



Optimizing Seed Germination and Growth of Seedlings in Persian Walnut

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ABSTRACT

The efficiency of conventional stratification methods for seed germination of walnut (*Juglans regia* L.), which uses long moist-chilling periods, is low. This experiment was conducted during winter to spring 2014 to optimize the seed germination of walnut ‘Chandler’ via changing growing media and removing seed mechanical dormancy. Fourteen treatments were tested. None of the seeds germinated without any treatment on shell or while they stored in manure. Germination percent of cracked seeds (CS) and cracked with toothpicks inserted seeds (CTS) without any stratification period reached up to 66.1% and 83.7% with mean germination time (MGT) of 0.73 and 0.98 day, respectively. Considering a 45-day cold storage period improved germination percentage of CS and CTS up to 88.3% and 94.1% with MGT of 1.02 and 1.12, respectively. Seedling growth parameters significantly increased after the cold storage period. Based on the results, mechanical dormancy was suggested as the main limiting factor for walnut seed germination. Therefore, rate and percentage of seed germination and seedling growth of walnut can be improved if the removal of mechanical dormancy is also considered in seed stratification.

Introduction

High nutritional value of walnut kernel makes it a popular nut all around the world. Nowadays grafting and budding have become appropriate methods in walnut propagation (Rezaee *et al.*, 2008; Farsi *et al.*, 2016 and 2018). Seedlings are usually used as rootstock for walnut cultivars. There are some problems for seedling propagation such as long stratification period requirement and relatively low germination rate of seeds. Seed germination and early growth of the seedlings are not homogenous, even after a long moist-chilling period (Einali and Sadeghipour, 2007; Lotfi *et al.*, 2009). Such problems reduce the propagation rate and uniformity of the plants (Sharma, 1984; Koyuncu *et al.*, 2000).

To achieve a proper seed germination and homogenous seedling growth, seed dormancy must be removed. Stratification, the standard method to overcome seed dormancy, involves storing seeds in moist medium for a period of time at a cold temperature. The effective temperature range for stratification is generally 0–10°C. A cold treatment at 5°C for 60 days suffices to overcome embryo dormancy in many plant species (Vahdati *et al.*, 2012). Protein mobilization and enzyme activation in response to such periods have been reported for several species including walnut (Einali and Sadeghipour, 2007). Protease and lipase enzymes

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have been shown to increase during chilling stratification (Vahdati *et al.*, 2012). Therefore, previous researchers mainly focused on seed stratification to break seed dormancy in walnut and related species. Parvin *et al.* (2015) showed that combination of stratification and gibberellic acid was effective in increasing seed germination percentage and rate as well as improving growth parameters of Eastern black walnut seedlings. The highest final germination percentage in their experiment reached only to 69.27%. Rawat *et al.* (2010) reported that stratification for 30 days at 5°C resulted in the highest germination percentage, root and shoot lengths, number of leaves and survival of pomegranate seedlings. The longest plumule, maximum collar diameter, highest shoot and root dry mass were recorded with 25 days of stratification. Koyuncu *et al.* (2000) reported 45-60% germination in some Persian walnut cultivars after natural chilling period. They reported that shell thickness has no significant effect on seed germination parameters and seedling early growth. Einali and Sadeghipour (2007) after evaluating chilling periods showed that Persian walnut seed needs at least a 40-day period of stratification in 5°C to reach germination up to 50%. They stated that cold stratification induced protein mobilization and amino acid accumulation in cotyledons and embryonic axes of walnut kernel.

Previous studies have shown germination of walnut seeds and related species are significantly low even after long moist-chilling stratification periods. It seems that there are other factors more than endogenous dormancy that control seed germination of this species. Current experiment was conducted to evaluate possible treatments of mechanical dormancy removing and sowing in different growing media to improve seed

germination and early growth of seedlings of Persian walnut.

Material and Methods

The experiment was conducted during winter to spring 2014 in the research greenhouse of Department of Horticulture, Aburaihan Campus of the University of Tehran in Pakdasht, Iran. Seeds of Persian walnut (*Juglans regia* L. 'Chandler') obtained from a 10 years old tree located in a commercial orchard located in Walnut Research Station, Hamadan, Iran. The pre-sowing seed treatments were included: (1) Soaking the seeds in a bucket of water for 48 hours and replacing water every 6 hours + sowing the seeds in perlite; (2) Soaking the seeds in a bucket of water for 48 hours and replacing water every 6 hours + 4 weeks chilling in a plastic bag at 4°C + sowing the seeds in perlite; (3) Soaking seeds in a bucket of water for 10 days and replacing water every day + 4 weeks chilling in a plastic bag at 4°C + sowing the seeds in perlite; (4) Soaking seeds in a bucket of water for 10 days (Vahdati *et al.*; 2009) and replacing water every day + cracking the seeds and sowing them in perlite; (5) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds and inserting a toothpick in the crack (Fig. 1) and sowing the seeds in perlite; (6) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds + 4 weeks chilling in a plastic bag at 4 °C and sowing the seeds in perlite; (7) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds and inserting a toothpick in the crack + 4 weeks chilling in a plastic bag at 4°C and sowing the seeds in perlite; (8) Soaking seeds for 48 hours in a bucket of water and replacing water every 6 hours + sowing the seeds in manure; (9) Soaking seeds for 48 hours in a bucket

of water and replacing water every 6 hours + 4 weeks chilling in a plastic bag at 4°C + sowing the seeds in manure; (10) Soaking seeds for 10 days and replacing water every day + 4 weeks chilling in a plastic bag at 4 °C + sowing the seeds in manure; (11) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds and sowing them in manure; (12) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds and inserting a

toothpick in the crack and sowing the seeds in manure; (13) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds + 4 weeks chilling in a plastic bag at 4°C and sowing the seeds in manure; (14) Soaking seeds for 10 days in a bucket of water and replacing water every day + cracking the seeds and inserting a toothpick in the crack + 4 weeks chilling in a plastic bag at 4°C and sowing the seeds in manure.

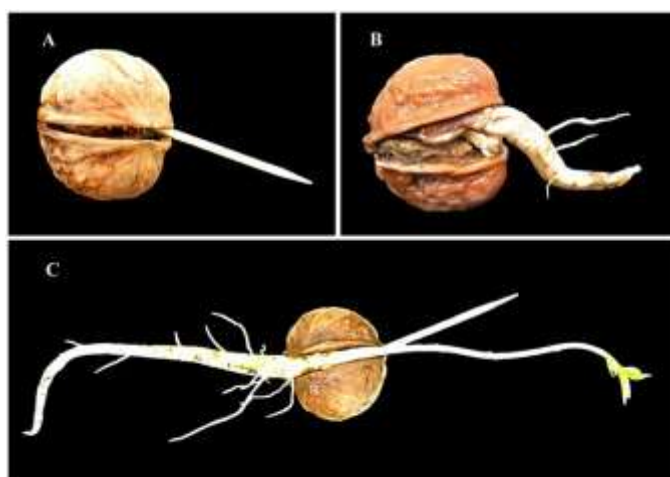


Fig. 1. Inserting a toothpick in the crack of walnut seed before sowing (A), and a germinated seed at 2 weeks (B) and 4 weeks (C) after sowing.

Five days after soaking, the seeds were treated with fungicide Captan 0.5% (Vahdati *et al.*; 2009) in the treatments 3-7 and 10-14. The seeds in all treatments also treated with the fungicide for 1 hour before sowing in the pots. The seeds were cracked by a gentle hammer hit to the middle part of the suture of the shell. Then they were placed in 6×20 plastic pots and after irrigation the pots were covered with a plastic bag. Then all treatments were conducted in a greenhouse at 26±1.0°C with 45±5.0% relative humidity, and 10-h dark/14-h light. The pots were irrigated for the second time at the 15th day after sowing the seeds. The plastic bags were removed after 30 days and the pots were irrigated with 5-day intervals.

Number of germinated seeds was determined every day in 45 days period after seed sowing. Total germination was recorded as the overall percentage of germinated seeds. Rate of germination was determined by calculation of mean germination time (MGT) using the following formula (Eq. 1) (Hartmann *et al.*, 1997):

$$\text{MGT} = \frac{\sum_{i=1}^n N_i T_i}{\sum_{i=1}^n N_i} \quad \text{Eq. 1}$$

Sixty days after sowing the seeds, growth parameters of the seedlings including shoot length, root length, leaf number, shoot fresh mass (FM), root FM, and stem diameter were measured. Shoot and root of the seedlings were dried in an oven at 80°C

for 72 hours and seedling dry weight, and shoot dry mass to root dry mass ratio were determined.

The experiments were arranged as a completely randomized design (CRD) consisting of 14 treatments with 10 replications. Statistical analysis was performed using the SAS program version 9.01 (SAS Institute, Cary, NC) and means were compared using the Duncan's multiple range test at $P \leq 0.05$. Pearson's correlation analysis was performed to analyze the correlations between the measured parameters.

Results

Final germination percentage of walnut seeds is shown in Fig. 2. Since no seed germinated in manure (treatments no. 8 to 14), these treatments were

excluded from the statistical analyses. Seed germination percentages in perlite ranged from 0 to 94.1%. No seed germination was observed in the treatments no. 1, 2, and 3 and these treatments also excluded from the statistical analyses. The highest germination percentage (94.1%) was found in treatment #7 which was statistically similar to that in treatment #6 and #5. The lowest seed germination percentage was found in treatment #4. Fig. 3 represents seed germination rate as mean germination time (MGT) after 60 days. MGT ranged between 0 to 1.12 days. The highest germination rate was observed in treatment #7 and seeds in treatment #4 had the lowest germination rate. However, the differences between the treatments was not statistically significant.

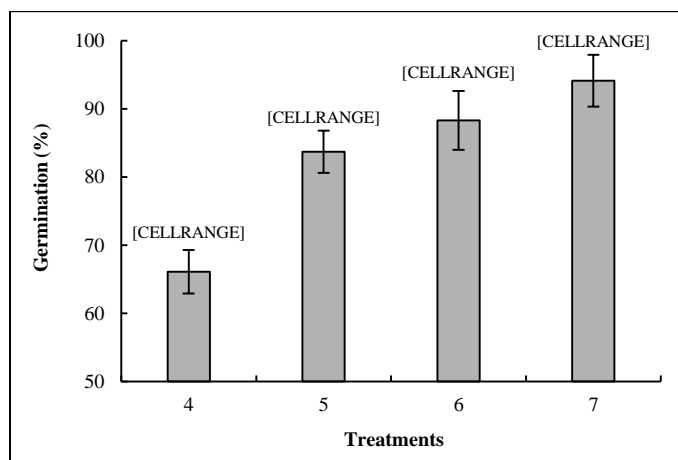


Fig. 2. Effect of seed pre-treatments on final germination percentage of Persian walnut seeds in perlite. The treatments included: 4) 10 days soaking + cracking; 5) 10 days soaking + cracking and inserting toothpick. 6) 10 days soaking + cracking + 4 weeks chilling; 7) 10 days soaking + cracking and inserting a toothpick + 4 weeks chilling.

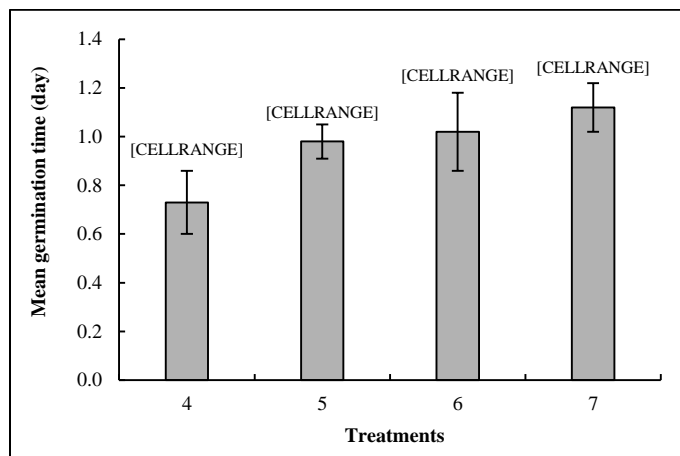


Fig. 3. Effect of seed pre-treatments on germination rate of Persian walnut seeds in perlite. The treatments included: 4) 10 days soaking + cracking; 5) 10 days soaking + cracking and inserting toothpick. 6) 10 days soaking + cracking + 4 weeks chilling; 7) 10 days soaking + cracking and inserting a toothpick + 4 weeks chilling.

Table 1 shows seedling shoot and root growth parameters in perlite at the end of the experiment. Walnut seeds did not germinate in the treatments No. 1 to 3 and these treatments were excluded from the statistical analyses. The highest values of plant height and root length were observed in the treatments #6 and #7. The highest leaf number (3.1

fully expanded leaves) was found in treatment #7, however no significant differences were found among the treatments No. 4, 5, 6 and 7. Stem diameter in treatment #7 was higher than the other treatments; however no significant differences were found between treatments No. 4, 5, 6 and 7.

Table 1. Growth of shoot and root of walnut seedlings at 60 days after seed sowing in perlite.

Treatment	Plant height (cm)	Root length (cm)	Leaf No.	Stem diameter (mm)
4	8.05 ^b	18.80 ^{ab}	2.8 ^a	4.77 ^a
5	8.20 ^b	14.88 ^b	2.6 ^a	4.77 ^a
6	12.42 ^a	21.99 ^a	2.7 ^a	5.02 ^a
7	13.85 ^a	21.97 ^a	3.1 ^a	5.11 ^a
EMS	87.11**	113.58*	0.466 ^{ns}	0.315 ^{ns}
Error	4.85	31.05	0.583	0.522

Treatments were included: 4) 10 days soaking + cracking; 5) 10 days soaking + cracking and inserting toothpick. 6) 10 days soaking + cracking + 4 weeks chilling; 7) 10 days soaking + cracking and inserting a toothpick + 4 weeks chilling.

** , * : significant effect at 0.01 and 0.05 probability level. ns: non-significant effect.

The highest fresh mass of shoot (4.31 g) and root (7.21 g) was found in treatment #7. Shoot fresh mass of plants in treatments #4 and #5 was significantly lower than that in their treatments (Table 2). Root fresh mass in treatments no 4, 5 and 6 was significantly lower than that in treatment #7. Plant biomass was significantly higher in treatment #7

(3.45 g). Shoot to root ratios were significantly higher in treatments #6 and #7 (0.58 g and 0.61 g, respectively) than the ratios in the treatments No. 4 and 5 (Table 2). Pearson's correlation analysis indicated that biomass of the plants had positive correlations with root fresh mass (Table 3).

Table 2. Seedling growth parameters at 60 days after seed sowing in perlite.

Treatment	Shoot FM (g)	Root FM (g)	Biomass (g)	Shoot / Root
4	2.15 ^c	5.36 ^b	2.19 ^b	0.41 ^b
5	2.27 ^c	5.97 ^b	2.57 ^b	0.45 ^{ab}
6	3.52 ^b	5.96 ^b	2.49 ^b	0.58 ^a
7	4.31 ^a	7.21 ^a	3.45 ^a	0.61 ^a
EMS	10.76**	6.01**	2.91**	0.096*
Error	0.566	1.04	0.632	0.032

Treatments were included: 4) 10 days soaking + cracking; 5) 10 days soaking + cracking and inserting toothpick. 6) 10 days soaking + cracking + 4 weeks chilling; 7) 10 days soaking + cracking and inserting a toothpick + 4 weeks chilling. **, *: significant effect at 0.01 and 0.05 probability level.

Table 3. The correlation coefficient between the measured parameters in Persian Walnut.

	Plant height	Root length	Leaf No.	Stem diameter	Shoot FM	Root FM	Biomass
Root length	0.85 ^{ns}						
Leaf No.	0.66 ^{ns}	0.65 ^{ns}					
Stem diameter	1.00 ^{**}	0.86 ^{ns}	0.68 ^{ns}				
Shoot FM	0.99 ^{**}	0.81 ^{ns}	0.72 ^{ns}	0.99 ^{**}			
Root FM	0.81 ^{ns}	0.45 ^{ns}	0.74 ^{ns}	0.81 ^{ns}	0.87 ^{ns}		
Biomass	0.76 ^{ns}	0.42 ^{ns}	0.77 ^{ns}	0.76 ^{ns}	0.84 ^{ns}	0.99 ^{**}	
Shoot / Root	0.99 ^{**}	0.78 ^{ns}	0.55 ^{ns}	0.98 [*]	0.97 [*]	0.80 ^{ns}	0.75 ^{ns}

ns, * and **: non-significant, significant at the 5% and 1% probability levels, respectively

Discussion

In this study, effects of different seed pre-treatments and media on walnut seed germination and early growth were evaluated. No seed germinated in manure probably due to high contaminations and seed spoilage in the media. Results shows that stratification alone may not remove seed dormancy of walnut 'Chandler' since the stratified seeds (treatments #2 and #3) did not germinate during the experiment period, however after 90 days, only 2.5% germination was recorded (data not shown). On the other hand, germination percentage after cracking (treatment #4) and using toothpicks (treatment #5) without stratification reached up to 66.1% and 83.7%, respectively. These results revealed that stratification period enhances germination parameters of seeds with removed mechanical dormancy. Changes in phytohormones

i.e. degradation of ABA, activation of gibberellins during stratification affects results in germination initiation (Shakarishvili *et al.*, 2013). Vahdati *et al.* (2012) reported that the mechanism of dormancy in walnut is both an intermediate physiological and a mechanical dormancy. This combined or double dormancy has developed in the walnut seeds to prevent premature germination of walnut seeds. In order to promote germination, these physiological and mechanical barriers should be removed. Soaking walnut seeds is known to improve their germination probably by washing the inhibitors out of the seeds (Memmedov, 1976). In this study, after a 10-days soaking period, cracking the hard shell without stratification induced seed germination. Soaking the seeds for 2 days did not stimulate walnut seed germination. It appears that a relatively long period

of seed soaking is needed to improve walnut seed germination. Dilution of internal germination inhibitors during soaking of seeds has shown to be involved in increasing seed germination (Hartmann *et al.*, 1997).

Since no intact seeds germinated after 4 weeks chilling (treatment #2) during the experiment period, the results suggested that chilling may improve germination of CS. On the other hand, 66.1% of CS germinated without any chilling period (treatment #4). The results suggested that chilling may not be essential for walnut seed germination and the primary factor that limits germination of walnut seed probably is the hard shell surrounding the seeds.

In this study, seed germination percentage ranged from 66.0% to 94.0%. The maximum seed germination percentages which had been reported in previous studies, particularly in 'Chandler' cultivar, are markedly lower. Sharma (1984) reported 50-60% of walnut seed germination. Çelebioğlu (1985) stated that germination percentage of non-stratified seeds (77%) can be increased up to 84% by stratification. Koyuncu *et al.* (2000) reported a germination percentage of stratified seeds between 22-82%. Mean of maximum walnut seed germination percentage reported in other studies is about 50% (Koyuncu *et al.*, 2000; Einali and Sadeghipour, 2007). Parvin *et al.* (2015) reported the highest percentage of seed germination of Eastern black walnut (69.27 %) with the combined treatment of two months chilling and application of gibberellic acid (400 mg L⁻¹). According to these results, it seems that obtaining a germination percentage of 66.1% by cracking (treatment #4) or 83.7% by opening up the seeds without any stratification treatment (treatment #5), during a 45-day period are most suitable. The results suggest that the major limitation of walnut 'Chandler' seeds for

germination is the shell which tightly surrounds the kernel and imposes mechanical dormancy. Removing mechanical dormancy in comparison to only chilling treatments not only reduces rootstock production time, but markedly reduces the production costs.

The results showed that stratification besides removing mechanical dormancy, not only improves walnut seed germination percentage (up to 94%) and MGT (to 1.0), but also increases seedling early growth. Vahdati *et al.* (2012) reported a 6-8 weeks stratification period is the most appropriate treatment to obtain the best germination percentage and best germination rate and to prevent physiological dwarfing. They also mentioned that the shell surrounding the walnut seed also plays an important role in regulating the seed germination. Chilling plays an important role in providing the stimulus required to overcome dormancy, increase germination, and produce normal seedlings in temperate zone species (Martinez-Gomez and Dicenta, 2001; Karam and Al-Salem, 2001; Jensen and Eriksen, 2001). Einali and Sadeghipour (2007) showed induced enzyme activation and mobilization in walnut seeds during chilling period. Enzymes by releasing seed growth promoters and storage help seeds to germinate (Hartman *et al.*, 1997). In the current study, seedling growth parameters, especially shoot growth, were significantly higher in the stratified seedlings. Increase in plant biomass and growth was correlated with root growth enhancement and increase in formation of leaves. As Ogawa *et al.* (2003) mentioned, increase in biosynthesis of growth stimulator phyto-hormones such as gibberellins after stratification may be involved in improving seed germination and early growth of the seedling. In agreement, our results also indicated that root growth and activity directly

influence growth and development of stem and leaves. However, stem diameter, the most important factor in walnut rootstock production, was not significantly different in stratified and non-stratified seeds. Therefore, it was concluded that after soaking walnut seeds for 10 days and removing the mechanical dormancy, there is no need for long stratification periods in production of walnut rootstock.

Conclusions

It is advised not to use manure as media for walnut seed germination and rootstock production due to high contaminations. Of course, comparing other kinds of manure or composts still is needed. Based on the results, soaking walnut seeds for 10 days prior to cultivation are recommended. Cracking walnut seeds may stimulate germination rate and percentage by increasing water absorption and improve the kernel expansion during the early stages of germination. Although, it is hard to remove the hard shell of walnut seeds without injuring the kernel but kernel may be easily subjected to soil born fungi after removing the shell. It seems cracking walnut seeds and keeping the shell open by inserting a toothpick in the crack is an easy and functional method to remove its mechanical dormancy. It was concluded that a stratification period in addition to opening up the seed shell is required to maximize walnut seed germination percentage and MGT, and improve seedlings early growth. The growth measurements showed that such seedlings are relatively vigorous and can be grafted after 60 days.

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References

- Çelebioğlu G (1985) Walnut cultivation. Bursa Technical Agriculture Directorate Publications. No. 1, Bursa.
- Einali AR, Sadeghipour HR (2007) Alleviation of dormancy in walnut kernels by moist chilling is independent from storage protein mobilization. *Tree Physiology*. 27, 519–525.
- Farsi M, Fatahi Moghadam M, Zamani Z, Hassani D (2018) Effects of scion cultivar, rootstock age and hormonal treatment on minigrafting of Persian walnut. *International Journal of Horticultural Science and Technology*. 5(2), 185-197.
- Farsi, M., Fatahimoghadam M, Zamani Z, Hassani D, Ahmadi A (2016) The histology of minigrafting of Persian walnut trees cv. Chandler. *International Journal of Horticultural Science and Technology*. 3(2), 167-177.
- Hartmann HT, Kester DE, Davies FT, Geneve RL (1997) *Plant propagation: Principles and practices*, 6th edition. Prentice Hall, USA. pp. 250-280.
- Jensen M, Eriksen EN (2001) Development of primary dormancy in seeds of *Prunus avium* during maturation. *Seed Science and Technology*. 29, 307-320.
- Karam NS, Al-salem MM (2001) Breaking dormancy in *Arbutus andrachna* L. seeds

- by stratification and gibberellic acid. Seed Science and Technology. 29, 51–56.
- King JE, Gifford DJ (1997) Amino acid utilization in seeds of loblolly pine during germination and early seedling growth: I. Argenin and arginase activity. Journal of Plant Physiology. 113, 1125–1135.
- Koyuncu FA, Kazankaya T, Tarilgac MA, Koyuncu S, Mehmet Sen (2000) Effect of shell thickness on the germination and seedling growth in walnut (*J. regia* L.). Fen Bilimle Enstitüsü Dergisi. 4(1), 111-114.
- Lotfi N, Vahdati K, Kholdebarin B, Najafian Ashrafi E (2009) Germination, mineral composition and ion uptake in walnut under salinity conditions. HortScience. 44(5),1352–1357.
- Martin CG, Mason IR, Forde HI (1960) Changes in endogenous growth substances in the embryos of *Juglans regia* during stratification. Journal of the American Society for Horticultural Science. 94(1), 13-17.
- Memmedov BA (1976) Preparation of walnut seeds for sowing - Temal. Sb. Tr. Azerb. N. II Sadovodstva, Vinigradarskai Subtropo. Kul'tur. 9, 23-28.
- Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during Arabidopsis seed germination. Plant Cell. 15(7), 1591-1604.
- Parvin P, Khezri M, Tavasolian I, Hosseini H (2015) The effect of gibberellic acid and chilling stratification on seed germination of eastern black walnut (*Juglans nigra* L.). Journal of Nuts. 6(1), 67-76.
- Rawat JMS, Tomar YK, Rawat SS (2008) Effect of stratification on seed germination and seedling performance of wild pomegranate. Journal of American Science. 6(5), 97-99.
- Rezaee R, Vahdati K, Grigoorian W, Valizadeh M (2008) Walnut grafting success and bleeding rate as affected by different grafting methods and seedling vigor. The Journal of Horticultural Science & Biotechnology. 83(1), 94-99.
- Sharma RM (1984) Walnut (*Juglans regia*) seed germination: effect of planting depth and seed position in soil. Progress in Horticulture. 16, 6-8.
- Shakarishvili N, Asieshvili L, Eradze N, Siradze M (2013) Effect of stratification on seed germination and epicotyl dormancy in *Arbutus andrachne* L. Bulletin of the Georgian National Academy of Sciences. 7, 1, 2013.
- Vahdati K, Lotfi N, Kholdebarin B, Hassani D, Amiri R, Mozaffari MR, Leslie C (2009) Screening for drought-tolerant genotypes of Persian walnuts (*Juglans regia* L.) during seed germination. HortScience. 44(7), 1815-1819.
- Vahdati K, Aslamarz AA, Rahemi M, Hassani D, Leslie C (2012) Mechanism of seed dormancy and its relationship to bud dormancy in Persian walnut. Environmental and Experimental Botany. 75, 74-82.

