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The Effect of Gibberellic Acid and Chilling Stratification on Seed Germination of Eastern Black Walnut (*Juglans nigra* L.)

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Abstract

Eastern black walnut (*Juglans nigra* L.) is used as a rootstock for the Persian walnut (*Juglans regia* L.) in some parts of the world and also has an important role in forestry and wood industry. Due to the deep physiological dormancy, the seed often shows an inconsistent or low germination percentage, making establishment difficult. This experiment was carried out as a completely randomized design with eight treatments and 16 replicates in a controlled greenhouse. The objective of this study was to determine the best treatment of breaking dormancy. Treatment groups consisted of seed priming with GA_3 (400 and 800 ppm) solution for 24 hours, chilling stratification (one month and two months) and the combined treatments of chilling stratification and GA_3. Results showed that the germination rate for separate application of both concentrations of GA_3 and one month chilling treatment was zero, as no seeds germinated. The highest percentage of seed germination (69.27 %) was recorded with the combined treatment of two months chilling and GA_3 (400 ppm). Also, this treatment showed significant differences for morphological, physiological and biochemical parameters compared to other treatments. It was found that the application of the combined treatment of chilling stratification and GA_3 was effective in increasing seed germination percentage and rate as well as improving growth parameters of Eastern black walnut seedlings.

Keywords: *Juglans nigra* L., Moist-chilling, Seed dormancy, Seed priming.

Introduction

Eastern black walnut (*Juglans nigra* L.) is one of the important species and the largest of the North American walnuts, reaching a height of 45m and a trunk diameter of 2 m. It is native to the deciduous forests of the eastern USA and Canada. Eastern black walnut bears nuts with hard, black shells and stronger flavored kernels than those of *J. regia*. The irregular grooves and ridges on the shell separate it from the other species native to the USA. Eastern black walnut is more resistant to frost than the Persian walnut and now planted in Europe as both a timber species and a rootstock. It is the most valuable hardwood produced in US and used in various industries (Mc Granahan and Leslie, 2009). The seeds of this species have deep physiological dormancy that is controlled by seed coat and embryo dormancy. Germination of healthy and live seeds may be delayed even in good environmental conditions such as light, oxygen, water and chemicals due to the seed dormancy (Hilhorst, 1995). Therefore, analyzing the causes of dormancy and evaluating methods of breaking dormancy in order to increase seed germination percentage and rate is necessary (Rajabiyan *et al.*, 2007).

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Seed germination is a complex process that started with the absorption of water and after a short pause; the enzyme is activated (Matilla and Matilla-Vazquez, 2008). Gibberellins (GAs) are the hormones proposed to control primary dormancy (the form of dormancy that is acquired during seed development) by inducing germination (Hilhorst and Karssen, 1992). Gibberellic acid (GA_3) is an exogenous growth regulator that promotes germination by stimulating the activation of food-mobilizing enzymes (Hartman and Kester, 1983). The use of GA_3 has been studied in fruit culture as a way to increase seed germination and therefore to obtain a uniform seedling size in the nurseries (Hore and Sem, 1993 and Dhupper, 2013). Chilling treatments is often practiced to enhance the germination of dormant seeds (Bello *et al*., 1998 and Hassan and Fetouh 2014). It is believed that moist-chilling treatment alters the inhibitor-promoter balance. Various dormancy breaking and germination stimulating treatments have been tried with seeds of many fruit species such as papaya (Nagao and Furutani, 1986), persimmon (Taha, 1987), peach (El-Khoreiby and Salem, 1985; El-Dengawy, 1997), pears (Pipinis *et al*., 2012a) and loquat (El-Dengawy and El-Refaey, 2005; Polat, 1997). Although application of GA_3 and moist-chilling treatments seem the most promising techniques in woody species (Powell, 1987 and Nasri *et al*., 2013), it has not been investigated on breaking dormancy of Eastern black walnuts seeds yet. Therefore, the aim of this study was to determine the effect of priming with GA_3 and chilling stratification on improving germination and growth of Eastern black walnut seeds.

Materials and Methods

Seed pretreatment

Seeds were collected from a vigor genotype of Eastern black walnut (*Juglans nigra* L.) tree at Botanical Garden, College of Agriculture and Natural Resources, Karaj, Iran. Seeds were collected in mid-October 2013, when the seeds had desiccated to about 12% moisture on a dry weight basis. The seeds were surface sterilized by soaking in 5% sodium hypochlorite solution for 10 minutes and subsequently rinsed thoroughly with sterilized water prior to germination or chilling. This experiment was carried out as completely randomized design in a controlled greenhouse at Shahid Bahonar University of Kerman, Iran, with eight treatments and 16 replicates. Each replicate consisted of four pots with one plant per pot. Seeds were subjected to one of the following treatments: GA_3 (400 ppm); GA_3 (800 ppm); 1 month chilling; 2 months chilling; 1 month chilling + GA_3 (400 ppm); 1 month chilling + GA_3 (800 ppm); 2 months chilling + GA_3 (400 ppm); 2 months chilling + GA_3 (800 ppm). Chilling temperature was set to 4±1 $^{\circ}$C. The soaking time in GA_3 solution was 24 hours.

The treated seeds were sown in pots contained cocopeat and perlite sterilized by autoclaving (2:1, respectively). After sowing, the plastic pots were watered regularly and shaded in a greenhouse.

Germination test

Seeds were considered germinated when the radicle reached to half the length of the seed. At the end of germination period (eight weeks), the germination percentage (GP) was calculated using the following formula:

$$GP = \sum G/N*100$$

Where GP is the germination percentage, G is the numbers of germinated seeds and N is the numbers of all seeds (Copeland *et al.*, 2001).

Germination rate (GR) was calculated using following equation:

$$Gr = \Sigma n/\Sigma (Dn)$$

Where n is the number of seeds that germinated on day D and D is the number of days counted from the beginning of the test (Copeland *et al.*, 1995).

Morphological and physiological parameters

At the end of experiment, seedlings were cut at soil surface and the roots washed free of soil. After measuring the length of shoot and root, shoot and root fresh weights and root volume were recorded. Root volume was determined by immersing root systems in a container of water placed on a balance. The displaced water (measured in grams) is equal to the volume (measured in cubic centimeters) of the root system in that 1 g of water equals 1 cm^3 at room temperature (Burdett, 1979). In order to estimate the dry weight, the samples were oven-dried at 75 ◦C for 48 hours, until constant weight was reached. Root area was determined using the method of Atkinson (1980):

$$Y\ (cm^2) = 2\{(\text{root length} \times \text{root volume})\}^{0.5}$$

$$Y\ (cm^2) = \text{root area}$$

The Chlorophyll index of leaf was measured by using Minolta (SPAD) Chlorophyll Meter (SPAD-502 Minolta Sensing Inc., Japan).

Biochemical parameters

The amount of photosynthetic pigment (chlorophyll a, b, total and carotenoids) was determined according to the method of Lichtenthaler *et al.* (1987). The pigment extract was measured vs. a blank of 80% (V/V) acetone at wavelengths of 646.8 nm and 663.2 nm for chlorophyll assays. Reducing sugars was measured by the method described by Somogyi (1952). The absorbance was measured at 600 nm. Glucose was used as standard solution.

Statistical analysis

Analysis of variance and comparison of means were performed using GLM procedure of SAS (SAS Institute, Inc., 2002). Data were subjected to transformation and normalization where necessary (Zar, 1999). Significant differences among the mean values were compared by Fisher's Least Significant Difference (LSD) ($P \leq 0.05$).

Results

Results showed that neither the seeds treated by GA_3 at 400 ppm and 800 ppm nor one month chilling stratification treatment germinated in the greenhouse. Therefore, data analysis and mean separation were performed for combined treatments. Seed germination percentage and rate were significantly different among treatments. Analysis of variance showed that there were significant differences in terms of morphological, physiological and biochemical parameters (Table 1). It was also found that two months chilling stratification in combination with GA_3 (400 and 800 ppm) resulted significant higher germination percentage and rate than other treatments (Fig. 1).

Table1. Analysis of variance of priming with GA_3 and chilling stratification on germination of Eastern black walnut seed (*Juglans nigra* L.)

S.O.V	df.	Germination percentage	Germination rate	Root length	Shoot length	Shoot diameter	Root volume	Root area	Total FW	Shoot FW
					MS					
Treatment	4	2815.20**	179.50**	34.08**	33.60**	0.26^{ns}	8.38**	17.36**	9.65**	4.83**
Error	50	2.96	33.53	10.40	6.81	0.13	1.18	3.51	1.92	0.42

S.O.V	df.	Root FW	Shoot DW	Root DW	Chlorophyll Index	Chlorophyll Total	Chlorophyll a	Chlorophyll b	Carotenoid	Reducing sugars
						MS				

Table 1. Continued

Treatment	4	3.22*	1.68**	1.60**	270.75**	271.28**	94.66**	45.91**	34.34**	0.001**
Error	50	1.23	0.09	0.11	0.33	0.42	0.19	0.14	0.09	0.00008

ns, **and * means non-significant, significant at 1% and 5% of probability respectively.

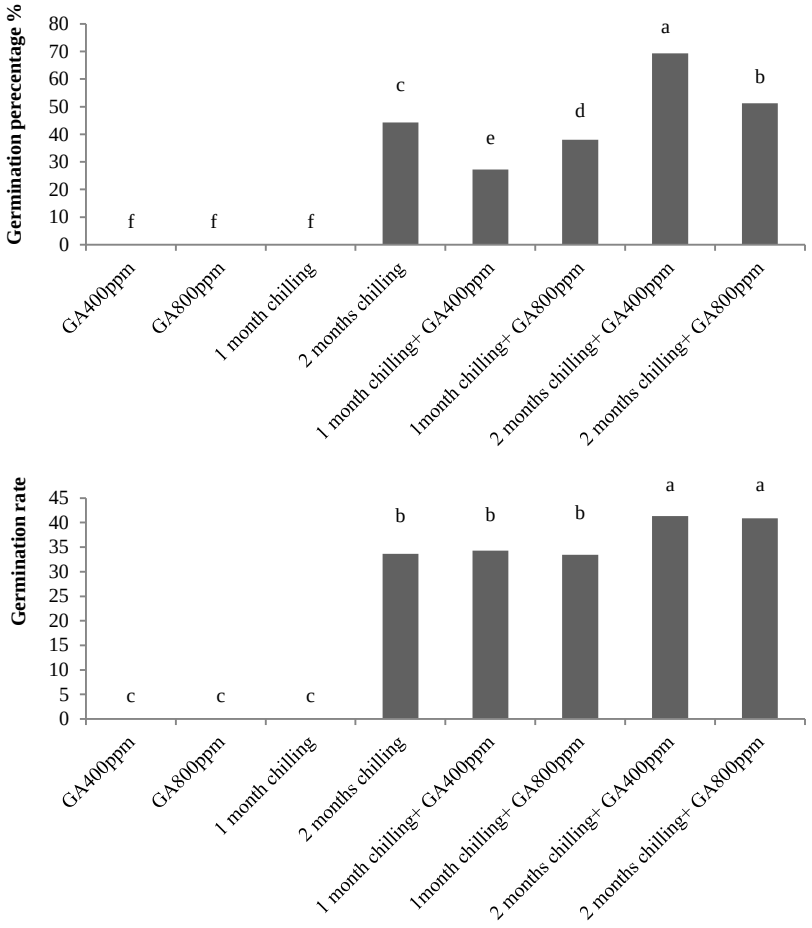

Fig. 1. The effect of priming with GA_3 and chilling stratification on germination percentage and rate of Eastern black walnut seed (*Juglans nigra* L.). Values are means ± SE and the means with the same letter are not significantly different at 5% LSD test.

Results showed that two months chilling stratification in combination with GA_3 (400 ppm) significantly improved seedling growth characteristics including seedling length, root length, root volume, root area, total fresh and dry weights as well as total chlorophyll and reducing sugars compared to other treatments (Tables 2, 3 and 4).

Table 2. The effect of GA_3 and chilling stratification on morphological parameters of Eastern black walnut seedling (*Juglans nigra* L.)

Treatments*	Stem length (cm)	Stem diameter (mm)	Root length (cm)	Root volume (cm^3)	Root area (cm^2)
2 months chilling	20.45±2.53b**	3.40±0.39ab	20.45±2.50b	7.18±1.2b	17.61±1.1 ab
1 month chilling+ GA_3 400ppm	19.99±2.90b	3.32±0.35ab	22.24±2.89ab	5.36±0.92c	15.33±1.35 c
1 month chilling+ GA_3 800ppm	21.86±1.62b	3.56±0.4ab	20.31±3.28b	6.63±1.20b	16.32±1.99 bc
2 months chilling+ GA_3 400ppm	24.58±3.06a	3.66±0.38a	24.58±3.61a	7.63±0.67a	18.64±2.29 a
2 months chilling+ GA_3 800ppm	21.36±2.67b	3.30±0.24b	23.07±3.66ab	6.27±1.27bc	16.88±2.28 bc

* Application of GA_3 at both concentrations (400 and 800 ppm) and also one month chilling stratification had no germination percentage, therefore, the analysis of mean comparison was carried out among other treatments.
** Values are means ± SE and the means with the same letter are not significantly different at 5% LSD test.

Table 3. The effect of GA_3 and chilling stratification on physiological parameters of Eastern black walnut seedling (*Juglans nigra* L.)

Treatments*	Total FW (g)	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root FW (g)	Chlorophyll Index
2 months chilling	12.25±1.42b**	4.88±0.96b	7.19±0.78b	1.38±0.36b	1.53±0.37b	37.70±0.39d
1 month chilling+ GA_3 400ppm	11.80±1.66b	4.57±0.43b	7.08±1.20b	1.29±1.27b	1.35±0.32b	34.59 ±0.77e
1 month chilling+ GA_3 800ppm	12.44±1.20b	4.67±0.68b	7.59±1.22ab	1.52±0.24b	1.37±0.31b	39.99 ±0.40c
2 months chilling+ GA_3 400ppm	14.23±0.96a	6.21±0.34a	8.46±0.98a	2.26±0.27a	2.28±0.30a	47.06 ±0.49a
2 months chilling+ GA_3 800ppm	13.03±1.56b	5±0.65b	7.58±1.27ab	1.52±0.34b	1.55±0.39b	44.04 ±0.70b

* Application of GA_3 at both concentrations (400 and 800 ppm) and also one month chilling stratification had no germination percentage, therefore, the analysis of mean comparison was carried out among other treatments.
** Values are means ± SE and the means with the same letter are not significantly different at 5% LSD test.

Table 4. The effect of GA_3 and chilling stratification on biochemical parameters of Eastern black walnut seedling (*Juglans nigra* L.)

Treatments*	Total Chlorophyll (mg/g FW)	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Carotenoid (mg/g FW)	Reducing sugars (mg/g FW)
2 month chilling	20.09 ±0.94d**	13.95 ±0.46d	6.13 ±0.48d	5.76±0.33d	0.11 ± 0.009b
1 month chilling+ GA400ppm	16.24 ±0.36e	13.95 ±0.55e	4.80 ±0.33e	3.13±0.37e	0.11 ± 0.008b
1month chilling+ GA800ppm	22.90 ±0.62c	15.26 ±0.38c	7.64 ±0.31c	5.76±0.33c	0.12 ± 0.008b
2 months chilling+ GA400ppm	29.33 ±0.68a	19.17 ±0.42a	10.16 ±0.41a	7.90±0.28a	0.14 ± 0.011a
2 months chilling+ GA800ppm	25.17 ±0.48b	16.94 ±0.31b	8.22 ±0.33b	6.68±0.19b	0.11 ± 0.008b

* Application of GA_3 at both concentrations (400 and 800 ppm) and also one month chilling stratification had no germination percentage, therefore, the analysis of mean comparison was carried out among other treatments.
** Values are means ± SE and the means with the same letter are not significantly different at 5% LSD test.

Discussion

Results showed that Eastern black walnut seeds display an endogenous dormancy that might be removed by priming with moist-chilling and GA_3 for a certain period of time. It was found that seeds treated with GA_3 at two concentrations (400 ppm and 800 ppm) for 24 hours and one month chilling treatment alone showed zero germination percentage. A similar trend was observed by Conner *et al.* (2008) on 'Fry' muscadine seed.

The effect of GA_3 and stratification on enhancing growth could be attributed to the solubility of fats and sugars due to stratification plus the increasing of gibberellins synthesizing enhanced the growth. In addition, the improving effect of GA_3 and stratification on seed germination may reflect on enhancing the shoot parameters. These results are in agreement with Dahkaei (2009) on *Danae racemosa*, Rawat *et al*. (2010) on *Punica granatum* and Hassan and Fetouh (2014) on seeds of *Magnolia grandiflora*.

The effect of GA_3 and stratification on root parameters followed the same trend of its effect on shoots. The promotion effect of GA_3 and stratification on root parameters might be explained through the role of GA_3 and stratification in enhancing gibberellins synthesis which also leads to increase the growth and root branching and overall increased roots fresh weight (Penfield *et al.* 2005). The results of the present experiment are in agreement with Rawat *et al.* (2010) on *Punica granatum* and Hassan and Fetouh (2014) on *Magnolia grandiflora* seeds.

It was also found that GA_3 (400 ppm) combined with two months chilling stratification increased the chlorophyll and carotenoid contents and reducing sugars. These results are in accordance with the findings of Amooaghaie (2009) on *Ferula ovina* seeds. The increase of reducing sugars could be explained by the possible conversion of fatty acids to sugars and the conversion of soluble sugars to reducing sugars. It is also possible that insoluble sugars were converted to soluble and reducing sugars during seed germination and seedling growth and development. Previous studies showed a preference of sugars to fatty acids for energy production during germination of oil seed crops (To *et al*., 2002; Tonguc *et al*., 2012).

It has been reported that seeds of the Persian walnut (*Juglans regia* L.) treated with two months of chilling had higher germination percentage and rate compared to one month chilling treatment and significantly improved seedling characteristics (Vahdati *et al*. 2012). Hassan and Fetouh (2014) and Pipinis *et al*., (2011b) reported a similar trends on seeds of *Magnolia grandiflora* and *Cercis siliquastrum* seeds, respectively. However, in the present study, one month of chilling treatment showed no impact on improving seed germination while combined treatment of two months of chilling and GA_3 (400 ppm) showed the highest seed percentage and rate. According to Khan (1977) and Hassan and Fetouh (2014), stratification affects metabolic processes including changes in hormones, disappearance of ABA, activation of GA_3 and initiation of germination. Previous studies have shown that after-ripening and moist (warm or cold) stratification affect metabolic and physiological changes in seeds that involve both the embryo and its covering layers (Leubner-Metzger, 2005; Bair *et al*., 2005 and Shakarishvili *et al*., 2013). It was also indicated that seed stratification causes a rapid decline in the abscisic acid (ABA) content and ABA sensitivity and increases GA_3 sensitivity of imbibed dormant seeds (Gubler *et al*., 2005).

Exogenous application of GA_3 has been reported to be effective in breaking dormancy and substituting for the chilling requirement in seeds of many species (Karam and Al-Salem, 2001; Pipinis *et al*., 2011a; 2012; Smiris *et al*., 2006 and Dhupper, 2013). Ghayyad *et al.* (2010) reported that GA_3 is effective in shortening the chilling requirement. However, in the present study, the

application of GA_3 treatments separately showed zero germination percentage. Since the highest percentage of seed germination and seedling growth parameters were recorded for a two months chilling period combined with GA_3 (400 ppm), it may be possible that higher concentration of GA_3 (800 ppm) could not shorten the chilling period.

It was found that GA increases the growth potential of embryo and promotes germination and is necessary to overcome the mechanical restraint conferred by the seed covering layers by weakening of the tissues surrounding the radicle (Finch-Savage and Leubner-Metzger, 2006).

The combination of chilling stratification and GA_3 pretreatment has been reported to improve germination in *Prunus* species (Imani *et al.*, 2011) and cherry seeds (Al-Absi, 2010). Moreover, GA_3 and chilling stratification affect physiological and metabolic activities of seeds resulting in early germination, which was previously reported (Amooaghaie, 2010; Zeinalabedini, 2009 and Pipinis *et al*., 2012b).

The combination of GA_3 (400 ppm) and chilling might be more effective in bringing a hormonal shift that not only enhanced germination but also sped it up. Such results are in accordance with those of Nasri *et al*. (2013), Amooaghaie (2009) and El-Dengawy (1997) on peach seeds and Chin *et al.* (1992) on kiwi fruit seeds. They concluded that the combination between a suitable moist-chilling period and an effective level of GA_3 would considerably enhance seed germination, which is in accordance with the present study.

In conclusion, it was found that the combined treatments of two months of chilling followed by soaking in GA_3 (400 ppm) solution for 24 hours might be recommended for improving the seed germination process and improving growth characteristics of the Eastern black walnut seedlings.

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