occurs in seeds (Taraseviciene et al, 2009) including, imbibition of water, inactivation of growth inhibitors by heat and cold (Pedriali et al, 2010), washing of inhibitors and solutes away, induction of cell division and cell growth by growth hormones promotion (Jobea et al, 1981), changes in cell ultra-structure, proteinases enzymes activation, food digestion, (AAs) synthesis, RNA & protein synthesis stimulation (Rodrıguez et al, 2008).

The current background knowledge on positive regulation of germination mechanism and

associated metabolic pathways as well as functions of organic compounds involved in this process still unclear in a given variety but only share some similarities with other plant varieties with a few exceptions that include plant models such as, *Arabidopsis thaliana*, and *Medicago truncatula*. Recently, many studies have investigated germination mechanism in many plant varieties to determine the effect of some biologically active compounds on plant seeds germination process including, seed coats, inhibitory factors, proteins, lipids, carbohydrates, phenolics and lignins contents and distributions in dormant and non-dormant seeds (El-Maarouf-Bouteau and Bailly, 2008). Regarding free AAs seeds storage, no detailed studies have reported their individual impacts on seed germination and development in higher plant including pomegranate species. Thus, our present study was designed to examine the changes in free amino acid contents and their change rates on germination of two genetically different pomegranate varieties (Khazemi edible variety and Automi wild type) in order to invest their potential in seed germination and/or seed dormancy processes. In addition, comparison between those varieties of pomegranate might provide us with knowledge basis of dormancy/germination phenomena related to seed storage free (AAs) contents and compositional changes in the course of seeds germination (Rawat et al, 2010) so that, we can evaluate the possible role and chemical pathway of each amino acid as well as interactive process involving a number of these (AAs) during various stages of germination and tissue development in pomegranate.

MATERIALS AND METHODS

Description of plant materials and local growth condition:

The Yemeni pomegranates two varieties used in the current study are; 1) Automi shrub wild variety that has a low yield and low fruit quality, but it has a high toleration capabilities to adverse physiological and environmental factors. It is grown in Automah region of Dhamar governorate in the southern part of Sana'a, at elevation up to 2,500 meters (m) upper sea level. Dhamar climate is generally characterized by hot during the day, and average temperature equal to 27.5 °C, but it is very cold during nights of winter months frost. Its annual rainfall range between 400 and 500 millimeters (mm) concentrated exclusively in the summer months. 2) Khazemi is a commercial selected edible variety known as the highest yield and fruit quality over all other Yemeni pomegranate varieties. It is grown in Sa'da governorate located in the northern part of Yemen on the border with Saudi Arabia at elevation up to 1,800 meters m upper sea level. Average temperature is about (26.7°C), Annual rainfall ranges from 300 to 500 millimeters (mm). Accordingly, both regions can be defined as sub tropical tract of Yemen which can be regarded as optimal regions for pomegranate cultivation worldwide (Morton, 1987) and there is no significant differences in prevailing conditions between the two regions (figure 1).

Seeds collections and storage:

The fruits were collected during Fall 2008. Seeds were hand extracted from the surround membranes, washed with a disinfectant water for removal of fruits residues and red juice. Finally, seeds were left uncovered at room temperature for a short period for drying before they in and were stored dry in sealed plastic bags. The collected seeds of Pomegranate were dry stored at low temperatures to maintain embryonic viability for a long time according to (Scharpf and Parmeter, 1962). Before using them for germination in the current trials, seeds were stored for about 8 months

at low temperatures to undergo certain after-ripening changes (Crocker and Barton 1953).

Surfaces sterilization and leaching:

Pomegranate seeds were firstly, rinsed in 95% Ethanol solution for 1 minute. Secondly, they were washed three times by sterile water. Then, they were sterilized in 25% bleach (chlorox) for 5 minutes and were washed again three times with sterile water for removal of growth inhibitors from seeds.

Seed Vernalization and Imbibition:

For breaking of seed dormancy, pomegranates seeds were per-chilled at 4°C for 10 days under dark conditions according to (Riley, 1981 and Baskin et al, 2001). 15 seeds in three replicates for each studied varieties that were transferred with a sterile tweezers into 20 centimeter (cm) sterile dishes of a 3 cm deep, lined with four sheets of watt-man paper towels to keep continual moisture around seeds. For ensuring water uptake require for seed imbibition and sprouting, seeds were moisten daily with sterilized water. The seed coat analysis for permeability to water were done to examine the presence of physical germination barriers in both varieties by noticing imbibition rates occurrence that were scored by subtracting seeds weight before and after imbibition.

Germination essay and growth conditions:

Seeds were transferred to plant growth chamber 'Bio-Gen' growth chamber, at of 25°C, for the period up to 12 h light and 12 h dark. Germination was also performed in triplicates. The germinated seeds of both pomegranate varieties were allowed to grow under these conditions for 40 days with continual watering.

Total free amino acid extraction from plant tissues:

The triplicate samples of pomegranates tissues from each variety were harvested and analyzed according to the schedule listed in (table 1). Samples were blotted dry with filter paper, weighed and immediately put into liquid nitrogen in a mortar for stopping biochemical processes. The control sample used was dry seeds of the two varieties. Finally, samples were stored at - 80°C to be ready for the next analytical experiments. The weighted samples were ground with a pestle and mortar till turned fine powder. Free (AAs) were extracted from the raw seed, imbed seeds, roots and shoots fine powders (100 mg from each sample) with 5% Trichloroacetic acid (TCA)/0.05 M HC1 solution containing 1,7-diaminoheptane and Nor-leucine as internal standards. The homogeneous suspension was centrifuged at 2500 g for 15 min. The supernatant were used directly for amino acid analysis by High-performance liquid chromatography (HPLC).

Separation and Determination of amino acids* (AAs) *by HPLC:

Separation and determination of free AAs of pomegranate extracts were done by (HPLC) in which, gradients and run time were $1 ml min^{-1}$ 100% methanol and $1 ml min^{-1}$ 90% acetonitrile. Each sample injection volume was $5\mu l$. (AAs) photometric detections and measurements were done by light fluorescence with automatic analyzer of (AAs) coupled to the HPLC column filled with fluorescent materials, ionite ostion LGANB. The colorimetric detection by HPLC is done for amino acid quantification by internal standard method according to (Villanueva et al, 2000). (AAs) were detected using a Micro mass Triple Quadrupole Quattro Ultima mass spectrometer (Waters, Milford, MA, USA) using an electrospray positive mode, 3.0kV capillary voltage, 25V cone voltage, 120°C source temperature, and 300°C desolvation temperature.

Statistical analysis:

The statistical analysis for testing amino acid concentrations variance used in our study was student T-test to show significant difference in the amounts of these AAs within and among germinated and non-germinated pomegranate varieties. Concentration determination of amino acid composition of the samples was made in three replicates and the data presented here are means of three replicates.

Bioinformatics databases analysis and application:

The AraCyc, publicly available data of the model plant *A. thaliana* amino acid metabolism which is the first plant metabolism database tools (Mueller et al. 2003) at (http:// www.arabidopsis.org/tools/aracyc/) along with another alternative databases covering other organisms at (http://www.genome.jp/kegg/) were used as a platform source for obtaining information of AAs metabolic pathways for creation of pathway database. Then created pathways were manually predicted and then verified on the light of major metabolic data surveyed in literature as well as resulted data presented in this study in order to validate them and then placing metabolite components within their hypothetical metabolic context in pomegranates tissues.

Table 1. Summary of tissue samples collected from the two cultivars, Automi and Khazemi, of Yemeni pomegranates at various harvesting times for (AAs) extraction and HPLC analysis.

Dry seeds	Automi + Khazemi	Samples were collected from dry seeds, before vernalization step
Imbed seeds	Automi + Khazemi	Samples were collected after 20 days of seeds imbibition (five days before initiation of seeds germination)
Roots	Khazemi	Samples were harvested after 2 and 10 days of germination respectively
Shots	Khazemi	Samples were harvested after 2 and 10 days of germination respectively

Table 2. Individual amino acids concentration correlates changes within and between Khazemi commercial cultivar and Automi wild type cultivar of Pomegranate measured at various stages prior to, during and after initiation of seed germination. Concentrations were measured in picomoles per milligram fresh weight samples.

Amino acid	Automi raw seeds	Automi Imbed seeds	Khazemi raw seeds	Khazemi 2-days roots	Khazemi 10-days roots	Khazemi 2-days shoots	Khazemi 10-days shoots
Asp	54.389	17.923	166	107.7487	36.875	140.2	5.8622
Ser	41.309	14.703	122.36	473.3606	44.436	500.68	45.39961
Glu	198.85	67.356	932.38	172.0975	124.11	404.14	142.7977
Gly	87.64	23.196	66.935	16.01268	4.8422	13.895	8.842364
His	313.29	42.288	83.922	2774.154	124.68	3307.7	85.55592
Arg	102.32	13.845	2500	90.88256	3.0252	3102.1	5.634341
Thr	75.872	15.087	88.433	148.948	35.488	275.26	20.06783
Ala	29.653	15.765	153.36	40.23759	23.877	33.956	22.44474
Pro	18.298	8.6475	37.404	295.7808	73.148	412.68	104.191
Tyr	46.098	23.984	47.964	107.3104	10.689	1014.9	176.5832
Val	24.347	8.298	40.987	76.87981	11.673	120.09	2.761037
Met	127.39	47.468	270.75	1.156211	0.1927	7.353	1.395723
Lys	10.982	4.677	34.665	25.62487	24.248	119.11	14.00266
Leu	12.389	3.2423	24.337	40.29559	3.3731	97.621	2.510207
Ile	67.349	23.462	130.39	14.76426	4.9658	107.61	4.706139
Phe	495.63	9.987	43.087	4.742657	0.1979	69.861	6.216318

Figure 1. Yemeni general map representing two collection regions of Automi cultivar from Automah region located in Dhamar governorate and Khazemi cultivar from Sa'da governorate.

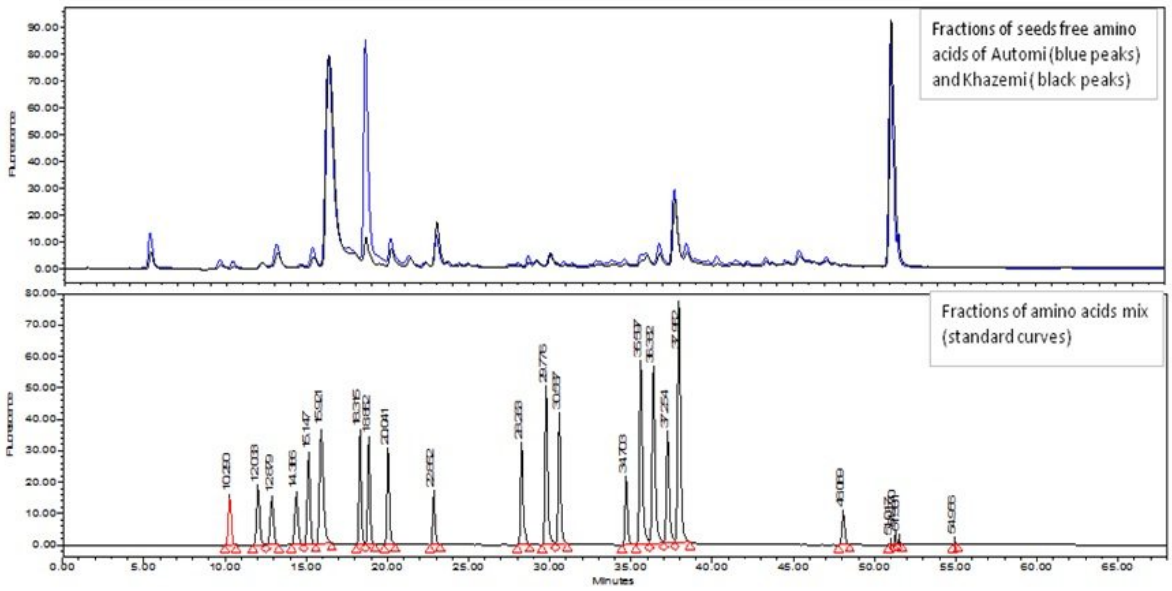

Figure 2. Chromatogram of standard amino acid mix including nor-leucine (Norl; internal standard) with total concentration equal to (200 pmol), by HPLC. Amino acids (AAs) are ordered according to elution retention times as follow: Asp; 12.033, Ser/Asn; 12.879, Glu; 14.386, Gly 15.147, His/Gln; 15.921, Arg; 17.82656, Thr; 18.315, Ala; 18.315, NH3; 18.852, allo-Thr; 20.041, Pro 22.852, Tyr; 28.263, Val; 29.776, Met;30.687, Lys; 35.597, Isl; 36.382, Leu; 37, 254, Norl; 37.952 Phe; 48.069. Peaks with other retention times are unidentified non-protene amino acids. Upper peaks shows the fractions of samples free amino acids stated as; the blue peaks belong to Khazemi edible cultivar while, black peaks belongs to Automi wild type.

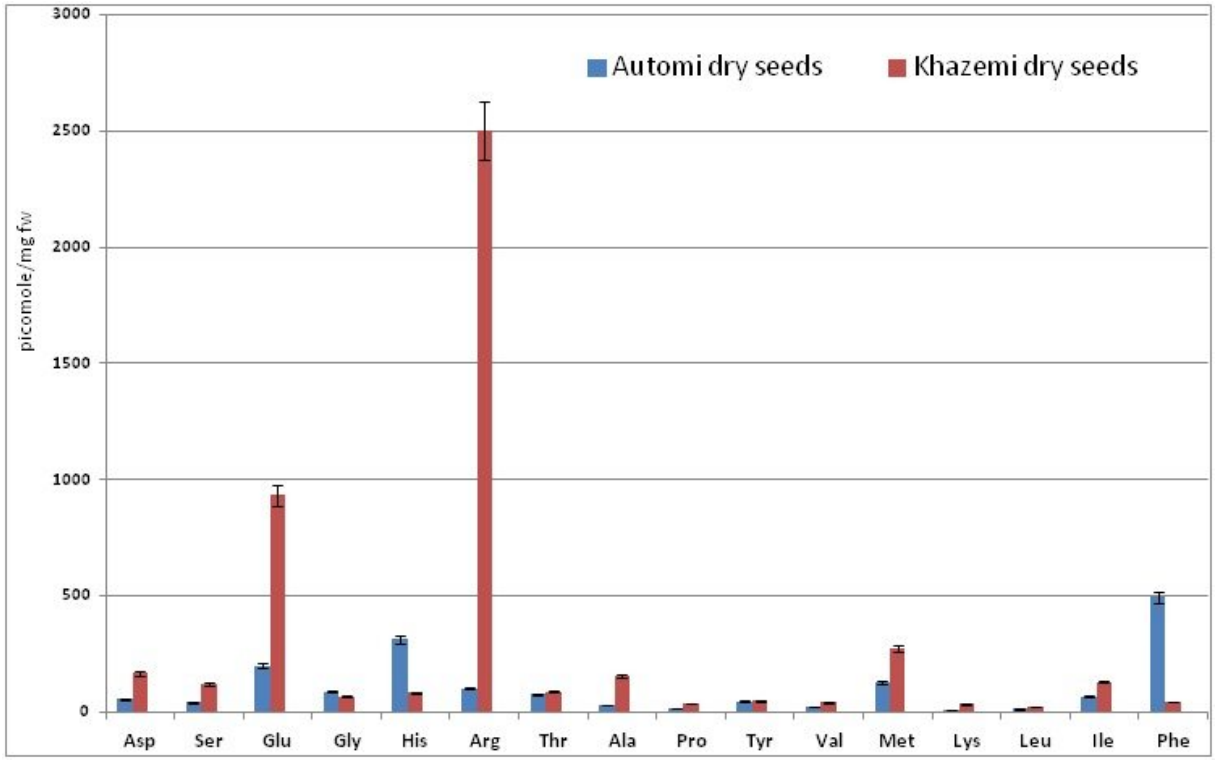

Figure 3. Composition of free protein amino acids in the raw seeds between the two pomegranate cultivars. Concentrations were measured in picomoles per milligram fresh weight samples. Standard deviation, three replicates. Mean ± SD of n = 3 to 4. *, P<0.05, Student's t test.

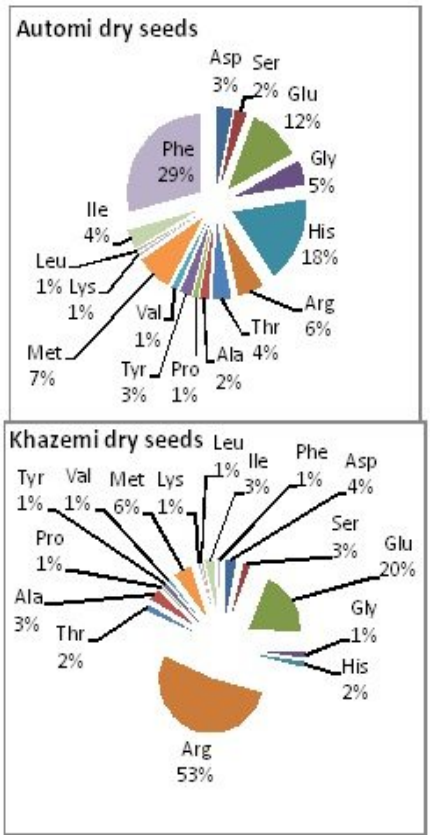

Figure 4. Amino acids (AAs) content percentage rates of the two different cultivars of Pomegranates raw seeds. A; is Automi wild type cultivar and, B; is Khazemi commercial cultivar.

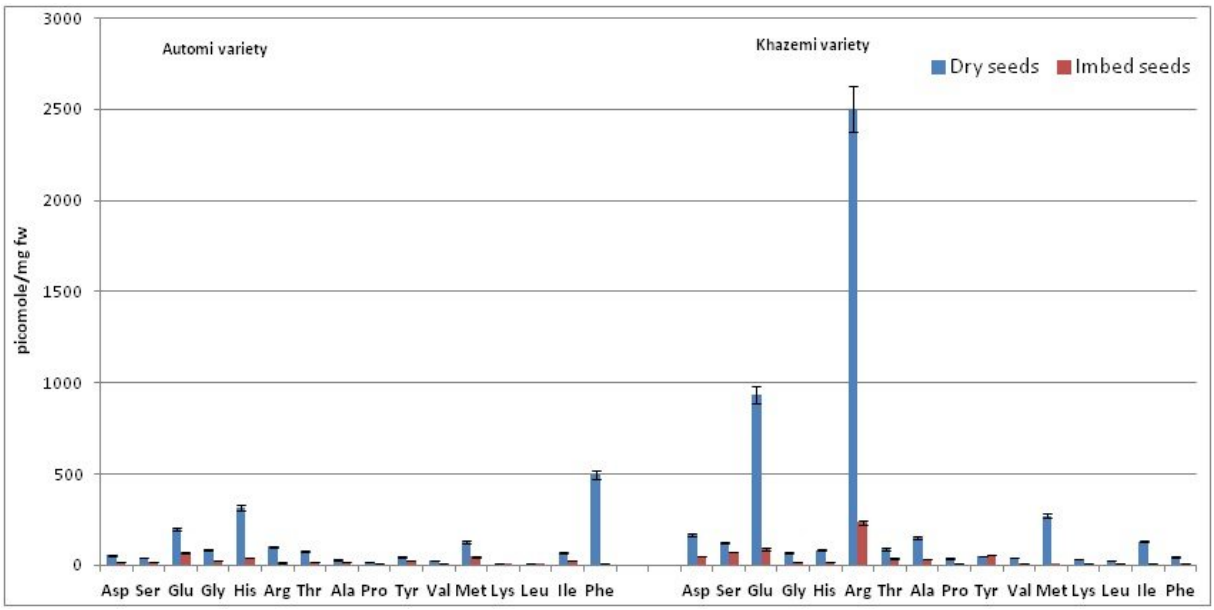

Figure 5. Amino acid compositional changes after seeds imbibitions of Automi wild cultivar (the left side), and Khazemi edible cultivar (the right side). Mean ± SD of n = 3 to 4. *, P<0.05, Student's t test. Concentrations were measured in picomoles per milligram fresh weight samples.

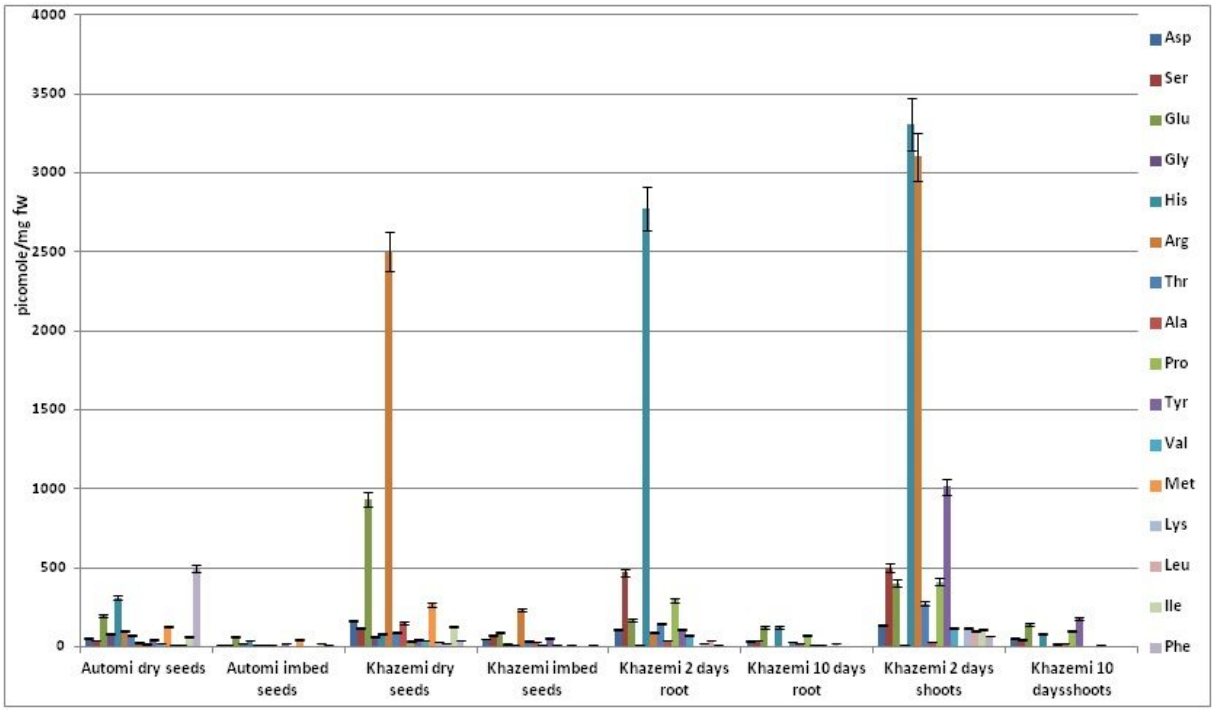

Figure 6. Time course analysis of free amino acids (AAs) variation rates during seeds germination process of Khazemi commercial cultivar and Automi wild type cultivar of pomegranate. Mean ± SD of n = 3 to 4. *, P<0.05, Student's t test. Concentrations were measured in picomoles per milligram fresh weight samples.

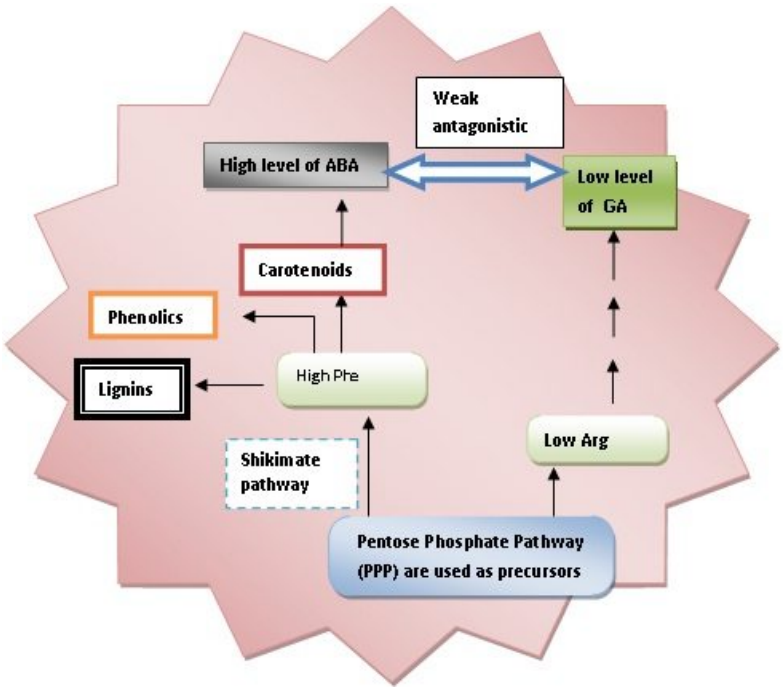

Figure 7. Illustration of possible antagonistic effect high concentration level of Phe on low concentration level of Arg presented in the dormant Automi raw seeds. High amount of Phe leads to synthesis a high amount of ABA, while low Arg level will result in low GA synthesis that is not sufficient to lower seed sensitivity to ABA, and hence fail to stimulate seed germination in Automi seeds.

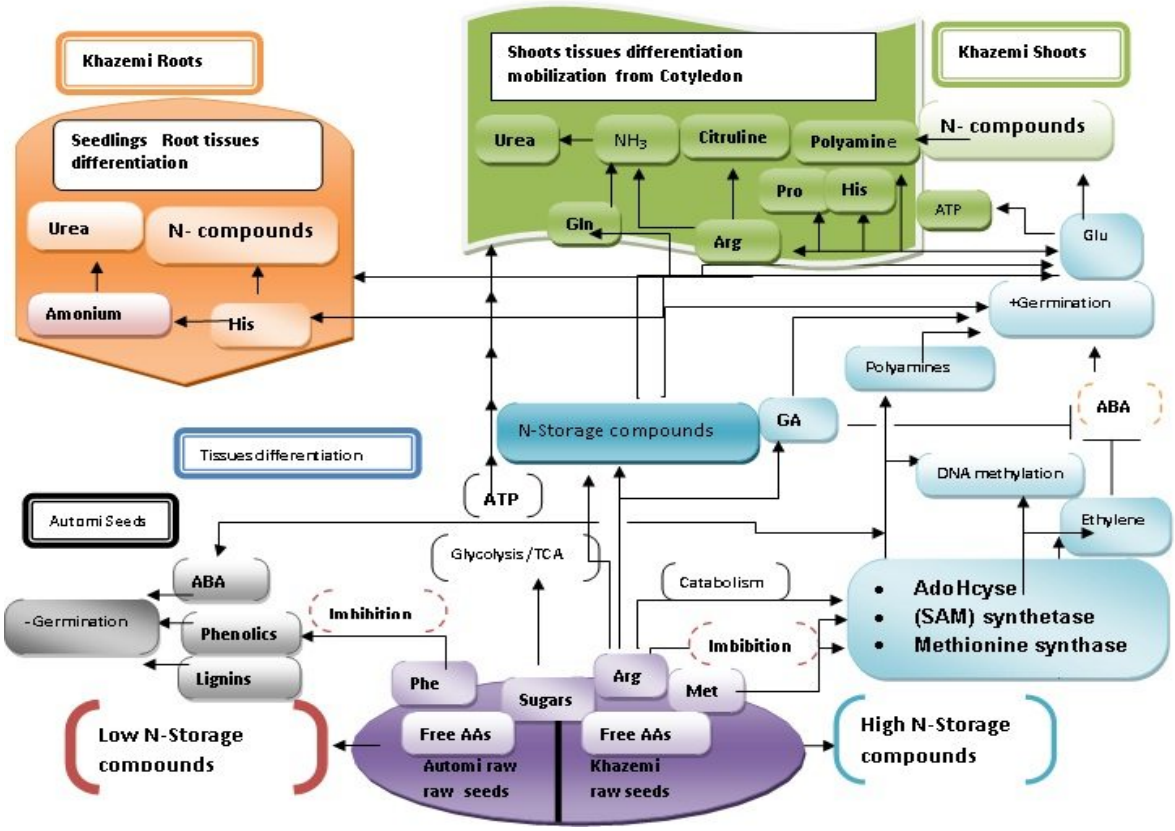

Figure 8. Model illustrating the hypothetical function of pomegranate seeds major amino acids and their probable metabolic routes during seeds germination and growth development processes.

RESULTS AND DISCUSSION

Seeds Germination/Dormancy Factors Analysis:

Our germination trials were designed to ensure the presence of all required factors involved in seeds germination of pomegranates including; (a) water; to allow seeds to take in significant amounts of water as seeds during desiccation period are often extremely dry. The weight differences of the imbed seeds relative to that of dry seeds indicates water passage into seeds in both varieties, which ensure that, seed coat constraint was removed by freezing the seeds at 4°C for 10 days. Consequently, air that is necessary for all plant seeds to allow them to respire aerobically to supply energy for growth when there is enough water. (b) optimum constant light and temperatures were supplied to enable enzymes to work optimally during imbibition and germination steps using artificial light in temperature controlled chambers. (c) The the state of the embryo and seeds of both Automi and Khazemi varieties were checked and were found healthy. This means that, there were no dead seeds or any other physical barriers responsible for any kind of seeds dormancy occurred in the present study.

Concentration and evolution of amino acids (AAs):

HPLC separation of free amino acids presented in various pomegranate tissue mentioned in (table 1) were identified based on symmetric comparison of our unknown samples with amino acid standards curves of the total amino acid standard mix composed of 200 p mole representing all 20 protein AAs. The results standard curves comparison with samples (AAs) fractions showed the presence of sixteen protein AAs along with NH4, allo-Thr, and a number of unknown components labeled as others (figure 2). The scored AAs of the two pomegranate varieties(Automi and Khazni) are; Alanine (Ala), Arginine (Arg), Aspartic acid (Asp), Glutamic acid (Glu), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tyrosine (Tyr), Valine (Val), while Cysteine (Cys) and Tryptophan (Trp) rottenly present as scarce in many plant species (Sulieman, et al, 2008 and Malaguti et al, 2001) are absent in pomegranates tissue samples. Similarly, Lys, Met, and Thr were noticed in limited amounts in seeds of many crop plants (Zhu and Galili, 2003). The unknown components that are non-protein constituents presented in seeds fraction are assumed to be metabolic intermediates in amino acid bio-synthesis such as, ornithine, homoserine) or in other primary metabolic pathways such as *S*-adenosyl methionine in methylation reactions as it was suggested by (Kuo, 1983).

Analysis of Seeds Germination Capacities of Automi and Khazemi pomegranates:

The major result found in the current study showed differences in germination capabilities between Automi and Khazemi under previously mentioned controlled conditions. Khazemi seeds started to germinate at the 26th day of imbibition and reached a maximum germination rate after 28 day where (100%) of the seeds were germinated while, Automi seeds failed to germinate during and after this period up to 40 days. On the other hand, free AAs pool found in the raw seeds were calculated by addition of measured concentrations of all free AAs that was estimated as; 1705.806 and 4742.974 (picomole/mg fw) in Automi and Khazemi respectively (Table). Generally, free amino acid content found in the raw seeds of Khazemin variety present in higher than that of Automi varieties by 4 folds. In addition, most of essential and non-essential free (AAs) rates were differed significantly between the two varieties. The major free AAs variability between and within two varieties were found in Arg, His, Glu Phe and Met. While Arg

followed by Glu are the major (AAs) constituting about 53% and 20% of the total free (AAs) found in the Khazemi raw seeds, they were found as low as 6% and 12% in Automi variety respectively. On the other hand, phenylalanine and His in Automi dry seeds was rated as 29% and 18% while they were 3% and 2% in Khazemi seeds respectively. The other amino acid existed in low rates ranged by 2-3% in both varieties. These findings fit with that found in loblolly pine (King and Gifford 1997), maritime pine (Allona et al, 1994) and eastern white pine, and Douglas fir (Feirer 1995). In case of proline, which is sensitive to heavy metal stress conditions (Ekpenyong and Borchers, 1982), it presents equal low rate as1% in both varieties(figure 3 and table 2).

The previous estimates and AAs rates differences shown in (figure 4) obviously reflect the presence of genetic differences between the two varieties in free amino acid levels. Generally, we expect that, high (AAs) content will contribute positively in germination of the Khazemi seeds, while low (AAs) content led to failure of Automi seeds germination. In particular, high levels of Arg, Glu, and Met presented in Khazemi raw seeds and a high level of Phe presented in wild type Automi variety are assumed to have direct effects on the different germination capacities between the two varieties tested in the current experimental trails. These finding is fitting with that found by (Baudoin and Maquet, 1999) who stated that, plant varieties with different genetic backgrounds showed different germination capability and seedling development under the same conditions that correlated with the metabolic evolution and composition of seeds free AAs. The overall attribution of such variability in germination behavior was assumed to be to genetic factors regulating metabolic evolution of protein (Khan and Minocha, 1984 and Koning, 1994) as well as mechanism of synthesis of selected AAs and amine compounds (Basha et al, 1980; Rozan et al, 2000). For example, these differences were also found in six peanut, *Hopea* and *Dipterocarpus* varieties that come to the conclusion that germination is mostly rely on seeds food storage accumulated in desiccation stage (Angelovici et al, 2010).

Growth Development Evaluation and Analysis:

Upon seed imbibition, amino acid analysis showed significantly fast reduction of all (AAs) imbed seeds of both pomegranates varieties(figure 5). This phenomenon has been noticed in many plant species including *Arabidopsis* model plant (Fait et al, 2006) and some lentil varieties, Rubatab, Nadi and Selaim, (Sulieman et al, 2008). This could explain the important role played by these amino acid in actively participation in germination initiation steps of pomegranates seeds. In fact, seeds imbibition is known as the most important phase for waking up plant seeds from their quiescent state, where it is usually associated with increase in water uptake, oxygen consumption, hexose sugars, and organic acids in rice seeds (Howell et al., 2006, 2009 and Nakamura and Ohtsubo, 2011). It is also known to be associated with alteration in transcript, protein, and hormone levels occur in order to set the stage for the later events (Weitbrecht et al, 2011) including seed storage proteins processing, assembly, and mobility (Miernyk and Hajduch, 2011).

Sprouting of Khazemi variety time course analysis, showed that, free AAs underwent two steps of changes (figure 6) in seedling tissues. During the first step, 2 days after tissue emergence, His, Ser, Pro and Glu were observed in high amounts in root, while they were found as either moderate or trace amounts in imbed seeds. In shoot tissues on the other hand, it was shown significant increase in His,

Arg, Tyr, Ser, Glu, Pro and Thr levels orderly ranked. There were also reasonable amount of other AAs were either present in moderate increase such as Asp, Thr, Val, Lys, Tyr, Leu, Ile, Phe, or scarcely exist such as Met, Ala and Gly at the same period. Prolonged germination period till 10 days (the second step) caused a dramatically decreasing in essential and non-essential AAs content in both, roots and shoots. The current findings of amino acid compositional changes fit with that previously found in sprouted lentil (Rozan et al, 2000), *Picea, Hopea* and *Dipterocarpus* variety (Huang and Villanueva 1993), coffee (Shimizu and Mazzafera 2000) and broccoli plants (Taraseviciene et al, 2009). In addition our results also supporting general findings stated that, total free amino acid content in the shoots is higher than that of the roots confirming previous findings by (Lesko and Simon-Sarkadi, 2002) on many other plant variety. The noticed fluctuation in amino acid concentrations within sprouting stage can be attributed to a second cycle of metabolism activation required for roots and shoots formation in which 1) hydrolysis of the storage protein as result of proteases activity rise with imbibition for energy production as well as cytoplasmic production of signaling molecules events (El-Mahdy and El-Sebaiy, 1985 and Khan et al, 2010), 2) mobilization of the protein from cotyledons to the newly emerged shoots and roots for sprout's growth (Rodrıguez *et al.* 2008), 3) metabolism activation of AAs bio-synthesis genes that were noticed in the early stage of sprouting, highly expressed and resulted in synthetic activity of these AAs (Ruuska et al., 2002 and Kanmegne et al, 2010).

In order to elucidate hypothetical impact(s) of major AAs such as (Arg. Glu, His, Phe, and Tyr) that prevail either of the two varieties, we excluded all environmental and physiological interference impacts on these total free AAs that resulted in the current differences in germination capabilities and morphogenesis between Khazemi and Automi varieties. Our discussion of putative function(s) will be justified on the light of gained results showed high level of compositional changes within and among various germination and development stages along the growing time course of both varieties (figure 6). To achieve this goal, firstly we included all possible biochemical pathways and relevant biochemical reactions involving AAs bio-synthesis, degradation, reactions and intermediates metabolites mentioned in related publicly available databases of model *A. thaliana* and other plant model organisms available online. The downloaded data were subjected to manual functional prediction and validation of single metabolic pathways according to our major findings in the present study and relevant literatures. Analysis of major AAs databases have also shown us high temporal compositional changes in germination/dormancy, growth and development of *A. thaliana* and other model plants in that, 1) Arginine is synthesized from glutamic acid, ornithine and citrulline, while its degradation result into ornithine and glutamic acid as well as proline and that require ATP source of energy. Arginine also involved in several reactions that result in urea. 2) In contrast Histidine competes arginine for the glutamic acid and glutamine as main substrate in their bio-synthesis route. 3) However, Phenylalanine and Tyrosine degradation is associated with glycolysis and needs energy and result in chrisomate synthesis that in turn needs glutamic acid to form phenylalanine again or to be converted to tyrosine or phenylalanine. Its degradation interfere with the glycolysis (pyruvate) and kreb cycle intermediates to form alanine and glutamate respectively. On the other hand, literatures surveys on the importance of these amino acid content in different germination

stages of various plant species and varieties have provided supporting findings to that of databases. Secondly, surveying information presented in the literatures to clarify existed switches in amino acid levels and possible cross-links associated activation/deactivation of corresponding cellular metabolism supporting germination/dormancy and tissues development to come finally with the possible metabolic routes as it is deducted in (figure 7 and 8).

The functions of AAs in germination and growth development of pomegranate seeds still largely unknown. Therefore the gained results of major AAs synthesis regulation and consequent compositional changes noticed during various germination stages were subjected to extensive analysis with respect to their response to the needs of seeds germination and development in each stage. Generally, AAs synthesis, degradation as well as their biochemical reaction occur via various branches of pathways.

Germination capability of plant seeds depends on desiccation stage (Angelovici et al, 2010) where concentrations of certain organic compounds including sugars and AAs increase to be involved in physiological factors and/or chemical promoters via various internal germination mechanisms (Footitt et al, 2002 and Lau and Deng, 2010) depending on plant species. In case of pomegranate species studied here, Phe is a dominating AA in Automi raw seeds while, Met was presented in a high rate in Khazemi raw seeds. Regarding other AAs factors previously mentioned that might contribute in seeds germination, we know that Abscisic acid (ABA) is the main inhibitor received the most attention in seeds dormancy imposition and maintenance (Bewley and Black, 1994). It is also known that ABA is regulated by Tyr which is required for inactivation of mitogen-activated protein kinase (MAPK) through its dephosphorylation (Luan, 2003 and Ghelis et al, 2008) and hence turning off ABA synthesis (Reyes et al, 2005). However, our trials haven't shown significant differences in Tyr concentration between Khazemi and Automi raw seeds so that, Tyr possible impacts on pomegranate seeds germination/dormancy can be excluded here. Regarding Phe, many others studies have found crosslinks between high level of Phe seeds dormancy factors. Phe was found as the highest level among the other AAs in the raw seeds of Automi variety, then it declined sharply in the imbed seeds. It was also found in a very low rate in Khazemi raw seeds as well as imbed seeds. We know that Sugar phosphates compounds produced in Pentose Phosphate Pathway (PPP) are used as precursors for shikimate pathway in which they are converted into aromatic (AAs) production like phenylalanine, tyrosine, and tryptophan via arogenate and chorismate (Herrmann and Weaver, 1999 and Tzin and Galili 2010a). These (AAs) serve as precursors for various secondary metabolites including carotenoids, phenolics, lignin, and hormones (Tzin and Galili 2010b). Knowing that dormant and non-dormant seeds highly vary varies in transcriptome levels and specificity during imbibition (Weitbrecht et al, 2011a). Also, it is found that ABA synthes in the imbibed state causes higher ABA contents (Nambara et al., 2010). Consequently plant hormones such as Abscisic acid (ABA), synthesized from carotenoids via 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) in the dormant Automi variety will act as an endogenous inhibitor for seeds germination along with its antagonistic effects on Gibberellins. In addition, lignification can slow germination by lowering germination inhibitors leaching within seeds (Weitbrecht et al, 2011a) as it is reported in wheat seeds coat (testa) (Debeaujon et al, 1988). Accordingly, production of ABA and other dormancy factors might provide a strong

evidence that, seeds dormancy of Automi variety is attributed to the presence of high level of Phe in raw seeds. Methionine on the other hand, is involved in synthesis of important enzymes such as Adenosyl homocysteinase (AdoHcyse) and methionine synthase which are involved in maintenance of DNA methylation in plants, along with S-adenosylmethionine (SAM) synthetase. SAM also responsible for controlling metabolism in the transition from a quiescent to a highly active state during *Arabidopsis* seed germination as well as the synthesis of polyamines and ethylene that promotes seed dormancy breaking and germination and counteracts ABA effects (Gallardo et al., 2002; Pawłowski, 2009 and 2010) confirming previous findings by (Manasis and Gaikwad, 2011) in *Simarouba glauca* DC. oil plant species. Moreover, Methionine can be used as precursor for 1-aminoeyclopropane-l-carboxylica acid oxidase (ACCoxidase) for ethylene synthesis which stimulates the germination of various dormant and non-dormant (Delatorre and Barros, 1996). It is obvious that, Phe and Met are might be the main AAs factors controlling seeds dormancy and seeds germination in pomegranate species respectively.

During imbibition, Arginine and Glutamate seems to play the main role in initiation of seeds germination of pomegranate. Starting with Arginine (4N atomes), presented in high rates in Khazemi raw seeds. Arg is a basic AA harboring the highest amount of Nitrogen (N) among other protein AAs (Canton et al, 2005). Catabolic activity of Arg during seeds imbibitions lead to transfer 2N out of 4 atoms to various forms of metabolite intermediates involved in bio-synthesis of nitrogenous compounds that are required for tissues differentiation and seedling growth (Jones and Boulter, 1968 and de Ruiter and Kollöffel 1983) such as, polyamines (putrescine, spermidine and spermine) and plant hormones (Gibberellic Acid; GA) (Hernandez-Sebastia et al, 2005 and Weitbrecht et al, 2011b). The sole biochemical pathway for polyamines bio-synthesis from Arg occur via decarboxylation of arginine and ornithine in plant seeds (Pukacka et al., 1991; Huang and Villanueva 1993a,b and Krawiarz, et al, 2008). On the other hand, plant hormones are known to serve as endogenous dormancy-breaking factors influencing initiation of seed germination and seedling morphogenesis (Bewley and Black, 1994; Brady and McCourt, 2003; Lau and Deng, 2010). In addition, they are classic signaling molecules, in the regulation of morphogenetic and adaptive processes along with Glutamatic acid as one of the novel signaling molecule many plant species (Lopez-Bucio et al, 2006). For example, high level of GA can antagonize inhibitory effect of Abscisic acid (ABA) in seeds germination (Pieruzzi et al, 2011). Also, Indole-3-acetic acid (IAA) is found in tobacco to trigger imbed seed germination upon 24 hours (Slavov et al, 2004). On the other hand, polyamines contribute in dormancy-breaking and germination initiation of many plant species (Szczotka et al., 2003; Minocha et al., 2004 and Swamy et al., 2004). They are also involved in cell division and the synthesis of macromolecules such as proteins and nucleic acids (Berta et al, 1997; Baudoin and Maquet, 1999 and Hajduch et al.,2005) and amino acid bio-synthesis (Rozan et al, 2000). This might explain dramatic decrease in Arg and His concentrations during imbibition stage in our trials where metabolic intermediates are necessary for seeds early germination. Accordingly, consequences of low Arg level was the failure in germination of Automi variety that can be attributed to 1) insufficient N, 2) low energy sources, 3) low ABA leaching activities during seed imbibitions of Khazemi and vice versa . This assume is supported by the trial conducted by (Light et al, 2005) who

found that heated AAs including Argine, gave high levels of germination when combined together is sugar solution prepared for imbibition of Grand Rapids lettuce seeds in the dark. It was assumed that, stimulation activity of these compounds was due to amino-carbonyl reactions of amino-containing compounds with Glucose. Similarly, Glu was found as scarce in the imbed seeds when compared to that in raw seeds that were noticed as much higher in Khazemi than Automi seeds in our trials which means that Glu is also highly required for pomegranate germination. Many studies on different plant species have reported that, Glu is the precursor of glutamine, arginine and proline (Buchanan et al., 2000) which are in turn the substrates for aspartate and alanine aminotransferases (AspAT and AlaAT) that are activated during imbibition and thought to participate in respiratory pathways (Rocha et al., 2010). In addition, Glu is subjected to rapid degradation into asparagine during germination in many legumes, supplying in part the energy requirements of their germination (Sivaramakrishnan and Sarma, 1956). This enzyme is also involved in re-assimilation of free ammonium within the plant into glutamate and glutamine is readily disseminated into plant metabolism, because these AAs donate nitrogen in the bio-synthesis of AAs, nucleic acids and other N-containing compounds from source organs to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth processes (Coruzzi and Zhou, 2001). Moreover, the previous events are enhanced by suitable energy sources provided by Glycolysis and Krebs cycles activities upon imbibition so that, they can facilitate early germination and energy-demanding processes increase for active metabolism resumes as well as for leaching growth inhibitors such as abscisic acid (ABA) out of the pomegranate seed (Pieruzzi et al, 2011).

During sprouting stage, inter-conversion activity between Arg/His and Glu reported in many plant species such as *M. truncatula,* seems to be within the most important physiological role contributing in nitrogen mobilization from raw and germinating seeds to sprouts till becoming nitrogen- and carbon-autotrophic seedlings (Glevarec et al, 2004 and Gaufichon et al, 2010). N-cycle in this process might be maintained by the three AAs inter-conversion via 2N atomes donations and ammonium (re)assimilation. In our trials, Arg and Glu were found among the highest rates over the rest of AAs in Khazemi dry raw seeds. However, after 2 days of sprouts emergence during early stages of pomegranate germinating, Glu concentration decreased while, Arg increased in shoots tissue only along with appearance of high level of His in both roots and shoots tissues. The noticed Arg 2 days shoots of developing pomegranate started to decline in the following days till 10 days of shoot formation that fit with the results gained by (de Ruiter and Kolloffel, 1983) in developing pea. Also our findings that, His concentration in roots during early stages of germination is prevailing which was similar to that found in rice (Taraseviciene et al, 2009) and two dipterocarp species (Huang and Villanueva, 1993a). Based on these observations, it can be assumed that, Glu is degraded into Arg and His as well as ammonium release in germinating seeds by glutamate/histidine dehydrogenase (Morot-Gaudry et al, 2001), while ammonium in is subjected for hydrolysis by urease (Todd et al, 2001), or incorporated glutamine as seen in Scots pine (Suarez et al. 2002). The fate of arginine in roots however, can be occurred through catabolism activity via the arginase-urease and ornithine aminotransferase pathways during the second step of

sprout development produces ammonium as it was noticed in loblolly pine (King and Gifford 1997). Therefore, these three AAs can be assumed to play same major role in shoot and root development during early stage of pomegranate sprouting. Histidine is particularly used for roots tissues. This is because of their N mobilization activities through both biochemical routes in this stage.

CONCLUSIONS:

Comparisons of amino acid levels between and within Khazemi and Automi pomegranate seeds varietiesand during seeds imbibitions, seeds germination initiation and early sprouting stages under same growth conditions resulted in remarkable compositional changes, depending on metabolism demands and growth requirements of each developmental stage. Seeds dormancy-breaking of pomegranate plant species might be controlled by Met and…. , while Phe might be the main AAs factors for dormant Automi seeds as its catabolism is part of ABA synthesis. It is concluded that genetic background differences reflected by amino acid contents variation is in relation with germination/dormancy capabilities. Significant change in AAs contents were observed upon seeds imbibition where metabolism is highly reactivated. This was associated with sharp decrease in AAs levels to switch on seeds germination that requires signal transduction, genes transcription, enzymes synthesis, energy metabolism, protease, protein synthesis, etc,. During seeds early germination, majority of AAs content decreas and the most dramatic changes occur during first 2 days of germination process, while after that period slightly decreases. Other AAs presented in high level of germinated seeds and low level in non-germinated seeds might be involved as precursors /stimulates in several different branches of metabolic pathways for synthesis of amides and other amino acid in seeds germination and plant development. In particularly, arginine, Glutamate that hve been reported in many metabolism networks for and synthesis of high content nitrogenous compounds along with other necessary organic compounds that might be involved stimulate cell division, tissue differentiation and plant development. The current study might provide progress in our understanding of physiological mechanisms and some AAs factors in pomegranate seeds germination/dormancy under controlled conditions.

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