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Effect of Cold Stratification and GA_3 on Deshelled Seeds Germination and Seedlings Growth of Bitter Almond

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ABSTRACT

Bitter almond rootstock can resist drought and soil pests. Therefore, it is used as a rootstock for almond trees. Seeds have two types of dormancy; endocarp and embryo dormancy, so before germination, some chemical and physiological treatments must occur in the seeds to contribute to the speed and regularity of germination, which is desirable, especially for commercial production. A nursery experiment was conducted during 2017/2018 & 2018/2019 at Kalubia governorate, Egypt to evaluate the effects of cold stratification (CS) periods (0, 2, 4, 6, 8 weeks) and gibberellic acid (GA_3) concentrations (0, 100, 200, 300 ppm) on de-shelled bitter almond seed germination rate, mean germination time, and growth of produced seedlings. The highest germination percentage (80.4%) and lowest mean germination time (13.8 days) were obtained by combining CS periods for 6 weeks with GA_3 at 300 ppm. The better seedlings growth parameters; stem and root length, diameter, fresh and dry seedlings weight, number of lateral shoots, number of roots and number of leaves/seedling were obtained from an application of the GA_3 at 300 ppm plus CS for 6 and 8 weeks without any significant differences between them at the same concentration. As a result, it was concluded that treating bitter almond seeds with a high concentration of gibberellin has shortened the stratification period required for seeds germination. Thus, CS for 6 weeks and applications GA_3 at 300 ppm can be recommended to break dormancy de-shelled seeds to increase seed germination rate and enhance the growth of produced bitter almond seedlings.

Keywords: Bitter almond, de-shelled, cold stratification, GA_3, seed germination, seedling growth.

1. Introduction

Bitter almond seedlings are the most important rootstocks of almond cultivars. It is more resistant to drought and soil pests than that obtained from sweet almond seeds (Parvaneh, *et al.,* 2011). Successful seed germination is hampered by many endogenous and exogenous factors that lead to different types of dormancy, as in *Prunus* species. Two main mechanisms of dormancy have been described in *Prunus* species: an external mechanism controlled by the shell (endocarp) and the testa (maternal tissue), and an internal mechanism controlled by the embryo, which affects the later growth of seedlings (Martínez-Gómez and Dicenta, 2001; García-Gusano *et al.,* 2004; García-Gusano *et al.,* 2009). Under natural conditions, the release of *Prunus* species dormancy generally occurs during stratification (imbibition at low temperature), is regulated by a combination of environmental and endogenous signals with both synergistic and competing effects (Imani, *et al.,* 2011). There are several methods of breaking seed dormancy and stimulating germination in *Prunus* species. The most common is the application of hormones such as gibberellic acid or the mechanical removal of shell or cold stratification, which is known to have a positive effect on seed dormancy (Rajjou *et al.,* 2012; Dhupper, 2013; Hassan and Fetouh, 2014).

Almond seeds are surrounded by a hard shell (endocarp) that has a mechanical effect, affecting gas exchange, preventing seed imbibition and washing out of the endocarp hormones, which can make the germination rate low (García-Gusano *et al.,* 2005). De-shelled (removal endocarp) contributes significantly to the acceleration of the germination process; possibly by removing the phenolic

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compounds and alkaloids contained in the seed coat, which inhibit seed germination, and also the entry of water in the reserves that allow the fast exit of the root and the starting of the metabolic reactions of the embryo and accelerate seed germination (Chebouti-Meziou *et al.,* 2014).

In addition, many studies have been carried out such as cold stratification and exogenous application of gibberellic acid and its roles effective in breaking seed dormancy and promoting germination (Acar, *et al.,* 2017; Chetouani *et al.,* 2017; Yasar and Acar, 2019).

Therefore, the purpose of this study was to evaluate the effect of interaction between cold stratification periods and different gibberellic acid concentrations on the germination percentage of bitter almond and seedling growth.

2. Materials and Methods

2.1. Study area

The study was carried out during the two successive seasons of 2017/2018 & 2018/2019 in a private greenhouse at Kalubia governorate, Egypt.

2.2. Seeds collection

Seeds were collected from mature bitter almond fruits (*Prunus amygdalus* L.) grown in Saint Catherine located at South Sinai of Egypt. Fruits were harvested in August during both seasons 2018 & 2019 and packed in plastic bags and transported to the laboratory. The hull (exocarp and mesocarp) was removed by hand, then seeds were stored in burlap bags in a dry place and well ventilated at room temperature till used in the experiment.

2.3. Treatments

De-shell seeds (without endocarp) were surface sterilized in 10 % chlorox for 10 min and rinsed with distilled water three times, afterwards were mixed with sand moister and stratified in plastic boxes (15 × 10 × 5 cm) at 5 ± 1°C for 0, 2, 4, 6, 8 weeks. During stratification periods, sand moisture was checked and added distilled water whenever necessary to keep it moist 60-70%.

At the end of every cold stratification periods seeds were soaked in GA_3 solutions at (0, 100, 200, 300 ppm for 24 hours. On 15th December (Thanaa, *et al.,* 2020) from each season, the seeds were disinfected in a 0.01% Rizolix fungicide solution for 10 min and sown at a depth equal to three times of the seed size in polyethylene bags (30 cm diameter, 50 cm height) containing sandy loam soil Table 1, then placed in the greenhouse and irrigated with water daily.

Table 1: Physical and chemical properties of the experiment soil

Soil pH	EC ds.m^{-1}	OM (%)	Sand (g/kg)	Silt (g/kg)	Clay (g/kg)
7.42	0.66	3.82	482.1	153.2	64.23
Soluble cation (meq/kg soil)			**Soluble anions (meq/kg soil)**		
Ca^{++}	**Mg^{++}**	**K^{+}**	**Na^{+}**	**HCO^{-3}**	**Cl^{-}**
3.5	1.05	0.25	1.1	1.58	0.3
Available macronutrients (mg/kg soil)			**Available micronutrients (mg/kg soil)**		
N	**P**	**K**	**Fe**	**Zn**	**Mn**
0.49	0.98	0.15	1.18	1.4	1.2

2.4. Germination percentage (GP)

Germinated seeds were counted every day for 30 days, from the beginning of the emergence of the cotyledons above the soil (Ghayyad, *et al.,* 2010). The germination percentage per treatment was calculated by the equation:

$$Germination\ percentage\ (GP\ \%) = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} \times 100$$

2.5. Mean germination time (MGT)

Mean germination time was calculated by the equation described by Isfendiyaroglu and Özeker (Isfendiyaroglu and Özeker, 2001):

$$MGT = \frac{T\ x\ n}{n}$$

Where t: time in days starting from day zero, n: number of seeds completing germination on day (t).

2.6. Seedling growth parameters

At the end of September from both seasons the seedlings vegetative properties were determined in terms of stem and root length (cm), diameter (mm), seedlings fresh and dry weight (g), number of lateral shoots, roots and leaves/seedling. Leaf chlorophyll contents index were measured by using a portable chlorophyll meter model (SPAD-502 Minolta Sensing Inc., Japan). Vigour index of seedling was measured according to the formula described by Abou Rayya *et al*., (2018) as follows:

Seedling vigor index= Germination percentage x seedling x seedling dry weight

2.7. Statistical analysis

The experimental design was a completely randomized design with five replications per treatment (ten plants per replicate). Data were subjected to analysis of variance (ANOVA) according to Gomez and Gomez (1984), using CoStat Software Program Version 6.303 (2004), and LSD at 0.05 level of significance was used for the comparison between means.

3. Results and Discussion

3.1. Effect of CS and GA_3 on germination percentage

Results in Table 2 confirmed that the interactions between different cold stratifications periods and GA_3 concentrations had an important effect on seed germinations percent than the control (Zero CS + Zero GA_3).

Concerning the effect of different concentrations of GA_3 during every CS period on GP, it can be noticed that GP increased as GA_3 concentrations increased. The highest GP occurred in 300 ppm followed by 200 and 100 ppm. There were no significant differences between concentrations 200 and 100 ppm, and that is true by the short period (2 weeks) of stratifications.

As for the effect of stratification periods, the results obtained from the study revealed a positive correlation between the time of CS periods on almond seeds de-shelled and GA_3 treatments and germination percent (Table 2). The highest GP (80.4%) was obtained by the combination between a suitable CS period (6 weeks) with the effect level of GA_3 300 ppm, followed by (8, 4 and 2 weeks) which recorded (78.0), (70.3) and (64.90%), respectively at the same concentration of GA_3 while the lowest GP was obtained by the control which recorded (52.60%). This result may be due to the separate applications of GA_3. It can be noticed that the cause of the significant differences between the positive values of GP by removing endocarp seeds, return to the different periods of CS.

As for the stratification period of (6 weeks), it can be noticed that there were no significant differences between the time of (8 and 6 weeks) at the same levels of GA_3 on increasing GP. It may due to the effect of GA_3 to shorten the periods of CS. Ghayyad *et al.* (2010) reported that GA_3 is effective in shortening the chilling requirement. Following two periods (2, 4 weeks), the stratification analysis showed that GP increases in the long period of CS (4 weeks), when the seeds were soaked by the same concentrations of GA_3. A shorter period of stratification was not enough to scarify the shell and long periods caused damage to the embryo (Aygun, *et al.,* 2009). The combination of GA_3 (300 ppm) and chilling might be more effective in bringing a hormonal shift that not only enhanced germination but also sped it up (Amooaghaie, 2009 ; Nasri, *et al.,* 2013) on peach and on Black walnut (Parvin, *et al.,* 2015).

The above results may be due to the role of removed endocarp of almond seeds with GA_3 treatments on GP. The stimulatory role of GA_3 on seed germinations was explained by at least two different mechanisms. First, induce certain hydrolytic enzymes to overcome mechanical resistance imposed by endosperm and seed coat. Second, GA_3 increase the growth potential of the embryo (Debeaujon and Koornneef, 2000; Zhang, *et al.,* 2010). The removal of endocarp seeds employ GA_3 to induce α-amylase which change the starch to sugar and transport to the embryo 15, and also observed that GA_3 at 1250 ppm after removal of the endocarp of seeds gave the highest GP followed by 1000 and

750 ppm, this insured the role of GA_3. Remove endocarp plus GA_3 application of *Pistacia* seeds gave 95.6% GP. Yasar and Acar, (2019) and Aygun *et al.,* (2009) concluded that GA_3 application accelerates the germinations and increases the percentage of germination, and GP increased with an increase of GA_3 concentration up to a certain point. A concentration of 75 ppm GA_3 is suggested for breaking dormancy and for achieving fast and highest GP. Suja *et al.* (2016) revealed that 100 and 50 ppm GA_3 showed maximum GP (98, 24 and 54.23 %), respectively. This increase in seeds GP might be related to the initial enzyme induction and to the activation of reserve food mobilizing systems by gibberellins which have also been used to enhance germination and stimulate early seedling emergence and growth (Hopkins and Hüner, 2004).

Table 2: Interaction effects of cold stratification and GA_3 concentrations on germination percentage and mean germination time of de-shelled bitter almond seeds.

Treatments		Germination percentage (%)	Mean germination time (days)
CS (weeks)	**GA_3 (ppm)**		
0	**0**	52.60^{g}	25.40^{a}
	100	53.00^{g}	25.80^{a}
	200	54.30^{fg}	25.00^{a}
	300	58.90^{f}	24.70^{a}
2	**0**	54.80^{fg}	23.00^{b}
	100	54.90^{fg}	23.91^{b}
	200	59.20^{f}	21.90^{c}
	300	64.90^{d}	19.50^{d}
4	**0**	59.00^{f}	23.85^{b}
	100	65.20^{d}	22.50^{c}
	200	65.90^{d}	19.90^{d}
	300	70.30^{c}	16.19^{e}
6	**0**	63.90^{d}	19.90^{d}
	100	76.00^{c}	18.30^{f}
	200	74.80^{b}	15.10^{g}
	300	80.40^{a}	13.80^{g}
8	**0**	66.00^{d}	19.20^{d}
	100	71.60^{c}	17.90^{f}
	200	75.00^{b}	14.20^{g}
	300	78.00^{a}	14.00^{g}
LSD 0.05		**4.71**	**1.63**

The same letter with column indicates that there is no significant difference ($p<0.05$)

These results were reported in the same finding by Parvin *et al.* (2015). who observed that the highest percentage of seed germination (69.27 %) was recorded with the combined treatment GA_3 400 ppm of two months chilling, similar results obtained by Hassan and Fetouh (2014). The combination of chilling stratification and GA_3 pretreated has been reported to improve germination in *Prunus* species (Imani, *et al.,* 2011) and cherry seeds (Al-Absi, *et al.,* 2010). GA_3 and chilling stratification affect physiological and metabolic activities of seeds resulting in early germination (Pipinis, *et al.,* 2011) and recorded the maximum sprouting (Suja *et al.,* 2016).

3.2. Effect of CS and GA_3 on mean germination time

It can be noticed from Table 2 that the shortest MGT of complete germination was obtained from the CS period (6 weeks) combined with 300 ppm GA_3 followed by CS (8, 4, and 2 weeks) and the same concentration, respectively than the control which recorded the longest mean time of complete germinations there were no significant differences between CS periods (8 and 6 weeks).

These results are going in line with those obtained by Abu-Qaoud *et al.* (2007) who reported that the longest MGT was obtained from the control. Similarly, Esmailpour, and Van Damme, (2016) mentioned that MGT significant decreased by increasing soaking time durations of seeds to 12 and 24 hours compared to 6 h. Isfendiyaroglu and Özeker, (2001) noticed that more rapid and completed germination in 15 days after they were exposed to cold stratification for 45 days.

3.3. Effect of CS and GA_3 on seedling growth parameters

The Interaction effects of cold stratification and gibberellic acid concentrations on some growth parameters of almond seeds showed that all treatments increased the stem length, stem diameter and root length than the control treatment (Table 3). The application of GA_3 at 300 ppm with stratification period for (8 weeks) recorded the maximum values of stem length (48.3 cm), stem diameter (4.80 mm) and root length (32.94 cm), followed by GA_3 at (300, 200 and 100 ppm) combined with the CS for (6, 4, 2 weeks), respectively. In contrast, the minimum values were obtained by the control (Zero CS + Zero GA_3).

Table 3: Interaction effects of cold stratification and GA_3 concentrations on stem length stem diameter and root length of bitter almond seedlings.

Treatments		Length (cm)		Stem diameter (mm)
CS (weeks)	GA_3 (ppm)	Stem	Root	
0	0	24.47^{g}	25.34^{f}	3.60^{c}
	100	25.24^{fg}	26.33^{ef}	3.69^{c}
	200	26.50^{fg}	27.97^{cde}	3.55^{c}
	300	28.33^{def}	27.33^{cdef}	3.51^{c}
2	100	38.33^{fg}	25.40^{f}	3.70^{b}
	100	28.90^{fg}	25.45^{f}	3.60^{b}
	200	31.17^{cd}	27.40^{cdef}	3.79^{b}
	300	34.00^{c}	27.95^{cde}	3.90^{b}
4	0	27.46^{efg}	27.92^{cde}	4.18^{b}
	100	34.00^{c}	27.90^{cde}	4.14^{b}
	200	39.07^{b}	28.64^{cd}	4.15^{b}
	300	47.60^{a}	28.00^{cde}	4.70^{a}
6	0	38.50^{b}	28.67^{cd}	4.10^{b}
	100	42.50^{b}	29.16^{bc}	4.00^{b}
	200	47.00^{a}	29.19^{bc}	4.15^{b}
	300	46.50^{a}	31.67^{a}	4.71^{a}
8	0	39.07^{b}	29.16^{bc}	3.90^{b}
	100	41.87^{b}	31.00^{ab}	4.00^{b}
	200	46.30^{a}	31.67^{a}	4.10^{b}
	300	48.33^{a}	32.94^{a}	4.80^{a}
LSD 0.05		**3.44**	**2.16**	**0.64**

The same letter with column indicates that there is no significant difference ($p<0.05$)

It can be concluded the high level of GA_3 was more affected in increasing seedling growth than the low concentration during the same period of stratification. Confirming the results reported by Parvin *et al.*, (2015) who showed that, two months of chilling stratification combined with GA_3 400 ppm significantly improved seedling growth characteristics including seedling length, root volume and root area. However, the application of GA_3 after CS was more effective on seedling growth of *Pistacia mutica*. GA_3 applied at higher concentrations 500 and 750 ppm increased the rate of growth, also the best root growth was obtained from a seedling of de-hull seeds (Acar, *et al.*, 2017). Rahemi and Baninasab (2000); Mobli, and Baninasab, (2008) reported that GA_3 applications during and after stratification increased the length and diameter of the stem. The increase in seedling growth parameter with GA_3 might be

related to the fact that GA_3 promotes stem and shoot elongation through both cell division and elongation in higher plants (Hopkins, and Hüner, 2004). Observation of the experiment showed in Table 4 that, a further increase about No. of leaves/seedling by all treatments than the control. The highest numbers (25 leaves/ seedling) were obtained by the interaction between stratification seeds without endocarp for (8 weeks) and GA_3 at 300 ppm. While the lowest No. of leaves/seedling (11.68) by (Zero CS plus Zero GA_3). It can be concluded the important role of the long period of stratification treatments. Furthermore, the enhancing effect of the interaction treatments on No. of leaves/seedling may be due to the high stem length and diameter of a seedling. Confirming the results reported by Acar *et al.* (2017) who mentioned that, seedling diameter had a positive and significant correlation with seedling height, stem and number of leaves. Nearly results were observed by Thanaa *et al*. (2020) who found that the large area of leaf due to good parameters of seedling growth (stem length and diameter), Rehemi *et al.* (2000) demonstrate the growth capacity can be determined by stem diameter.

Table 4: Interaction effects of cold stratification and GA_3 concentrations on the number of leaves, lateral shoots and roots of bitter almond seeding.

Treatments		No of leaves/ seedling	No. of lateral	
CS (weeks)	GA_3 (ppm)		Shoots	Roots
0	0	11.68^{gh}	0.00^{i}	0.00^{i}
	100	13.79^{ef}	1.00^{i}	4.00^{h}
	200	15.00^{de}	1.67^{gh}	5.00^{gh}
	300	16.01^{d}	2.00^{gh}	6.00^{fg}
2	0	14.33^{def}	3.00^{fg}	7.00^{ef}
	100	13.33^{def}	2.00^{gh}	8.00^{de}
	200	15.00^{de}	2.00^{gh}	9.00^{cd}
	300	15.00^{de}	3.00^{fg}	10.00^{dc}
4	0	19.30^{c}	2.00^{gh}	8.00^{de}
	100	18.00^{c}	3.00^{fg}	8.00^{de}
	200	19.00^{c}	3.00^{fg}	9.00^{cd}
	300	22.09^{b}	4.67^{cd}	10.00^{bc}
6	0	23.16^{b}	4.00^{ef}	10.00^{bc}
	100	22.00^{b}	4.00^{ef}	11.00^{ab}
	200	24.00^{a}	5.00^{bc}	11.00^{ab}
	300	24.33^{a}	5.67^{ab}	12.00^{a}
8	0	22.00^{b}	4.00^{ef}	10.00^{bc}
	100	23.33^{ab}	5.33^{bc}	11.00^{ab}
	200	24.00^{a}	5.00^{bc}	11.00^{ab}
	300	25.00^{a}	6.00^{a}	12.00^{a}
LSD 0.05		**1.89**	**1.62**	**1.83**

The same letter with column indicates that there is no significant difference ($p<0.05$)

The number of lateral shoots and roots was enhanced by the combination of stratification long time and high GA_3 concentration until a certain point more or less. Therefore, there were no significant differences between (6 and 8 weeks) periods of CS at the same GA_3 concentrations in this respect (Table 4).

Parvin *et al.* (2015) showed that two months to chilling stratification combined with GA_3 at 400 ppm improved seeding growth characteristics including root volume. Dhupper, (2013) reported that the use of GA_3 has been studied culture as a way to obtain a uniform seedling size in the nurseries.

The fresh and dry weight of seedlings produced from almond seeds without endocarp showed in Table 5 that fresh weight has the same trend as dry weight. Significant results by the combined treatments (different periods of stratification and GA_3 levels were achieved in removal endocarp than the control. The biggest fresh and dry weight was found by GA_3 300 ppm by the two long periods (8

and 6 weeks) without significant differences at the same concentration. These results are in agreement with those obtained by Mobli and Baninasab, (2008), Rahemi *et al.,* (2011) who mentioned that application of most plant growth regulators significantly increased shoot fresh and dry weight.

Statistical analysis shows that vigor index and chlorophyll content index Table 5 has the same trend, which observed that significant increase by increasing cold stratification periods with the levels of GA_3 increased in this respect GA_3 at 300 ppm more effective than 200 and 100 ppm respectively at all the different periods of stratifications.

Table 5: Interaction effects of cold stratification and GA_3 concentrations on fresh and dry weight, vigor index and chlorophyll content of bitter almond seedlings

Treatments		Seedling fresh weight (g)	Seedling dry weight (g)	Seedling vigor index	Chlorophyll content index
CS (weeks)	**GA_3 (ppm)**				
0	**0**	6.00^{b}	4.67^{d}	247.51^{gh}	43.28^{f}
	100	6.50^{b}	4.33^{d}	227.76^{h}	46.31^{def}
	200	6.15^{b}	5.00^{d}	271.50^{fgh}	46.35^{def}
	300	7.00^{b}	5.67^{cd}	333.96^{def}	47.33^{de}
2	**0**	6.67^{b}	5.33^{d}	292.08^{efg}	43.61^{f}
	100	7.00^{b}	5.70^{cd}	312.93^{ef}	47.00^{def}
	200	7.29^{b}	6.37^{bc}	377.10^{de}	47.30^{de}
	300	7.18^{b}	6.00^{bc}	389.40^{de}	48.50^{cde}
4	**0**	7.20^{b}	5.33^{d}	314.47^{de}	47.40^{de}
	100	7.67^{b}	5.70^{cd}	371.64^{de}	46.31^{def}
	200	7.33^{b}	6.67^{ab}	439.55^{d}	46.58^{def}
	300	6.50^{b}	6.00^{bc}	421.80^{d}	47.33^{de}
6	**0**	13.00^{a}	9.67^{ab}	617.91^{cd}	49.01^{bcd}
	100	12.88^{a}	10.00^{ab}	760.00^{bc}	48.54^{cde}
	200	13.00^{a}	11.00^{ab}	822.80^{ab}	51.90^{ab}
	300	12.68^{a}	11.20^{a}	873.60^{ab}	51.70^{ab}
8	**0**	12.67^{a}	10.00^{ab}	660.00^{cd}	51.26^{ab}
	100	13.00^{a}	10.00^{ab}	716.00^{bc}	52.25^{ab}
	200	12.83^{a}	11.00^{ab}	825.00^{ab}	52.50^{ab}
	300	13.33^{a}	11.33^{a}	910.93^{a}	54.93^{a}
LSD 0.05		**1.94**	**2.68**	**211.75**	**3.71**

The same letter with column indicates that there is no significant difference ($p<0.05$)

4. Conclusion

The results showed the suitability of combining cold stratification for 6 weeks and gibberellic acid at 300 ppm to breaking dormancy of de-shelled bitter almond seeds for optimization seed germination percentage and enhance the growth of seedlings, which could have a practical application in nurseries management for shortening the time required to reach the appropriate transplanting size.

Significance Statement

This study reveals the effect of applying cold stratification for different periods followed by soaking in different concentrations of gibberellic acid that can be beneficial to break the dormancy of de-hulled bitter almond seeds. The high concentration of GA_3 had a positive effect in reducing the duration of cold stratification and these have economic and biological importance. This study will help the producers of bitter almond rootstock seedlings to reach the appropriate concentration of GA_3 and cold stratification period to obtain the highest germination percentage in the shortest possible time. Thus, it can have practical application in nursery management to increase uniformity and earlier seed germination.

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