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GA_{4+7} Soak Before Cold Stratification Enhances *Juglans nigra* Seedling Production

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Abstract

Eastern black walnut (*Juglans nigra* L.) seeds typically require a long period of stratification and often have low germination. A study was conducted to evaluate the effect of three formulations of gibberellic acid at 250 mg·L^{-1} or tap water as a soaking treatment for 'Thomas' black walnut seeds for 24 h before stratification for 30, 45, 60, 75, or 90 d. Gibberellic acid treatments included 1) GA_3 (ProGibb®); 2) GA_{4+7} (Provide®); and 3) 6-benzyladenine (BA) + GA_{4+7} (Promalin®). Percent walnut shoot emergence 60 days after planting, days to 20% and 80% shoot emergence (E20 and E80), and early seedling growth from black walnut seeds were evaluated. Percent shoot emergence was always higher for seeds soaked in GA_{4+7} or BA + GA_{4+7} when compared with other treatments. Shoot emergence for some seeds soaked in GA_{4+7} and BA + GA_{4+7} occurred with 30 d stratification and percent emergence increased with longer stratification periods. Seeds soaked in GA_3 had higher percent shoot emergence than those soaked in tap water only. Also, seeds soaked in GA_3 had fewer days to 20% shoot emergence when stratified for 45 or 60 d than those soaked in tap water and stratified for the same period of time. Addition of BA at 250 mg·L^{-1} apparently did not enhance percent shoot emergence, E20, E80, or seedling height or weight. With timely harvest, hulling, seed selection, and soaking walnuts with 250 mg·L^{-1} GA_{4+7} followed by 90 d stratification, 82% shoot emergence (i.e., germination) was attained.

Eastern black walnuts are recalcitrant and often have a low germination percentage (Dorn and Mudge, 1985; Flores et al., 2016). Immediately after harvest, intact black walnut seeds generally require stratification for 90 to 120 d, resulting in only about 50% germination (Brinkman, 1974). Early workers recommended immediate hulling after harvest, air-drying, and storage in moist peat at 1 to 3 °C for five to six months to promote seed germination (Muenscher and Brown, 1943). Later propagation methods included selecting large black walnut seeds for stratification and floating hulled nuts in water to remove small walnuts with shriveled (i.e., stenospermocarpic) kernels (Brinkman, 1974; Chase, 1947; Warmund and Van Sambeek, 2014). Cracking hulled black walnuts before stratification slightly improved germination percentage compared with the untreated controls (64% vs. 54%) when evaluated 270 days after planting, but cracking sometimes damaged kernels or increased kernel susceptibility to pathogenic microorganisms (Gaur, 1980). *Penicillium*, *Mucor*, *Phompsis*, *Fusarium*, and *Papulaspora* spp. were isolated from cotyledonary surfaces of kernels when walnuts were cracked (Kessler, 1978). Stratification and soaking intact walnuts in 10 or 20% sulfuric acid solutions for 30 min also reduced germination compared with untreated stratified controls (Gaur, 1980).

Exogenous application of gibberellic acid to recalcitrant seeds promotes germination (Frankland, 1961). For black walnut, stratification and GA_3 treatments at 125 or 250 mg·L^{-1} enhanced germination after plant-

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ing in outdoor nursery beds in Butan (Gaur, 1980). Dorn and Mudge (1985) reported conflicting results for enhanced black walnut germination when shells were notched, seeds were subsequently vacuum-infiltrated with GA_3 and placed in a greenhouse at 21°C under mist. Soaking intact black walnuts in GA_3 at 400 mg·L^{-1} and stratification for two months resulted in 69% germination in a shaded greenhouse in Iran (Parvin et al., 2015).

The use of other gibberellins and cytokinins to promote germination of seeds, including *Juglans microcarpa* (C.A. Leslie, personal communication) and *Corylus avellana* (Frankland, 1961), has been studied. However, results of early experiments investigating the use of growth regulators to replace or reduce stratification are unclear due to the paucity of information regarding time of fruit harvest and hulling, as well as storage of nuts before stratification was initiated. Thus, the objective of this study was to evaluate percent shoot emergence, days to 20 and 80% shoot emergence (E20 and E80), and early seedling growth of hulled 'Thomas' black walnuts soaked in either of two forms of gibberellins alone (GA_3, GA_{4+7}), BA + GA_{4+7}, or tap water for 24 h before stratification for 30, 45, 60, 75, or 90 d.

Materials and Methods

Seeds from seven 'Thomas' black walnut trees grafted to seedling Thomas rootstock and planted in the clonal repositories at the Horticulture and Agroforestry Research Center, New Franklin, MO, were used for this study. Trees were selected based on age (20 years-old) and their genetic identities confirmed by DNA fingerprinting, using a series of ten single sequence repeat microsatellite markers (Warmund and Coggeshall, 2010). Trees were spaced 12.1 x 12.1 m apart and were growing without irrigation or pesticides in a Menfro silt loam (fine-silty, mixed, superactive, mesic typic hapludalfs). Pelletized ammonium nitrate (34N-0P-0K) was applied annually with 67 kg·ha^{-1} and 45 kg·ha^{-1} in late April and late October, respectively. Ground cover was mowed as needed. When ~ 20% of the walnuts were on the ground, those remaining on trees were harvested with a tree shaker (Model 2138, Savage Equipment, Madill, OK) and a collection device (Nut Wizard, Louisville, KY) was used to gather fruits from the ground on 11 Oct. 2016. Immediately after harvest, fruits from each tree were hulled with a locally produced machine (Lane, 2000). The following day each nut was weighed and those > 31 g were soaked in growth regulator solutions (250 mg·L^{-1} gibberellic acid) or water. Soaking treatments included GA_3 (ProGibb®; Valent BioSciences, Walnut Creek, CA), GA_{4+7} (Provide®; Valent BioSciences, Walnut Creek, CA), BA + GA_{4+7} (Promalin®; Valent BioSciences, Walnut Creek, CA), and tap water. For the five replications of each treatment, 125 walnuts were soaked in 19 L-plastic containers using a 5 L solution. After walnuts were soaked for 24 h, they were air-dried at 21 °C for 15 min. Next, 25 of the 125 walnuts were placed in 3.8 L polyethylene bags (Pactiv Corp., Lake Forest, IL) and stored at 5 °C for 30, 45, 60, 75, or 90 d for stratification. After each stratification period, five replications of 25 walnuts of each treatment were planted in 40 x 40 x 15-cm (depth) polyethylene flats (Stuewe & Sons, Tangent, OR), using potting medium (ProMix; Premier Tech Horticulture, Québec, Canada) moistened with 1 L tap water. For each stratification period, flats were arranged in randomized complete block design in the greenhouse maintained at 26 °C under natural light and uniformly irrigated as needed. Shoot emergence (i.e., seed germination) was recorded every other day for 60 d. For germinants, the mean number of days to 20% (E20) and 80% (E80) shoot emergence during the 60-day greenhouse period was calculated. Germinants were then harvested, roots were washed free of potting media, and plant tissue (excluding nut shells) was oven-dried at 65 °C for 48 h to determine

seedling dry weights. Non-germinated seeds were cut transversely on a bandsaw to assess the cotyledons. Stenospermocarpic seeds and those with decayed cotyledons were omitted from statistical analyses.

Because no water-soaked control seeds germinated with 30 d of stratification, these data were omitted from statistical analyses. For all other stratification periods (45, 60, 75, and 90), the odds (i.e., probability) of shoot emergence of each growth regulator treatment were calculated, using the GLMMIX procedure of the SAS statistical analysis software (SAS Institute, Cary, NC) with a link = logit function for a binomial distribution due to the non-normal distribution of data. Odds were calculated from the antilog of the logit value and back-transformed [% shoot emergence = odds / (1 + odds)] for reporting shoot emergence percentage by stratification time for each growth regulator treatment. Mean differences among odds were determined using the LSMEANS statement ($P \leq 0.05$). Days to E20 and E80, seedling height, and dry weight were subjected to analysis of variance (ANOVA) using the PROC GLMMIX procedure of SAS and means were separated by Fisher's protected LSD test ($P \leq 0.05$). Orthogonal contrasts were performed to evaluate linear, quadratic, and cubic responses to varying stratification times, using the PROC GLM procedure of SAS.

Results

Percent shoot emergence varied among growth regulator treatments and the stratification times, but there was no interaction between these variables (Table 1). After 60 d in the greenhouse, the average percent shoot emergence for BA + GA_{4+7}, GA_{4+7}, and GA_3 seeds stratified for 30 d was 20, 18, and 6, respectively, whereas none of the water-soaked control seeds germinated at this time (data not shown). For all other stratification periods, seeds were more likely to emerge when soaked in BA + GA_{4+7} (62% emergence) or GA_{4+7} (60% emergence) than when soaked in GA_3 (34% emergence) (Table 1). Walnuts soaked in water alone were the least likely to germinate with only 27% shoot emergence across all stratification periods. For 45, 60, 75 and 90 d of stratification,

Table 1. Percent shoot emergence of *Juglans nigra* seed treated with selected growth regulators and stratification periods.[z]

	Stratification time (d)[y]				
Treatment	45	60	75	90	Mean (treatment)
BA + GA_{4+7}	34	52	73	87	62 a
GA_{4+7}	33	47	78	82	60 a
GA_3	10	23	42	60	34 b
Water only	4	18	33	52	27 c
Mean (stratification time)	20 d	35 c	57 b	70 a	
Significance					
Treatment (T)	<0.0001				
Stratification time (ST)	<0.0001				
T x ST	0.3840				

[z] Walnut seeds were soaked in solutions of each growth regulator (250 $mg \cdot L^{-1}$ gibberellic acid) before stratification and were subsequently grown in a greenhouse for 60 d.

[y] Values represent 5 replications per treatment with 25 seeds sown per replication. PROC GLIMMIX using a logit link for binomial distributions was used to analyze germination data. Back transformed data [% shoot emergence = odds (1+odds)] are presented. Mean differences among odds were determined using the LSMEANS statement. Within a column or row, means followed by the same letter are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

percent shoot emergence across all treatments increased significantly for each period of time (20, 35, 57, and 70%, respectively).

When days to E20 were calculated for 30 day stratification periods, seeds soaked in BA + GA_{4+7} averaged 46 d and those in GA_{4+7} averaged 50 d (data not shown). Since only 6% of the shoots of seeds in GA_3 treatments stratified for 30 d emerged, E20 (and E80) values were not calculated. For all other stratification periods, there was a significant interaction of treatment and stratification time for days to E20, and a linear response to stratification time (Table 2). Walnuts soaked in water only and stratified for 45 or 60 d had greater E20 values than all other treatments and stratification times. When the stratification period was 75 days, seeds soaked in GA_3 and water required 34 and 37 d for 20% germination, respectively, whereas those soaked in BA + GA_{4+7} or GA_{4+7} required ~ 29 to 30 d. E20 values for walnuts soaked in BA + GA_{4+7} or GA_{4+7} were also lower than that of water–soaked seeds after 90 d of stratification, but E20 values were similar for walnuts soaked in BA + GA_{4+7} or GA_3.

E80 values also differed among treatments (Table 3). Walnuts soaked in BA + GA_{4+7} or GA_{4+7} had lower mean E80 values (49 d) than seeds treated with GA_3 (51 d) or soaked in water alone (53 d). E80 values exhibited a quadratic response to stratification time. For 45, 60, 75, and 90 d of stratification, E80 values for walnuts were 58, 56, 50, and 38 d, respectively. There was no interaction of treatment and stratification time for E80 values.

Seedling heights of walnuts soaked in BA + GA_{4+7} or GA_{4+7} and stratified for 30 days averaged 10.8 and 13.6 cm, respectively, after 60 d in the greenhouse. Seedling dry weight for BA + GA_{4+7}-soaked walnuts stratified for 30 d was 1 g and that for GA_{4+7} was 1.6 g. For all other stratification times, seedling heights and dry weights for seeds soaked in BA + GA_{4+7} or GA_{4+7} were greater than those receiving other treatments (Table 4). However, seedling dry weight of GA_3-

Table 2. Days to 20% (E20) shoot emergence of *Juglans nigra* seed treated with selected growth regulators and stratification periods.[z]

	Stratification time (d)[y]				
Treatment	45	60	75	90	Mean (treatment)
BA + GA_{4+7}	41.4	33.4	29.6	24.0	32.1 c
GA_{4+7}	38.8	32.8	29.2	21.8	30.7 c
GA_3	42.0	41.8	34.4	26.4	36.2 b
Water only	52.0	47.4	37.4	28.4	41.3 a
Mean (stratification time)	43.6 a	38.9 b	32.7 c	25.2 d	
Significance[y]					
Treatment (T)	<0.0001				
Stratification time $(ST)_L$	<0.0001				
ST_Q	0.0682				
T x ST_L	0.0147				
T x ST_Q	0.1279				

[z] Walnut seeds were soaked in solutions of each growth regulator (250 mg·L^{-1} gibberellic acid) before stratification and were subsequently grown in a greenhouse for 60 d. Means represent 5 replications per treatment with 25 seeds sown per replication. For germinants, the mean number of days to 20% (E20) shoot emergence was calculated. Within a column or row, means followed by the same letter are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

[y] Linear (L), quadratic (Q), and cubic orthogonal contrasts were performed to test the trend of different stratification times for E20.

Table 3. Days to 80% (E80) shoot emergence of *Juglans nigra* seed treated with selected growth regulators and stratification periods.[z]

	Stratification time (d)				
Treatment	45	60	75	90	Mean (treatment)
BA + GA_{4+7}	57.6	53.4	48.2	34.8	48.5 c
GA_{4+7}	57.6	54.6	47.8	35.2	48.8 c
GA_3	58.0	57.4	50.6	38.6	51.2 b
Water only	59.0	58.2	53.6	41.2	53.0 a
Mean (stratification time)	51.8 a	55.9 b	50.1 c	37.5 d	
Significance[y]					
Treatment (T)	<0.0001				
Stratification time $(ST)_L$	<0.0001				
ST_Q	0.0001				
T x ST_L	0.0913				
T x ST_Q	0.8109				

[z] Walnut seeds were soaked in solutions of each growth regulator (250 mg·L^{-1} gibberellic acid) before stratification and were subsequently grown in a greenhouse for 60 d. Means represent 5 replications per treatment with 25 seeds sown per replication. For germinants, the mean number of days to 80% (E80) shoot emergence was calculated. Within a column or row, means followed by the same letter are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

[y] Linear (L), quadratic (Q), and cubic orthogonal contrasts were performed to test the trend of different stratification times for E80.

soaked walnuts was greater than that of water-soaked controls. Seedling height and dry weight exhibited quadratic responses to stratification time (Table 4). For 45, 60, 75 and 90 d of stratification, seedling height increased at each period of time. However, walnuts receiving 90 d stratification had greater seedling dry weight than those receiving fewer days of stratification.

Discussion

Plant hormone concentrations fluctuate in walnut seed tissues during stratification and the period immediately afterwards when exposed to warm temperatures (Somers et al., 1989). The highest concentrations of GA_3 and GA_{4+7} were recovered from the embryonic axis of seeds during the first 60 d of stratification, decreased when sampled at 120 or 180 d of chilling, but increased when seeds were removed from cold storage and exposed to ambient temperatures. The highest concentrations of abscisic acid and cytokinins were recovered in the embryonic axes when analyzed at 180 d of stratification, but decreased when removed from cold storage. In seeds, gibberellins induce enzymatic activity which degrades cell walls in the endosperm and subsequently hydrolyzes starches and protein into compounds needed for cellular activity and embryonic growth (Somers and Van Sambeek, 2003; Weaver, 1972). Genetic control of gibberellin biosynthesis, metabolism, and signaling has been studied in *Arabidopsis* and cereal crops, but this has yet to be explored in woody plants (Hedden and Thomas, 2016).

Exogenous applications of gibberellin have been used successfully to break seed dormancy and enhance seed germination of many plant species (Krishnamoorthy, 1975; Weaver, 1972). In our study, soaking walnut seeds in GA_3, GA_{4+7}, or BA + GA_{4+7} before stratification enhanced shoot emergence and required fewer days to attain 80% shoot emergence when compared with a tap water only soak (Tables 1 and 3). GA_{4+7} treatments were more effective in promoting seed

Table 4. Height and dry weight of *Juglans nigra* seedlings after 60 d in a greenhouse.[z]

Main effect	Seedling ht. (cm)	Seedling dry wt. (g)
Treatment		
BA + GA_{4+7}	24.9 a	1.96 a
GA_{4+7}	23.9 a	1.82 a
GA_3	20.0 b	1.47 b
Water only	19.3 b	1.13 c
Stratification time (d)		
45	31.3 d	1.27 b
60	50.0 c	1.35 b
75	75.3 b	1.48 b
90	94.3 a	2.28 a
Significance[y]		
Treatment (T)	<0.0001	<0.0001
Stratification time $(ST)_L$	<0.0001	<0.0001
ST_Q	<0.0001	<0.0001
T x ST_L	0.5056	0.0696
T x ST_Q	0.8387	0.0247

[z] Walnut seeds were soaked in solutions of each growth regulator (250 mg·L^{-1} gibberellic acid) before stratification and were subsequently grown in a greenhouse for 60 d. Means represent height and weight of germinants from 5 replications per treatment with 25 seeds sown per replication. Within each column, means followed by the same letter are not significantly different, according to Fisher's protected LSD test ($P \leq 0.05$).

[y] Linear (L), quadratic (Q) orthogonal contrasts were performed to test the trend of different stratification times. *P* values for ANOVA.

germination than GA_3 (Table 1). These results agree with others where a GA_{4+7} treatment resulted in greater seed germination of *Galeopsis pyrenaica*, *Lycopus europaeus* (Thompson 1969) and cereals (Mayer and Poljakoff-Mayber, 1989) than a GA_3 treatment. Additionally, Thompson (1969) reported that GA_{4+7} promoted germination at lower concentrations than GA_3, which may explain the lower percent shoot emergence for GA_3-soaked seeds than GA_{4+7}-soaked seeds in our study using one concentration of gibberellin (250 mg·L^{-1}).

Although cytokinins are commonly associated with the promotion of cell division, there are reports of enhanced germination following their exogenous application to some seeds or their embryos after pericarp and testa tissues were removed (Frankland, 1961; Weaver, 1979). In experiments with *Juglans microcarpa*, intact seeds soaked in BA + GA_{4+7} (Promalin®) at 62.5, 125 or 250 mg·L^{-1} had 70% to 90% germination, but treatments without BA were not included in the study (Leslie et al., 2014). In our study, percent shoot emergence, days to shoot emergence, and seedling heights and dry weights were similar for GA_{4+7} soaking treatments with or without BA. These results indicate that BA at 250 mg·L^{-1} did not enhance black walnut germination and there would be no additional benefit derived from its use, especially due to the higher product cost of BA + GA_{4+7} relative to that of GA_{4+7}. Shoot emergence from BA + GA_{4+7} and GA_{4+7} –soaked seeds occurred with as little as 30 d stratification and percent emergence increased with longer stratification periods (Table 1). About 50% shoot emergence occurred in the present study when seeds were soaked in either GA_{4+7} treatment and stratified for 60 d, whereas those in tap water required

an additional month of stratification to attain the same emergence percentage. After a 90 d stratification period, GA_{4+7}-treated black walnuts had over 80% germination (i.e., shoot emergence) while seed soaked in water had 52%, which is close to the typical 50% germination reported by Brinkman (1974). Days to 20% shoot emergence for seeds treated with GA_{4+7} were also reduced by 17 d and E80 values were reduced by 22 days when stratification time was increased from 45 to 90 d. The taller seedling heights and plant dry weights resulting from GA_{4+7} treatments compared with the tap water soak are likely due to the more rapid seed germination which allowed more time for growth in the greenhouse.

In conclusion, high percentages (73% to 87%) of shoot emergence (i.e., germination) were attained in this study, which may be attributed to timely harvest, immediate hulling of fruits, selection of sound walnuts with high fresh weight, immediate soaking in 250 $mg \cdot L^{-1}$ GA_{4+7} followed by 75 to 90 d stratification, and exposure to relatively high temperatures after cold storage. The increased seed germination derived from a GA_{4+7} soak and rapid early seedling growth provides nurserymen with a more efficient method for black walnut seedling production. This may be especially significant for nurserymen who start black walnut seeds in greenhouses for rapid germination and early seedling growth, resulting in larger plants than those grown in outdoor nursery beds.

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