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Mechanism of seed dormancy and its relationship to bud dormancy in Persian walnut

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ABSTRACT

Stratification, chilling, and heat requirements for both seeds and buds of five Persian walnut genotypes were compared. In experiments carried out between November 2006 and March 2008, 1-year-old twigs were collected after leaf-fall, placed in plastic bags, and kept at 4 ± 1 °C to provide chilling periods ranging from 400 to 1500 h. After chilling, twigs were transferred to a greenhouse for determination of chilling requirement and the number of growing degree hours (°C) needed for bud break. For germination experiments, mature seeds were stratified at 4 ± 1 °C for treatment periods ranging from 0 to 8 weeks and then grown in a greenhouse under natural conditions. Germination rate and time to radicle emergence were recorded weekly and seedling heights were recorded after 2 months growth. Results indicated genotypes could be classified into three dormancy groups based on their chilling and heat requirements: low ('Ronde de Montignac' and 'Serr'), medium ('Z_{53}'), and high ('Lara' and 'Z_{63}') dormancy. Stratification for 6–8 weeks was most appropriate to overcome walnut seed dormancy, to obtain the best germination percentage and germination rate, and to prevent physiological dwarfing. A relationship between the chilling and heat requirements for bud-break and the stratification requirement for germination of seeds was observed and data showed that the nutshell is a mechanical barrier to germination in walnut seeds with intermediate physiological dormancy.

1. Introduction

Dormancy is a general term used to indicate a period of temporary halt in visible growth of a plant structure (Lang, 1987), including shoot and root dormancy (Young and Werner, 1984; Young, 1987) and seed dormancy (Hartmann et al., 1997). Nikolaeva (1977) defined dormancy based primarily upon physiological controls and Lang (1987) defined the terms eco-, para- and endo-dormancy to simplify terminology.

Seed dormancy is an adaptation in various species to delay germination after the seed has been shed from the tree until the appropriate time for germination. Dormancy results from physiological, morphological and physical characteristics of the seeds (Baskin and Baskin, 1998) and affects both germination and subsequent seedling growth (Du Toit et al., 1979; Mehanna et al., 1985; Frisby and Seeley, 1993). There are two types of seed dormancy: exogenous and endogenous (Leadem, 1997). Temperature is the main factor affecting the length of the seed dormancy period (Baskin and Baskin, 1998). Stratification, the standard method used to overcome seed dormancy (Bewley and Black, 1994), involves storing seeds with an equal volume of moist medium for a period of time at a cold temperature. The effective temperature range for stratification is generally 0–10 °C (Crocker and Barton, 1957; Stokes, 1965). A cold treatment at 5 °C (Stokes, 1965) for 60 days suffices to overcome embryo dormancy in many plant species (Bewley and Black, 1994). Dormancy release is indicated by the ability of seeds to germinate at an optimal temperature. The time required to achieve a fully non-dormant state is indicated by the ability to germinate over a range of temperatures (Baskin and Baskin, 1988).

In deciduous woody plants, the date of shoot bud-break reflects both the chilling and the heat requirements of a given plant in a specific environment (Spiegel-Roy and Alston, 1979; Petropoulou, 1985). Each tree species and cultivar has a specific chilling requirement that is related to the accumulated hours below a chilling-temperature threshold or to a cumulative amount of chill units, defined as hours weighted for temperature effective for breaking dormancy (Weinberger, 1950; Erez et al., 1979). Understanding the mechanism of dormancy set and release is prerequisite to developing strategies for manipulation of the dormant period in order to avoid spring frost damage (Faust et al., 1997). This, combined with characterization of the heat requirements of cultivars,

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will provide us with information useful for managing cultivars with low chilling requirements but high heat requirement in relatively cold areas (Citadin et al., 2001).

Walnut (*Juglans regia*, L., *Juglandacea*) species are monoecious, i.e. the pollen male (staminate) flowers and the nut-producing female (pistillate) flowers are born separately on the same tree. Individual staminate flowers are small, relatively inconspicuous, and grouped in hanging clusters called catkin. These develop laterally on the previous season's growth. Pistillate flowers are formed most often in pairs at the tip of current season's shoots. In all cultivars the pistillate flowers emerge from terminal buds shortly after the emergence of the young leaves (Lin et al., 1977).

It is clear that increasing the inbreeding coefficient of the parent(s) or using full sibs instead of half sibs will result in less segregation among the progeny. On the other hand, control of only one parent and harvesting half sib seeds is much easier, more economical and more practical. In several studies of the relationship between seed and bud dormancy in fruit trees, half sib progeny have been used (Kester et al., 1977; Garcia-Gusano et al., 2004).

There are several studies regarding the chilling requirements for termination of bud dormancy in walnut (Chandler et al., 1937; Konarli and Celebioglu, 1977; Mauget, 1988, 1990; Aslani Aslamarz et al., 2009) and more on methods to enhance walnut seed germination (Crocker, 1948; Molchenko et al., 1977; Gaur, 1980) but data on the mechanism of dormancy in walnut and its relation to the chilling and heat requirements of buds are scarce. Therefore, the aims of this study were to: (1) exam stratification affects on walnut seed germination and growth; (2) determine dormancy mechanisms in walnut seed; (3) calculate chilling and heat requirements needed to break bud dormancy; and (4) find the relationship between seed and bud dormancy in several walnut genotypes.

2. Materials and methods

2.1. Source of seeds and twigs

Experiments were carried out during two successive years between November 2006 and March 2008. Mature seeds and 1-year-old twigs were collected from eight 14-year-old trees of five walnut genotypes ('Serr', 'Lara', 'Ronde de Montignac', 'Z_{63}' and 'Z_{53}') grafted onto *J. regia* seedling rootstocks as is standard practice for propagating walnut varieties. These trees were growing in the Kamal Shahr Experimental Orchard of the Seed and Plant Improvement Institute Horticulture Department at Karaj, Iran, located at 35.51 N, 50.51 E., 50 km west of Tehran and 1312 m above sea level. This location is characterized by 240-mm average annual rainfall, a relatively short (2 months) dry period in the summer, cold winters (minimum temperature −21 °C), and 6 months of frost danger (late November to April).

2.2. Estimation of chilling and heat requirements for buds

The collected twigs were cut into 288 stem pieces per genotype (1404 total pieces), each 20 cm long, and transferred to the laboratory. Only one lateral, one terminal, and one catkin bud were retained. All other buds were eliminated. After disinfection with 4000 ppm Captan (Bayer Co.), groups of 20 cuttings each were wrapped in moistened cheese cloth, placed in plastic bags to prevent dehydration during treatments, exposed to low temperature (4 ± 1 °C) for periods varying in 100 h increments from 400 to 1500 h. After chilling, the excised twigs were placed with their basal ends in distilled water and placed in a greenhouse under natural photoperiod and temperatures that varied from 18 to 27 °C. Approximately 3–4 mm were cut from the basal ends of the cuttings three times a week and the water was replaced daily (Citadin et al., 1998). Twigs were evaluated three times a week for the number of buds that had reached the balloon or green tip stage (Citadin et al., 2001). The accumulated growing degree hours (GDH) was calculated for the time interval from twig transfer to the greenhouse until 50% of buds reached the balloon or green tip stage (Richardson et al., 1974). One GDH is defined as 1 h at the temperature 1 °C above the base temperature of 4.5 °C. Total GDH was calculated using hourly temperatures between 4.5 and 25 °C and all temperatures above 25 °C were considered equal to 25 °C.

2.3. Stratification of seeds

Seeds were air-dried after collection, cleaned, and stored at room temperature (15–20 °C and 60% RH) for 2 months. Immature seeds and those damaged by insects were eliminated. Remaining seeds were allowed to imbibe distilled water at ambient temperature for 24 h, then surface-sterilized using a 5 min soak in 4000 ppm Captan (Bayer Co.) followed by a thorough rinse with sterilized water prior to applying treatments (Vahdati et al., 2009).

Seeds were placed on a double layer of peat moss moistened to field capacity and held in 40 cm × 25 cm × 15 cm perforated plastic boxes to allow gas exchange and drainage of excess water. These were then exposed to low temperature (5 ± 1 °C) in the dark for periods ranging from 1 to 8 weeks. Because seeds that require cold stratification need water imbibition during the process (Baskin and Baskin, 1998), samples were inspected periodically to avoid excessive drying or poor aeration during chilling. To reduce potentially damaging microorganisms, the nuts were washed in 4000 ppm Captan (Bayer Co.) for 1 min, every week during stratification.

To determine the influence of the shell on germination, shells were carefully and completely removed from half of the samples, while the remaining seeds were kept intact. The in-shell and shelled nuts were planted in containers of perlite in the greenhouse and kept under natural photoperiod, temperatures that varied between 18 and 27 °C, and 75% RH. The containers were watered three times a week. Seeds were considered germinated when the tip of the radicle had grown from the seed. Germination percentage was calculated and a germination index (GI), used as a measure of germination speed, was calculated by $\sum$(Gt/Dt) (Yang et al., 2007), where Gt is the number of germinated seed(s) after t days (Dt). Seedling heights were measured 2 months after the seeds were transferred to the greenhouse. All experiments using shelled and in-shell nuts were conducted using three replications of 15 seeds per treatment.

2.4. Experimental design and statistical analysis

Correlation coefficients between chilling and heating requirements of genotypes were determined using Pearson ranked-order correlation. Student's t analysis was performed using SPSS 13.0 Windows (Chicago, IL, USA). Data were analyzed using SAS Software (SAS Institute, Inc., 2002). Means with significant differences were compared using Duncan's multiple range tests at $P \leq 0.01$.

3. Results

3.1. Chilling and heat requirement of buds

The calculated (or estimated) chilling and heat requirements for walnut genotypes in 2 consecutive years are shown in Table 1. The chilling and heat requirements were considered to be satisfied when 50% of buds reached the balloon or green tip stage. The chilling requirements of catkins were lower than lateral and terminal buds and were highest for lateral buds. The chilling requirements ranged from 450 to 750 h for catkins, from 600 to 800 h for terminal buds, and from 650 to 900 h for lateral buds. 'Serr' and 'Ronde de

Table 1
Chilling ± standard error (4 °C) and heat requirement (GDH °C ± standard error) for 50% of lateral and terminal buds and catkins of Persian walnut genotypes to reach the balloon or green tip stage in 2006 and 2007.

Genotype	Years	Lateral bud	Mean	Terminal bud	Mean	Catkin	Mean
Chilling requirement							
Serr	2006	700 ± 35c	650 ± 25	600 ± 20c	600 ± 25	400 ± 30e	450 ± 30
	2007	600 ± 20d		600 ± 30c		500 ± 30d	
Lara	2006	900 ± 45a	900 ± 30	800 ± 20a	800 ± 30	700 ± 15b	750 ± 20
	2007	900 ± 20a		800 ± 40a		800 ± 30a	
Ronde de Montignac	2006	700 ± 15c	650 ± 25	600 ± 10c	600 ± 10	500 ± 40d	500 ± 30
	2007	600 ± 40d		600 ± 10c		500 ± 20d	
Z_{53}	2006	800 ± 45b	800 ± 35	700 ± 30b	700 ± 35	700 ± 15b	650 ± 15
	2007	800 ± 20b		700 ± 40b		600 ± 20c	
Z_{63}	2006	900 ± 10a	850 ± 15	800 ± 50a	750 ± 30	600 ± 30c	600 ± 35
	2007	800 ± 10b		700 ± 10b		600 ± 40c	
Heat requirement							
Serr	2006	10600 ± 380d	10430 ± 223	10850 ± 95d	10753 ± 122	7075 ± 212c	7165 ± 156
	2007	10268 ± 97d		10656 ± 122d		7255 ± 35bc	
Lara	2006	12150 ± 265bc	12457 ± 290	11936 ± 167c	12620 ± 176	7480 ± 84bc	7672 ± 56
	2007	12765 ± 366b		13305 ± 274b		7856 ± 34b	
Ronde de Montignac	2006	10220 ± 69d	10225 ± 87	10452 ± 224d	10552 ± 154	6955 ± 225c	7052 ± 134
	2007	10330 ± 158d		10652 ± 330d		7150 ± 53c	
Z_{53}	2006	10360 ± 114d	11064 ± 110	12764 ± 224bc	12020 ± 267	7930 ± 35b	8432 ± 56
	2007	11768 ± 143c		11276 ± 345 cd		8534 ± 73b	
Z_{63}	2006	15033 ± 89a	14636 ± 78	15267 ± 345a	15186 ± 234	8270 ± 35b	9071 ± 22
	2007	14240 ± 128a		15105 ± 274a		9423 ± 96a	

Mean ± SE in each column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

Montignac' had the lowest chilling requirements (650 and 650 h). 'Z_{53}', 'Z_{63}' and 'Lara', with 800, 850 and 900 h, respectively, showed intermediate and high chilling requirements. The range of chilling requirements for breaking bud and catkin dormancy in the genotypes studied varied from 450 to 900 h. The heat requirement for lateral and terminal buds and catkins was calculated by the method of Richardson et al. (1974). The genotypes were classified into three groups in accordance with their heat requirements: low ('Z_{30}' and 'Ronde de Montignac'), medium ('Z_{53}' and 'Lara'), and high heat requirement ('Z_{63}'). Our results indicated that the heat requirements for catkins were lower than for lateral and terminal buds and the GDH accumulation for terminal buds was higher than for lateral buds.

3.2. Seed germination

Our data confirmed a clear effect of the length of the stratification period on enhancement of seed germination. According to Fig. 1, all genotypes showed some germination, even without a stratification treatment. The mean germination percentage of in-shell and shelled seeds in the control treatment (0 week) for the first and second years varied as follows: 42–48% ('Serr'), 33–38% ('Lara'), 35–44% ('Ronde de Montignac'), 29–31% ('Z_{53}') and 32–34% ('Z_{63}'). Similar relatively low germination percentages were observed after a short period of stratification (3 weeks) but longer stratification (6–8 weeks) resulted in seed germination of 70–90%. During the longest stratification treatment (8 weeks) the germination percentages reached 84–91% ('Serr'), 82–92% ('Lara'), 90–93% ('Ronde de Montignac'), 62–70% ('Z_{53}') and 89–91% ('Z_{63}'). Regardless of whether seeds had shells or not, genotypes 'Serr' and 'Ronde de Montignac' with a low chilling and heat requirement, took less time to achieve 80% germination (5–7 weeks) than the higher chilling and heat requirement genotypes 'Lara' and 'Z_{63}' which needed 7 and 8 weeks, respectively.

3.3. Germination rate

According to our results, stratification increased GI values for both in-shell and shelled seeds. Furthermore, we found that when the seed of genotypes with low chilling and heat requirement ('Serr' and 'Ronde de Montignac') were stratified, the GI values, both in seeds with and without shell, were suddenly increased after 4–5 weeks in comparison to genotypes with higher chilling and heat requirements (Table 2).

A relationship between the combined chilling/heat requirement of genotypes and the time required for seeds to germinate was observed. Table 3 shows that the mean time needed for germination of low chill/heat genotypes ('Serr' and 'Ronde de Montignac') was less than the time required for the high chill/heat requiring genotypes ('Lara' and 'Z_{63}').

3.4. Interaction between chilling and heat requirement in the seeds and buds

The chilling requirements for breaking bud and seed dormancy were found to be correlated with the heat requirements (Table 4). For seeds, the genotypes 'Z_{53}' and 'Ronde de Montignac' had the highest correlation coefficients while the coefficients for 'Z_{53}' and 'Serr' were highest for buds. Except for 'Ronde de Montignac', the degree of correlation found in buds was consistent with the degree of correlation in seeds, indicating similar interaction between chilling and heat requirement in the seeds and buds of these genotypes. Among the genotypes examined, chilling and heat requirements were most strongly correlated in 'Serr' and 'Z_{53}'.

3.5. Seedling height

The correlation between mean height of seedlings after 2 months and the stratification treatments for seeds, either with shell (y1) or without shell (y2) in 2 successive years, along with regression functions and coefficients of determination, are shown in Fig. 2. Based on the linear regression functions, the mean height of seedlings at 2 months increased for all genotypes as the amount of stratification increased from no chilling to 8 weeks of cold. The increase in height of seedlings after 8 weeks of stratification in walnut genotypes with and without shell in 2 successive years was 1.9–2.5 fold ('Ronde de Montignac'), 2.3–3.2 fold ('Serr'), 2.1–2.3 fold ('Lara'), 1.9–2.2 fold ('Z_{63}') and 1.8–2.3 fold ('Z_{53}'). The greatest mean coefficient of determination (R^2) for 2 years was exhibited by 'Ronde de Montignac' (0.96) while 'Lara' showed the lowest coefficient of determination (0.90). No significant difference was observed among genotypes, indicating the chilling requirement of

Table 2
Effect of stratification at 4 ± 1 °C for 0–8 weeks on the seed germination index (GI) of several walnut genotypes grown with or without shells in a greenhouse at temperatures varying between 19 and 24 °C.

Genotypes	Germination index (GI)								
Stratification treatments at 4 ± 1 °C (week)									
	Year	0	1	2	3	4	5	6	7	8
In shell										
Serr	2006	1.8 ± 0.32p-t	1.9 ± 0.32o-t	2.7 ± 0.23m-t	3.1 ± 0.2 j-r	3.2 ± 0.21j-r	3.7 ± 0.15g-o	5.5 ± 0.08b-g	6.8 ± 0.8ab	7.9 ± 0. 4a
	2007	1.6 ± 0.28m-q	1.71 ± 0.3l-q	2.1 ± 0.32k-q	2.3 ± 0.2j-p	2.9 ± 0.14g-n	4.3 ± 0.12e-h	5 ± 0.28c-f	6.4 ± 0.7a-c	7.8 ± 0. 8a
Lara	2006	0.9 ± 0.31t	1.1 ± 0.12st	1.1 ± 0.32p-t	1.6 ± 0.08q-t	1.9 ± 0.34o-t	2.2 ± 0.14n-t	2.8 ± 0.18k-s	3 ± 0.36j-r	4 ± 0.1f-n
	2007	0.79 ± 0.19q	0.9 ± 0.2o-q	1 ± 0.1n-q	1.3 ± 0.2m-q	1.7 ± 0.12l-q	1.88 ± 0.1l-q	2.6 ± 0. 15i-p	3.1 ± 0. 8g-m	3.9 ± 0.06f-k
Ronde de Montignac	2006	1.5 ± 0.28q-t	2.1 ± 0.11o-t	2.8 ± 0.1 l-t	3.1 ± 0.3j-r	3.5 ± 0.11h-p	4.6 ± 0.1d-l	5.4 ± 0.47b-h	5.7 ± 0.07b-e	6.5 ± 0.11a-c
	2007	1.3 ± 0.3m-q	1.8 ± 0.29l-q	2.7 ± 0.1h-m	3 ± 0.3g-m	3.5 ± 0.35f-l	4.7 ± 0.1c-g	6 ± 0.028b-d	4.5 ± 0. 7d-h	6.9 ± 0.12ab
Z_{53}	2006	0.9 ± 0.18t	1.1 ± 0.31st	1.3 ± 0.31r-t	2.4 ± 0.31n-t	3.7 ± 0.12g-o	4.4 ± 0.5e-m	4.7 ± 0.15c-k	6.2 ± 0.16a-e	6.3 ± 0. 7a-d
	2007	0.9 ± 0.27o-q	1.1 ± 0.2n-q	0.8 ± 0.33pq	2.1 ± 0.25k-q	2.7 ± 0.18h-o	4 ± 0.08e-j	4.2 ± 0.11e-i	5.1 ± 0.26c-f	5.8 ± 0.06b-e
Z_{63}	2006	0.97 ± 0.34st	0.87 ± 0.02t	1.4 ± 0.1r-t	2.6 ± 0.2m-t	3.4 ± 0.2l-q	2.5 ± 0.22n-t	4.6 ± 0.1d-l	4.8 ± 0.38c-j	5.1 ± 0.15b-i
	2007	0.95 ± 0.3o-q	1 ± 0.14n-q	1.3 ± 0.2m-q	1.8 ± 0.24l-q	2.5 ± 0.09i-p	2.6 ± 0.12i-p	2.9 ± 0.21g-n	3 ± 0.04g-m	4.1 ± 0. 7e-j
Shelled										
Serr	2006	2.4 ± 0.2m-t	2.4 ± 0.2m-t	3 ± 0.1k-t	3.8 ± 0.33h-o	3.8 ± 0.3h-o	4.4 ± 0.1h-l	6.5 ± 0.6b-e	8 ± 0.5ab	9 ± 0.1a
	2007	2 ± 0.17k-o	2 ± 0.14k-o	2.6 ± 0.3k-o	2.8 ± 0.1k-o	3.5 ± 0.1f-k	5 ± 0.15d-f	5.9 ± 0.3c-e	7.4 ± 0.1ab	8.9 ± 0.5a
Lara	2006	1.9 ± 0.25t	1.3 ± 0.32r-t	1.7 ± 0.26q-t	2 ± 0.14o-t	2.2 ± 0.12n-t	2.6 ± 0.14l-t	3.3 ± 0.32j-r	3.5 ± 0.52i-q	4.5 ± 0.6f-k
	2007	1 ± 0.3o	1.1 ± 0.28no	1.2 ± 0.1no	1.6 ± 0.5k-o	2.1 ± 0.4k-o	2.2 ± 0.2k-o	3 ± 0.33g-o	3.58 ± 0.5f-k	4.5 ± 0.3f-k
Ronde de Montignac	2006	1.9 ± 0.3o-t	2.5 ± 0.1l-t	3.3 ± 0.2j-s	3.7 ± 0.1h-p	4.4 ± 0.1h-m	5.4 ± 0.2d-l	6.4 ± 0.15b-f	6.5 ± 0.42b-e	7.4 ± 0.7ab
	2007	1.7 ± 0.14k-o	2.2 ± 0.1k-o	3.3 ± 0.09f-l	3.6 ± 0.1f-k	4.2 ± 0.5e-i	5.6 ± 0.08c-e	5.6 ± 0.4c-e	5.2 ± 0.17d-f	7.8 ± 0.2ab
Z_{53}	2006	1 ± 0.0.2t	1.4 ± 0.2r-t	1.3 ± 0.1st	2.8 ± 0.2k-t	4.4 ± 0.6h-l	5.2 ± 0.4e-i	5.6 ± 0.4c-g	7.2 ± 0.2a-d	7.2 ± 0.5a-d
	2007	1 ± 0.15no	1.3 ± 0.15l-o	1.1 ± 0.3no	2.6 ± 0.1k-o	3.3 ± 0.1f-m	4.8 ± 0.2d-g	4.9 ± 0.3d-f	5.97 ± 0.3c-e	6.5 ± 0.2b-e
Z_{63}	2006	1.1 ± 0.4t	1 ± 0.12t	1.7 ± 0.2p-t	3 ± 0.1k-t	3 ± 0.14k-t	2 ± 0.4k-t	5.4 ± 0.2c-h	5.5 ± 0.2c-h	5.8 ± 0.2c-g
	2007	1.1 ± 0.2no	1.3 ± 0.1m-o	1.6 ± 0.2k-o	1.4 ± 0.4 l-o	3 ± 0.2g-o	3 ± 0.3g-n	3.4 ± 0.4f-k	3.65 ± 0.3f-j	4.7 ± 0.4d-g

Each value is the mean of three replications of 15 seeds per each treatment.
Mean ± SE in table followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

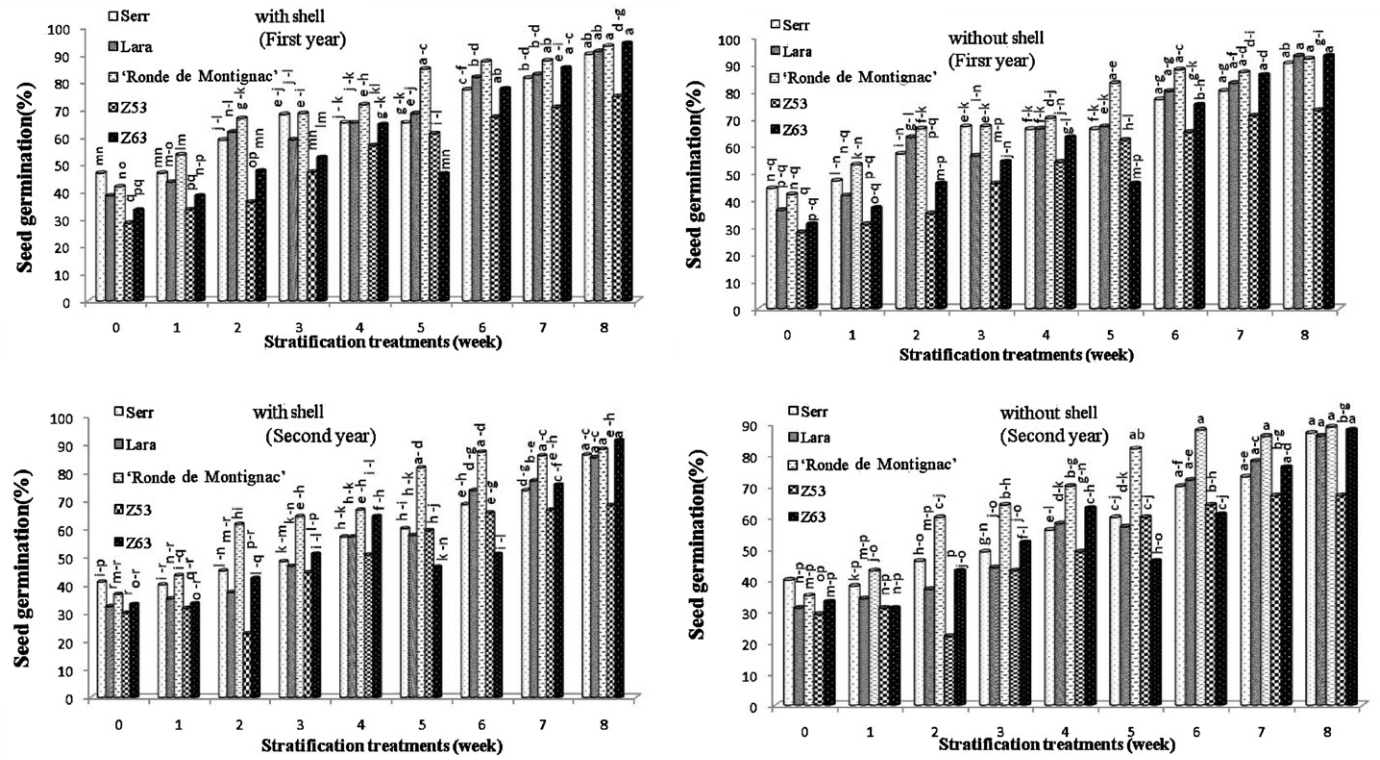

Fig. 1. Seed germination percentages of walnut genotypes with or without shells stratified at 4 ± 1 °C for 0–8 weeks. Seeds were planted in containers of perlite in the greenhouse in 2006 and 2007 under natural photoperiod and temperatures between 19 and 24 °C, and 75% RH. Means indicated by the same letter are not significantly different ($P \leq 0.01$).

Table 3
Seed germination index (GI) of several walnut genotypes grown with or without shells in a greenhouse at temperatures varying between 19 and 24 °C.

Genotype	Year	Mean of germination index (GI)
Serr	2006	4.09 ± 0.6a
	2007	3.8 ± 0.2a
Lara	2006	2.15 ± 0.3d
	2007	1.99 ± 0.1c
Ronde de Montignac	2006	3.92 ± 0.2ab
	2007	3.85 ± 0.2a
Z_{53}	2006	3.46 ± 0.4b
	2007	3 ± 0.3b
Z_{63}	2006	2.93 ± 0.3c
	2007	2.26 ± 0.3c

Means in column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

seed and buds had no relationship to the height and vigor of the seedlings.

3.6. Difference in the response to stratification of seeds with or without shells

Means analyses for testing differences between seeds with and without their shells were conducted using Student's *t*-test (Table 5). The *t*-value was only significant for the germination rate (index) which confirms the inhibition effect of shell on release from dormancy and germination.

3.7. Year to year variation

Year to year variation in data for combined experiments in 2006 and 2007 is shown in Table 6. The mean value of all treatments

Table 4
Correlation coefficients between the combined chilling and heat requirements needed for 50% of the buds of walnut genotypes to reach the balloon or green tip stage and the combined stratification/growth time needed to achieve 50% germination in seeds with or without shells in 2 successive years.

Genotype	Year	Correlation coefficient		
		Bud	Seed with shell	Seed without shell
Serr	2006	−0.65**	−0.58**	−0.61**
	2007	−0.63**	−0.62**	−0.64**
Lara	2006	−0.33ns	−0.41ns	−0.45ns
	2007	−0.62**	−0.38ns	−0.39ns
Ronde de Montignac	2006	−0.45ns	−0.75**	−0.77**
	2007	−0.48**	−0.63**	−0.66**
Z_{53}	2006	−0.87**	−0.82**	−0.85**
	2007	0.95**	−0.86**	−0.88**
Z_{63}	2006	−0.47ns	−0.51**	−0.52**
	2007	−0.32ns	−0.47ns	−0.57**

ns and ** non significant and significant at 1% statistical level, respectively ($P \leq 0.01$).

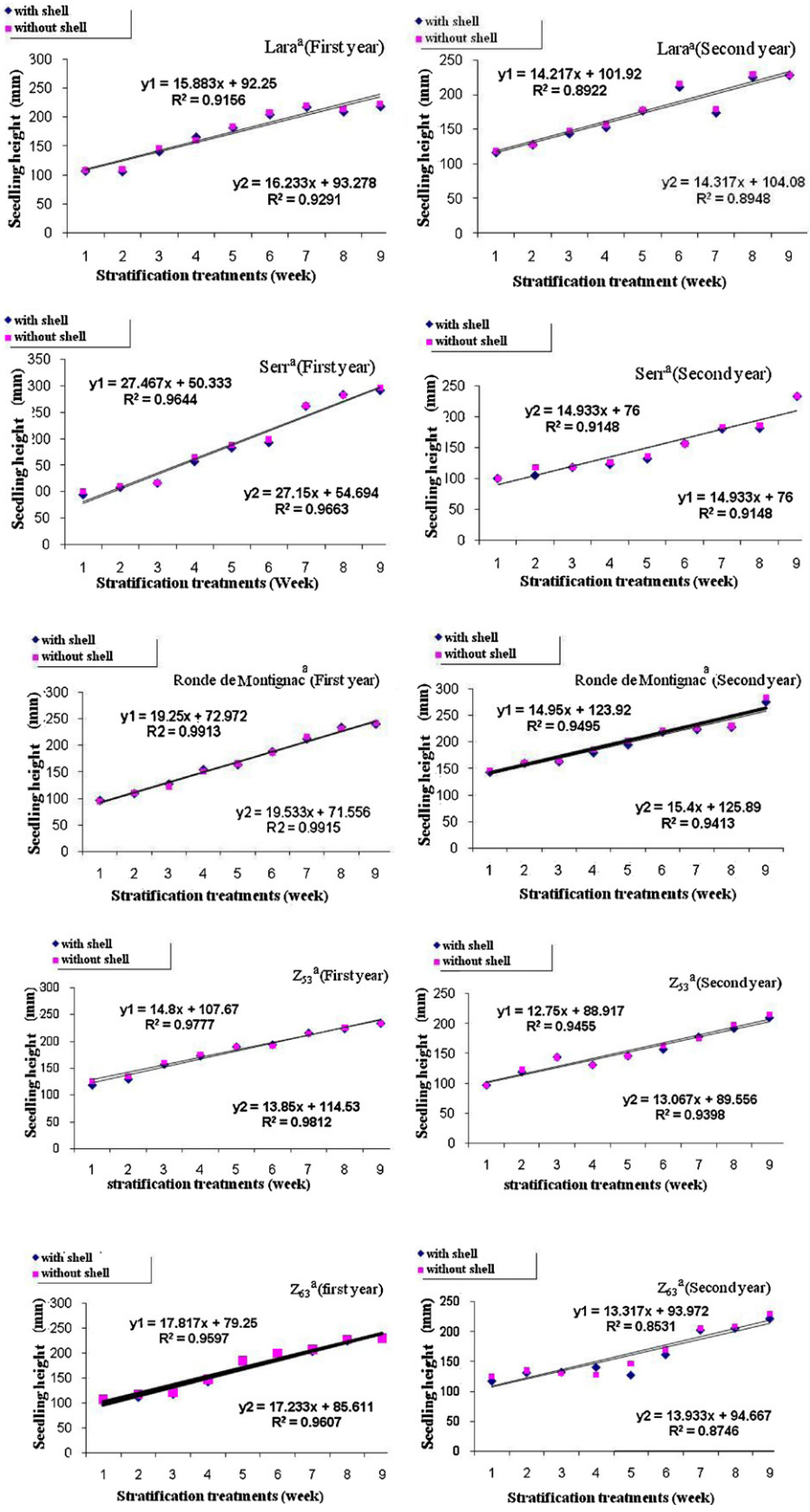

Fig. 2. Correlation between seedling height after 2 months growth and seed stratification treatments of walnut genotypes with (y1) and without (y2) shells in 2 successive years, including the regression functions and coefficient of determination. Seeds exposed to chilling temperatures for times from 0 to 8 weeks were then grown at 20 °C. Seedling heights measured after 2 months. Genotypes indicated by the same letter are not significantly different from each other ($P \leq 0.01$).

Table 5
Student's *t*-test comparison of seeds with and without shells for walnut genotypes collected in 2006 and 2007.

Paired differences between seed with and without shell	Mean	Std. deviation	Std. error mean	*t* value	d.f.
Seedling height	−5.1	44.79	2.72	−1.87ns	269
Germination percent	0.83	9.14	0.55	1.5ns	269
Germination rate	−0.52	0.85	0.51	−10.17**	269

Table 6
Year to year variation in the chilling and heat requirement of buds, seedling height, germination percent, and seed germination index acquired from analysis of combined experiments in 2006 and 2007.

Year	Germination percent (%)	Germination (GI)	Seedling height (mm)	Chilling requirement (h)	Heat requirement (GDH)
2006	62a	3.9a	180a	780a	12482a
2007	56b	3.4b	174a	750b	12143b

Year in column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.01$).

and genotypes in first and second years, respectively, were 62% and 56% for germination percentage, 3.9 and 3.4 for germination index (GI), 180 mm and 174 mm for seedling height, 780 h and 750 h for chilling requirement, and 12,482 GDH and 12,143 GDH for heat requirement. Only seedling height did not differ between years. Results for chilling-heat requirement, germination percent and germination rate (index) differed in the 2 years with $P \leq 0.01$.

Means analysis using Student's *t*-test for differences in temperature, rainfall and relative humidity between the first and second years based on data acquired from the meteorology station at Karaj, Tehran are presented in Table 7. Our data show that climatic conditions (temperature, rainfall and humidity) in 2 years were significantly different.

4. Discussion

The results for chilling requirements are in accordance with the suggestion of Chandler et al. (1937), who estimated the chilling requirement for walnut genotypes to be between 400 and 1500 h below 7 °C. Unfulfilled chilling can lead to losses in yield or low production when genotypes with higher chill requirements are grown in warmer regions (Erez, 2000). Based on our results, 'Serr' and 'Ronde de Montignac' appear to be more suitable for cultivation in warmer climates. Alternatively, 'Lara' may be more appropriate for cultivation in colder regions, especially those with a risk of late spring frosts. Among the Iranian genotypes, 'Z_{63}' seems to be best adapted to colder winter climates. The data support the hypothesis that terminal buds require less chilling than lateral buds (Scalabrelli and Couvillon, 1986). Although late-flowering cultivars usually had higher heat requirements, the flowering time was mainly determined by chilling requirements. Therefore, in breeding cultivars for areas with cold winters and early spring or late winter frost, parents exhibiting high chilling requirements like 'Lara' and 'Z_{63}' should be used to produce suitable progeny. Our data regarding the role of heat requirement in bud break supports the observations of Citadin et al. (2001) and Gariglio et al. (2006). Arnold (1959) and Citadin (1999) found that time of bloom of genotypes depends on their heat requirement (GDH C accumulation) during endodormancy. Among the walnut genotypes examined in this study, 'Z_{63}' seems to be most appropriate for cultivation in the regions with late winter or early spring frosts. 'Serr' and 'Ronde de Montignac', with the lowest heat requirements, appear least suitable for such a climate.

Large variability in seed germination rate was observed among genotypes. This could be due to differences in depth of dormancy or in the chilling/heat requirements of the seeds.

The increase in GI values with increased stratification seen in walnut seeds, both with and without shells, suggests a change in embryo growth potential during stratification. This may be due to a change in membrane fluidity at chilling temperatures or differential enzyme activity for storage reserves (Bewley and Black, 1994). Protease and lipase enzymes have been shown to increase during chilling stratification (Lewak and Rudnicki, 1977). Alteration in lipids, sugars and amino acids could explain the increase in growth potential seen in the embryo following chilling treatments and subsequent accelerated breaking of dormancy.

The relationship between chilling-stratification and heat requirement of buds and germination time of seeds is consistent with previous studies by Grigorian (1972), Kester et al. (1977) and Garcia-Gusano et al. (2004). The correlation coefficients between chilling treatment and heat requirement for breaking dormancy in buds and seeds were negative, and imply that prolonged exposure to chilling temperatures could reduce the heat requirement for seed germination and bud break. These results confirm that resting or partially chilled trees require extensive heat accumulation before they are able to bloom than trees which have their chilling fully satisfied (Samish, 1954; Richardson et al., 1975; Swartz and Powell, 1981). These results also agree with those obtained in peach by Seeley et al. (1998), who indicated an interaction between chilling and heat requirement for seed germination also confirmed the previous study about interaction between heat and chilling on bud dormancy in walnut (Mauget, 1988).

Stratification length strongly influenced the later growth of the seedlings. For chilling treatments shorter than 5–6 weeks a rosette-type of growth was observed in all genotypes; a phenomenon called physiological dwarfing by Hartmann et al. (1997). This effect was also observed by Grigorian (1972) in some almond cultivars and by Martinez-Gomez and Dicenta (2001) in peach. The high coefficient of determination (R^2) seen in all genotypes confirmed that stratification has an important effect on the height of seedlings.

The negative impact of an intact shell on walnut germination is consistent with other observations of shell impact on

Table 7
Differences between the first and second years of data collection in temperature, rain fall, and relative humidity during the growth period.

Paired difference	Mean	SD	SE	*t* value	d.f.
Temperature of first–second years	3.36	2.57	0.17	19.43**	215
Rain fall of first–second years	1.17	3.48	0.24	4.87**	215
Relative humidity of first–second years	2.5	3.17	0.27	9.42**	215

** Significantly different ($P \leq 0.01$) according to Student's *t*-test.

germination. Seed-enclosing structures, such as walnut shells (Crocker, 1948) and stone fruit pits (Nikolaeva, 1977; Crisosto and Sutter, 1985) restrict embryo expansion during germination. Mehanna and Martin (1985) and Gaur (1980) concluded that shells provide mechanical resistance to germination while Taylorson and Hendricks (1977) described both mechanical and hormonal effects of the shell during this process. Additional discussions of the role shell plays in germination were published by Du Toit et al. (1979), Hartmann et al. (1997) and Martinez-Gomez and Dicenta (2001).

Previous studies indicate dormancy can be affected by a variety of atmospheric and soil conditions including air temperature (Welling, 2003), nutrition (Almond and Young, 1990), rainfall (Buchanan et al., 1977) and other factors impacting seed development while maturing on the mother plant (Baskin and Baskin, 1975, 1995). Our climatic conditions (temperature, rainfall and relative humidity) statistically differed (by Student's *t*-test) during the 2 years of the study. Therefore one or more of these climatic factors could have had an impact on the observed year to year variation in seed and bud dormancy. The main difference in bud dormancy of walnut between 2 years in the natural condition has been shown previously (Mauget, 1990).

Our results are in agreement with Erez and Couvillon (1987) who demonstrated that the mechanism for breaking dormancy in seeds is similar to the mechanism for completing bud dormancy. Powell (1987) attributed this phenomenon to a similar hormonal mechanism in both. Experimental evidence supports the involvement of specific endogenous plant growth substances in the control of dormancy in both organs. Most of the information focuses on abscisic acid (ABA), gibberellins and cytokinins, whereas auxin and other compounds have been shown to have little or no effect on the dormancy. ABA concentration declines in both buds and seeds during cold treatment from the initiation to the breaking of dormancy (Powell, 1987; Rodriguez et al., 1991; Hilhorst and Karssen, 1992). A relationship between chilling and the appearance of cytokinins also has been demonstrated (Thomas et al., 1992; Mok and Mok, 1994) and previous studies have also shown changes in the levels of endogenous gibberellins in buds and seeds during stratification (Takahashi et al., 1991; Wieble et al., 1992).

Our data are consistent with earlier work indicating that seeds displaying intermediate physiological dormancy usually require stratification for release from dormancy, as distinguished from seeds with deep physiological dormancy and a shorter chilling stratification requirement (Hartmann et al., 1997). The seed covering (coat or shell) of seeds with an intermediate physiological dormancy can also have an impact on breaking rest (Hartmann et al., 1997). Hence our results confirm that the mechanism of dormancy in walnut is both an intermediate physiological and a mechanical dormancy (hard seed dormancy). This combined or double dormancy acts as a safety mechanism for walnut seeds in preventing premature germination. In order to promote germination, both blocking conditions (physiological and mechanical) must be removed. This dormancy is the most common type found in seeds of trees, shrubs and some herbaceous plants of the temperate zone possessing hard seed coats (Crocker, 1916; Young and Young, 1992; Fambrini et al., 1993).

5. Conclusion

Chilling treatments increase the percentage of bud break and decrease the heat requirement in all genotypes of walnut studied, but the responses of genotypes and the various bud types to chilling differ. The estimated chilling and heat requirements are good predictors for time of bud break. Stratification periods of 6–8 weeks were the most appropriate to overcome walnut seed dormancy, to obtain the best germination percentage and best germination rate, and to prevent physiological dwarfing. The walnut shell also plays an important role in regulating dormancy. These results confirm a relationship between the dormancy breaking mechanism in buds and in seeds. Finally, walnut is a good example of double dormancy in seeds and its various bud types differ in their chilling and heat requirements.

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References

Almond, M.A., Young, E., 1990. Growth and protein content of apple in response to shoot and root temperature following chilling. HortScience 22, 371–377.
Arnold, C.Y., 1959. The determination and significance of the base temperature in a linear heat unit system. Proc. Am. Soc. Hort. Sci. 74, 430–445.
Aslani Aslamarz, A.A., Vahdati, K., Rahemi, M., Hassani, D., 2009. Estimation of the chilling and heat requirement of some Persian walnut cultivars. HortScience 44, 697–701.
Baskin, J.M., Baskin, C.C., 1995. Variation in the annual dormancy cycle in buried seeds of the weedy winter annual *Viola arvensis*. Weed Res. 35, 353–362.
Baskin, J.M., Baskin, C.C., 1975. Year-to-year variation in the germination of freshly-harvested seeds of *Arenaria patula* var. Robusta from the same site. J. Tenn. Acad. Sci. 50, 106–108.
Baskin, C.C., Baskin, J.M., 1988. Germination ecophysiology of herbaceous plant species in a temperate region. Am. J. Bot. 75, 286–305.
Baskin, C.C., Baskin, J.M., 1998. Seeds—Ecology, Biogeography, and Evolution of Dormancy and Germination. Academic Press, San Diego.
Bewley, J.D., Black, M., 1994. Seeds: Physiology of Development and Germination. Plenum Press, New York, USA.
Buchanan, D.W., Bartholic, J.F., Biggs, R.H., 1977. Manipulation of bloom and ripening dates of three Florida grown peach and nectarine cultivars through sprinkling and shade. J. Am. Soc. Hort. Sci. 102, 466–470.
Chandler, W.H., Kimball, M.H., Phillip, G.L., Tufts, W.P., Weldon, G.P., 1937. Chilling requirements for opening of buds on deciduous orchard trees and some other plants in California. Calif. Agric. Exp. Bull. 611.
Citadin, I., 1999. Necessidade de calor para antese e brotação em pessegueiro [*Prunus persica* (L.) Batsch]. Msc. Diss., Univ. Federal de Pelotas, Pelotas-Brazil.
Citadin, I., Raseira, M.C.B., Quezada, A.C., 1998. Substrato para conservacxa̋o de ramos destacados de pessegueiro, *Prunus persica* L. (Batsch). A gropecuaïria de clima Temperado 1, 55–59.
Citadin, I., Raseira, M.C.B., Herter, F.G., da Silva, J.B., 2001. Heat requirement for blooming and leafing in peach. HortScience 36, 305–307.
Crisosto, C., Sutter, E.G., 1985. Role of the endocarp in 'Manzanillo' olive germination. J. Am. Soc. Hort. Sci. 110, 50–52.
Crocker, W., 1916. Mechanics of dormancy in seeds. Am. J. Bot. 3, 99–120.
Crocker, W., Barton, L.V., 1957. Physiology of Seeds. Chronica Botanica, Waltham.
Crocker, W., 1948. Growth of Plants. Reinhold, New York.
Du Toit, H.G., Jacobs, G., Strydom, D.K., 1979. Role of various seed parts in peach seed dormancy and initial seedling growth. J. Am. Soc. Hort. Sci. 104, 490–492.
Erez, A., 2000. Bud dormancy; phenomenon, problems and solution in the tropic and subtropic. In: Erez, A. (Ed.), Temperate fruit crops in warm climates. Kluwer Academic Publisher, Dordrecht, The Netherlands, pp. 17–48.
Erez, A., Couvillon, G.A., 1987. Characterization of the influence of moderate temperatures on rest in peach. J. Am. Soc. Hort. Sci. 112, 677–680.
Erez, A., Couvillon, G.A., Hendershott, C.H., 1979. The effect of cycle length on chilling negation by high temperatures in dormant peach leaf buds. J. Am. Soc. Hort. Sci. 104, 573–576.
Fambrini, M., Pugliesi, C., Vernieri, P., Giuliano, G., Baroncelli, S., 1993. Characterization of a Sunflower (*Helianthus annus* L.) mutant, deficient in carotenoid synthesis and abscisic-acid content, induced by in-vitro tissue culture. Theor. Appl. Genet. 87, 65–69.
Faust, M., Erez, A., Rowland, L.J., Wang, S.Y., Norman, H.A., 1997. Bud dormancy in perennial fruit trees: physiological basis for dormancy induction, maintenance and release. HortScience 32, 623–629.
Frisby, J.W., Seeley, S.D., 1993. Chilling of endodormant peach propagules: seed germination and emergence. J. Am. Soc. Hort. Sci. 118, 248–252.
Garcia-Gusano, M., Martinez-Gómez, P., Dicenta, F., 2004. Breaking seed dormancy in almond (*Prunus dulcis* (Mill.) D.A. Webb). Sci. Hort. 99, 363–370.
Gariglio, N., Gonzalez, D.E., Mendow, M., Reig, C., Agusti, M., 2006. Effect of artificial chilling on the depth of endodormancy and vegetative and flower budbreak of peach and nectarine cultivars using excised shoots. Sci. Hort. 108, 371–377.
Gaur, N.V.S., 1980. Effect of gibberellins, sulphuric acid and hand cracking on the germination and growth of seedlings in walnut. Indian J. Hort. 37 (1), 16–19.
Grigorian, V., 1972. L'embryogenèse chez l'Amandier (*Prunus amygdalus* Batsch) étude comparé de la dormancies des graines et de la dormances des bourgerons végétatifs. Ph.D. Dissertation. University of Bordeaux, Bordeaux, France, 144 pp.

Hartmann, T.H., Kester, E.D., Davies, F.T., Geneve, R.L., 1997. Plant Propagation: Principles and Practices. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 770 p.
Hilhorst, H.W.M., Karssen, C.M., 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant Growth Reg. 11, 225–238.
Kester, D.E., Raddi, P., Assay, R.N., 1977. Correlation of chilling requirements for germination, blooming and leafing within and among seedling populations of almond. J. Am. Soc. Hort. Sci. 102, 145–148.
Konarli, O., Celebioglu, G., 1977. Chilling requirement of domestic olive and walnut cultivars. Yalova-Bache Kulturleri Arastirma Enstitusu 8, 7–18.
Lang, G.A., 1987. Dormancy, a new universal terminology. HortScience 22, 817–820.
Leadem, C.L., 1997. Dormancy-Unlocking seed secrets. In: Landis, T.D., Thompson, J.R. (Tech. coords.), National Proceedings, Forest and Conservation Nursery Associations. Gen. Tech. Rep. PNW-GTR-419. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, OR, pp. 43–52.
Lewak, S., Rudnicki, R.M., 1977. After-ripening in cold-requiring seeds. In: Khan, A.A. (Ed.), The Physiology and Biochemistry of Seed Dormancy and Germination. North Holland Publishing, Amsterdam, The Netherlands, pp. 193–217.
Lin, J., Shabnay, B., Ramos, D., 1977. Pistillate flower development and fruit growth in some English walnut cultivars. Journal of the American Society for Horticultural Science. 102, 702–705.
Martinez-Gomez, P., Dicenta, F., 2001. Mechanics of dormancy in seed of peach (*Prunus persica* (L.) Batsch) cv.GF305. Sci. Hort. 91, 51–58.
Mauget, J.C., 1988. Main characteristics of bud dormancy in some walnut cultivars: consequence on date of bud break and branching of the tree. Fruits 43, 391–398.
Mauget, J.C., 1990. Bud dormancy in the cold deprived walnut tree. Acta Hort. 279, 303–310.
Mehanna, H.T., Martin, G.C., 1985. Effect of seed coat on peach seed germination. Sci. Hort. 25, 247–254.
Mehanna, H.T., Martin, G.C., Nishijuma, C., 1985. Effects of temperature, chemical treatments and endogenous hormone content on peach seed germination and subsequent seedling growth. Sci. Hort. 27, 63–73.
Mok, D.W., Mok, M.C., 1994. Cytokinins, Chemistry, Activity and Function. CRC, Boca Raton, FL.
Molchenko, L.L., Shevchuk, N.S., Yatsyshin, V.M., Krushelnitskii, A.F., 1977. Rapid preparation of *Juglans nigra* seeds for sowing. Kef. Zlutrnal 6, 1025.
Nikolaeva, M.G., 1977. Factors affecting the seed dormancy pattern. In: Khan, A.A. (Ed.), The Physiology and Biochemistry of Seed Dormancy and Germination. North-Holland Publishing, Amsterdam, pp. 51–76.
Petropoulou, S.P., 1985. Temperature related factors as selection criteria in apple breeding. Ph.D. Thesis, University of London.
Powell, L.E., 1987. Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. HortScience 22, 845–850.
Richardson, E.A., Seeley, S.D., Walker, D.R., 1974. A model for estimating the completion of rest of 'Redhaven' and 'Elberta' peach trees. HortScience 9, 331–332.
Richardson, E.A., Seely, S.D., Walker, D.R., Anderson, M.J.L., Ashcroft, G.L., 1975. Phenoclimatography of spring peach bud development. HortScience 10, 236–237.
Rodriguez, A., Canal, M.J., Sanchez-Tames, R., 1991. Seasonal changes of plant growth regulators in Corylus. J. Plant Physiol. 138, 29–32.
Samish, R.M., 1954. Dormancy in woody plants. Plant Physiol. 5, 183–204.
SAS Institute, Inc. 2002. SAS User's guide: Statistics. Version 9.1. Gray, N.C.
Scalabrelli, G., Couvillon, G.A., 1986. The effect of temperature and bud type on rest completion and the GDH requirement for bud break in 'Redhaven' peach. J. Am. Soc. Hort. Sci. 111, 537–540.
Seeley, S.D., Ayanoglu, H., Frisby, J.W., 1998. Peach seedling emergence and growth in response to isothermal and cycled stratification treatments reveal two dormancy components. J. Am. Soc. Hort. Sci. 123, 776–780.
Spiegel-Roy, P., Alston, F.H., 1979. Chilling and post-dormant heat requirement as selection criteria for late-flowering pears. J. Hort. Sci. 54, 115–120.
Stokes, P., 1965. Temperature and seed dormancy. In: Ruhland, W. (Ed.), Encyclopedia of Plant Physiology, 15/2. Springer-Verlag, Berlin, Heidelberg and New York, pp. 746–803.
Swartz, H.J., Powell, L.E., 1981. The effect of long chilling requirement on time of bud break in apple. Acta Hort. 120, 173–178.
Takahashi, N., Phinney, B.O., Macmillan, J., 1991. Gibberellins. Springer-Verlag, New York.
Taylorson, R.B., Hendricks, S.B., 1977. Dormancy in seeds. Annu. Rev. Plant Physiol. 28, 331–354.
Thomas, T.H., Kaminek, M., Mok, D.W.S., Zimalova, E., 1992. Physiology and Biochemistry of Cytokinins in Plant. SPB. Academic Publishing, Hague, the Netherlands.
Vahdati, K., Lotfi, N., Kholdebarin, B., Hasani, D., Amiri, R., 2009. Screening for drought tolerant genotypes of Persian walnuts (*Juglans regia* L.) during seed germination. HortScience 44, 1815–1819.
Weinberger, J.H., 1950. Chilling requirements of peach varieties. Proc. Am. Soc. Hort. Sci. 56, 122–128.
Welling, A., 2003. Overwintering in Woody Plants: Involvement of ABA and Dehydrins. Institute of Biotechnology and Department of Biosciences, Division of Genetics Faculty of Science, University of Helsinki, Finland, pp. 1–59.
Wieble, J., Downton, W.J.S., Chacko, E.K., 1992. Influence of applied plant growth regulators on bud dormancy and growth of mangosteen (*Garcinia mangostana* L.). Sci. Hort. 52, 27–35.
Yang, Q.H., Ye, W.H., Yin, X.J., 2007. Dormancy and germination of *Acera trianda* seeds. Sci. Hort. 113, 107–111.
Young, E., 1987. Effects of 6-BA, GA4+7 and IBA on growth resumption of chilled apple roots and shoots. HortScience 22, 212–213.
Young, E., Werner, D.J., 1984. Effects of rootstock and scion chilling during rest on resumption of growth in apple and peach. J. Am. Soc. Hort. Sci. 109, 548–551.
Young, J.A., Young, C.G., 1992. Seeds of Woody Plants in North America. Oreg. Dioscorides Press, Portland.