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In Vitro Conservation and Germination of Encapsulated Somatic Embryos of "Barhi" Date Palm

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ABSTRACT

Synthetic seeds technique is a tool for mass propagation of elite plant species with high commercial value. Somatic embryos (1.5 mm in length) of date palm cv. Barhi were used in two experiments, the first experiment was designed to study the effect of sodium alginate concentrations at 4%, 5% and 6% and Abscisic acid concentration at 0.0, 0.5, 1.5 and 2.0 mg l^{-1} on conservation and germination of encapsulated somatic embryos of date palm cv. Barhi after four months. The second experiment aims to get the highest germination percentage and growth vigor of synthetic seeds which conserved for a period of four months at the same condition of the best treatment from the previous experiment, it has been used three combinations of plant growth regulator as follows: I- (0.1 mg l^{-1} α-naphthalene acetic acid (NAA) + 0.05 mg l^{-1} 6-benzylaminopurine(BA)), II- (0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kinetin (Kin)) and III- (0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kin + 0.5 mg l^{-1} Gibberellic acid). The results showed that, the best treatment which gave the highest percentage of synthetic seeds germination (71.6%) and a maximum degree of embryos growth (= 4) was 5% of sodium alginate +1.5 mg l^{-1} Abscisic acid. While in the second experiment, the best result of percentage of conversion (80.1%) and (80.6%) were achieved at using of second and third treatments combinations respectively.

Keywords: Date palm, Encapsulated synthetic and In vitro conservation seeds.

Abbreviations: MS: Murashige and Skoog (1962) basal medium; ABA: Abscisic acid; NAA: α-naphthalene acetic acid; Kin: Kinetin (N^6-furfuryladenine); BA: 6-benzylaminopurine; GA_3: Gibberellic acid.

INTRODUCTION

Synthetic seeds technique is a powerful tool for mass propagation of elite plant species with high commercial value. Synthetic or artificial seeds have been defined as somatic embryos engineered for use in the commercial propagation of plants **(Gray and Purohit, 1991)**. Synthetic seeds can also be defined in other ways, such as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and those possess the ability to convert into a plant under *in-vitro* or *ex-vitro* conditions and retain that potential even after storage **(Capuano *et al*., 1998)**.

Synthetic seed technology is currently considered as the most effective and efficient alternative technique for propagation. This technology also facilitates the way of handling cells and tissues, protecting them from external gradients, short-term and long-term storage under low temperature and ultra-low temperature, respectively and as an efficient system of delivery **(Roy and Tulsiram, 2013).**

A typical synthetic seed has the following parts such as: (a) Plant propagule (apical shoot tips, axillary buds, nodal segments, embryogenic calli, somatic embryos, and protocom-like bodies) (b) Matrix, is a gelling material encapsulating plant propagules which incorporate nutrients, plant growth regulators, antibiotics or other essential additives. (c) Seed hull these are the artificial seed coats prepared with complex mixture of alginate-gelatin which was used to develop the coat system for encapsulation. Synthetic seeds offer many advantages and promising opportunities as easy handling, inexpensive transport and solving the propagation and preservation of highly valuable plants to enable the synchronized planting of commodities to produce a huge number of plants which are identical in there uniformity without being restricted to agricultural season. **(Sahoo *et al.*, 2012)**

Das *et al*. (2016) reported that synthetic seeds can be used for germplasm maintenance, clonal propagation and storing for a long time and its genetic constitution could remain the same.

The date palm, *Phoenix dactylifera* L., is one of the most economically important perennial plants in arid areas of the Middle-East and the North Africa. Date palm cv. Barhi is an important variety, where it increases demand for food and many other commercial purposes. It is known that propagation of palm is very slow; therefore we need to find a protocol for increasing and acceleration its propagation in a large quantity and its quality to enough our demand. Synthetic seeds as possible be employed for this purpose.

AlKhateeb and Alturki (2014) mentioned that the use of plant tissue culture technique for propagation of date palm is considered an alternative method to the conventional methods, whereas seed propagation is not common due to heterozygosis and dioeciously nature of the date palm which may result in producing off type plant and offshoot propagation is a slow method and its mortality is usually high. **Mazri and Meziani (2015)** reported that somatic embryogenesis and organogenesis are the two pathways of choice for rapid and large-scale propagation of date palm.

Somatic or asexual embryogenesis is the production of embryo-like structures from somatic cells without gametes fusion. During their development, somatic embryos pass through stages similar to those observed in zygotic embryogenesis **(Dodeman *et al*., 1997 and Von-Arnold *et al*., 2002)**.

Somatic embryo germination can be achieve by using plant growth regulators, **Zouine and El Hadrami (2007)** were used a combination of BAP, IBA and NAA to promote embryos germination in date palm cvs. Jihel and Bousthami Noir. **Al-Khayri (2003)** reported that the concentration of NAA, IBA as well as the MS medium strength influence somatic embryo germination in date palm.

The objective of this work was to determine the effect of Sodium alginate and Abscisic acid on conservation, germination and growth vigor of somatic embryos of date palm cv. Barhi through synthetic seeds *in vitro*. An additional objective of this study was to investigate the effect of some plant growth regulators on germination and growth vigor of the somatic embryos.

MATERIAL AND METHODS

I- **Plant material:**

This study was conducted at the Central Laboratory for Date Palm Researches and Development, Agriculture Research Center, Giza, Egypt. The shoot tips (meristem tips and leaves primordial) used as explant materials isolated from offshoot apex of date palm cv. Barhi. Shoot-tip sections were cultured individually on sterilized **Murashige and Skoog (1962)** (MS) basal nutrient medium supplemented with 10 mg l^{-1} dichloro-phenoxyacetic acid (2,4-D), 3 mg l^{-1} N6-(2-iso-pentenyl adenine) (2-iP), 40 mg l^{-1} adenine sulfate, 200 mg l^{-1} $KH_2P0_4.2H_20$, 170 mg l^{-1} $NaH_2P0_4.2H_20$, 200 mg l^{-1} glutamine, 100 mg l^{-1} myo-inositol, 1.5 g l^{-1} activated charcoal, 40g l^{-1} sucrose and 6 g l^{-1} bacteriological agar **(Tisserat (1982) and Sidky *et al.* (2007).** The medium pH was adjusted to 5.8. and dispensed on small jars 200 ml at rate of 30 ml per jar and autoclaved under 1.2 kg/cm^2 pressure at 121°C for 20 minutes. Cultured jars were incubated at 25±2°C in total darkness and re-culture until formation of the embryogenic callus. After that cultures were transferred to fresh medium supplemented with 0.1 mg l^{-1} NAA for two months to obtain the somatic embryos. After that the somatic embryos with opaque white of 1.5 mm size were harvested and used for encapsulation.

First experiment:

Somatic embryos were encapsulated in calcium-alginate capsules (4,5 and 6% alginic acid-sodium salt (Sigma) gel (w/v) supplemented in basal MS medium free of calcium salt + 3% sucrose + 100 mg l^{-1} KH2P04.2H20, 100 mg l^{-1} NaH2P04.2H20, 200 mg l^{-1} glutamine, 100 mg l^{-1} myo-inositol, and Abscisic acid (ABA) concentration at 0.0, 0.5, 1.5 and 2.0 mg l^{-1} and exposed to 100 mM calcium chloride (CaCl2.2H2O) solution), the sodium alginate solution and calcium chloride solution were autoclaved under 1.2 kg/cm^2 pressure at 121°C for 20 minutes. Isolated somatic embryos were suspended in sodium alginate solution which contains the previous components for 5 min. and dropped one by one through an eppendorf pipette into CaCl2 being mixed continuously and kept for 20 min for hardening of the beads. Encapsulated embryos were

washed with sterile water to remove remnants of CaCl2.2H2O. These beads (Plate1) used as the source of explants in the first experiment that was designed to study the effect of sodium alginate concentrations at 4%, 5% and 6% combined with Abscisic acid (ABA) concentration at 0.0, 0.5, 1.5 and 2.0 mg l^{-1} on conservation and germination of encapsulated somatic embryos. The encapsulated somatic embryos were conserved in Petri dishes in total darkness at 15± 2°C for four months. After that the synthetic seeds were soaked in sterile distilled water for six hours and then were cultured in pyrex glass test tubes O.D. × L 24 mm × 150 mm on MS medium supplemented with 0.1 mg l^{-1} NAA + 0.05 mg l^{-1} BA for germination. Firstly, the cultures were incubated under total darkness for 15 days. Then, the cultures were incubated under the normal conditions (16-h photoperiod day/night condition for another 4 weeks).The germination percentage and growth vigor were recorded after six weeks.

Second experiment:

Aims to investigate the effect of different growth regulators on germination of the encapsulated somatic embryos after four months from conservation at the same conditions of the previous experiment, The best treatment from first experiment were repeated on three combination treatments of plant growth regulators as follows: M1- (0.1 mg l^{-1} NAA + 0.05 mg l^{-1} BA), M2- (0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kin) and M3- (0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kin + 0.5 mg l^{-1} Gibberellic acid (GA_3).

For each conservation treatment, three Petri dishes sealed with Para film, each dish contain ten encapsulated somatic embryos. While for each germination treatment ten tube, each tube contains one synthetic seed. Data were calculated in our experiments visually for growth vigor according to **Pottino (1981)** as follow:

Negative results (-)	=1
Below average results (+)	=2
Average results (++)	=3
Good results (+++)	=4

Statistical analyses

All experiments were arranged in a Randomized Complete Block Design with three replicates, each replicate was contained ten beads. The mean values were compared using LSD method at 5% level according to **Snedecor and Cochran (1980).**

RESULTS AND DISCUSSION

First experiment:

I- Effect of Sodium alginate and Abscisic acid concentrations on germination percentage of somatic embryos after four months from conservation:

Table (1): Effect of Sodium alginate and Abscisic acid concentrations on germination percentage of somatic embryos after four months from conservation:

Sodium alginate (w/v)	ABA (mg l^{-1})				Mean of Sodium alginate
	0.0	**0.5**	**1.5**	**2.0**	
4%	54.3	60.0	63.4	50.8	**57.1**
5%	56.8	62.0	71.6	53.7	**61.0**
6%	53.7	56.8	60.0	50.8	**55.3**
Mean of ABA	**54.9**	**59.6**	**65.0**	**51.8**	
L S D at 0.05 of Sodium alginate					**0.6**
L S D at 0.05 of ABA					**0.6**
L S D at 0.05 of Interaction					**1.2**

** Data have been transformed according to **Snedecor and Cochran (1980)**

Data presented in Table (1) referring to Sodium alginate concentrations, revealed that the conversion percentage of somatic

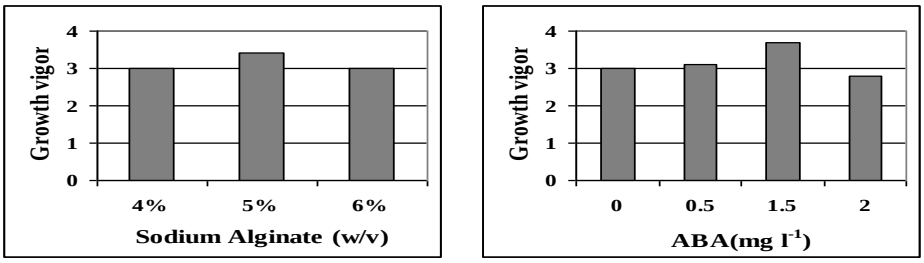
embryos of date palm cv. Barhi after four months from conservation and after six weeks from culture on germination medium was significantly affected by Sodium alginate concentration. The highest conversion percentage (61%) was obtained with Sodium alginate at 5%, followed by Sodium alginate at 4% (57.1%), but in case of 6% Sodium alginate the conversion percentage decreased significantly to (55.3%). These data are in accordance with **Nesreen, 2011** who mentioned that, different concentrations of sodium alginate ranging from 1.5 % to 6 % have been used for different systems of synthetic seeds, and to produce hydrated synthetic seeds, the somatic embryos are mixed with sodium alginate gel (0.5–5.0% w/v).

With respect to ABA concentrations, data showed that the addition of ABA had a significant effect on germination percentage of somatic embryos after four months from conservation. When ABA concentration increased from 0.0 to 0.5 and 1.5 mg l^{-1}, the conversion percentage increased significantly from 54.9 to 59.6 and 65.0%, respectively. But in case of 2 mg l^{-1} ABA the conversion percentage decreased significantly to 51.8%. Our results are in accordance with observations of a number of researchers **(Senaratna *et al*., 1990; Schuller *et al*., 2000; Nieves *et al*., 2001)** who reported that, Abscisic acid is crucial in all the stages of somatic embryos development and maturation.

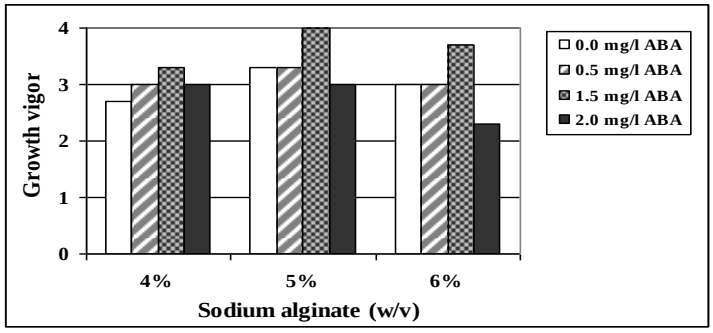
The interaction between Sodium alginate and ABA concentration had a significant effect on the conversion percentage of somatic embryos after four months from conservation, in case of 5% Sodium alginate combined with 1.5 mg l^{-1} ABA (Plate2) were most effective combination treatment to produce the highest significant percentages (71.6%) of conversion. While the lowest significant percentage (50.8%) was noticed with combination treatment containing 4 or 6 % Sodium alginate and 2 mg l^{-1} from ABA. A number of researchers have tried to improve the quality and quantity of somatic embryos via modification of culture conditions, such as, medium composition, growth regulators (types and concentrations), physical state of the medium, as well as incubation conditions like temperature, illumination, etc. So the coating material is

considered an influential factor on the success of the synthetic seed technology.

II- Effect of Sodium alginate and Abscisic acid concentrations on growth vigor of converted somatic embryos after four months from conservation:



a- It is clear from data in Fig. (1-a) that there were significant differences between the treatments as a result of the use of Sodium alginate where the growth vigor in the case of Sodium alginate at 5% =4 (the highest degree of growth vigor) followed significantly by using Sodium alginate at 4,6% where the growth vigor = 3 in both cases. **Pintos *et al.*, (2008)** reported that the mature somatic embryo of Cork oak which encapsulated with 5 % (w/v) sodium alginate converted to plantlets and formed a well developed root.



c- Effect of interaction between sodium alginate and ABA

Fig. (1): Effect of Sodium alginate and Abscisic acid concentrations on growth vigor of converted somatic embryos after four months from conservation

b- Growth vigor of converted somatic embryos of date palm cv. Barhi was significantly affected by ABA concentration. In case of ABA at 1.5 mg L^{-1}, the growth vigor of converted somatic embryos was = 3.7 followed significantly by using ABA at 0. 5 mg l^{-1} where the growth vigor = 3.1. In the case of free-ABA the growth vigor decreased to = 3, these results agree with those obtained by **Ammirato, (1983)** who found that, ABA promote the normal development of both somatic and zygotic embryos *in- vitro*. **Nieves *et al.* 2001** reported that survival of encapsulated somatic embryos of sugarcane was achieved in presence of ABA which induced an increase in protein, polyamines, free proline levels and starch levels.

c- Growth vigor of converted somatic embryos as affected by interaction between Sodium alginate and ABA concentration Fig. (1-c) showed the highest record was =4 at 5% Sodium alginate combined with ABA at 1.5 mg L^{-1}. As ABA and Sodium alginate concentration increased ultimately to 2.00mg L^{-1} and 6% respectively, growth vigor deteriorated significantly to =2.3.

Second experiment:

I - Effect of combination treatments of plant growth regulators on conversion percentage and growth vigor of encapsulated somatic embryos of Date Palm cv. Barhi after four months from conservation:

Table (2). Effect of different plant growth regulators concentration on germination percentage of encapsulated somatic embryos after six weeks from culture ***

Treatment					Germination percentage
No.	Concentration mg l^{-1} NAA	BA	Kin	GA_3	
M1	0.1	0.05	0.0	0.0	71.6

M2	0.1	0.02	0.03	0.0	80.1
M3	0.1	0.02	0.03	0.5	80.6
L S D at 0.05					**3.5**

*** Data have been transformed according to Snedecor and Cochran (1980)

It is clear from data in **Table (2)** that there were significant differences between the treatments (M2 and M3) and (M1). The conversion percentage of encapsulated somatic embryos of Date Palm cv. Barhi in case of M1 was 71% increased significantly to 80.1 and 80.6 % in case of M2 and M3 respectively. This reflects the promoted effect of both the Kin and GA_3 for conversion of encapsulated somatic embryos. These results are in accordance with observations of **Das *et al*. (2016)** who reported that gibberellic acid has a significant influence on the germination rate of the encapsulated somatic embryos (ESEs) of *Litchi chinensis* sonn.

II- Effect of combination treatments of plant growth regulators on growth vigor of converted somatic embryos of Date Palm cv. Barhi after six weeks from culture:

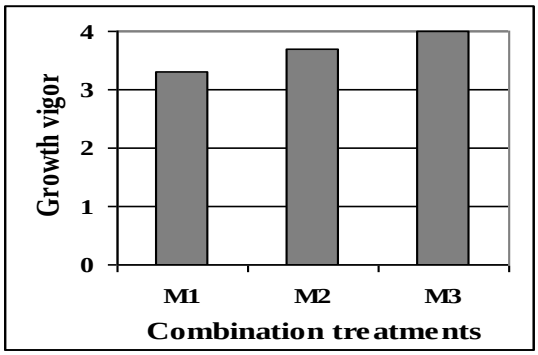


Fig. (2): Effect of combination treatments of plant growth regulators on growth vigor of converted somatic embryos of Date Palm cv. Barhi after six weeks from culture. (M1= 0.1 mg l^{-1} NAA + 0.05 mg l^{-1} BA, **M2=** 0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kin and **M3=** 0.1 mg l^{-1} NAA + 0.02 mg l^{-1} BA + 0.03 mg l^{-1} Kin + 0.5 mg l^{-1} GA_3)

Data presented in **Fig. (2)** Show the effect of combination treatments of NAA, BA, Kin. and GA_3 on growth vigor of converted somatic embryos of Date Palm cv. Barhi. In case of M1 the growth vigor was =3.3 which increased to 3.7 and 4 in case of M2 and M3, respectively.

Conclusion

The present work represented an efficient protocol for *in vitro* conservation using synthetic seeds of the most economically importance date palm cultivar, Barhi.

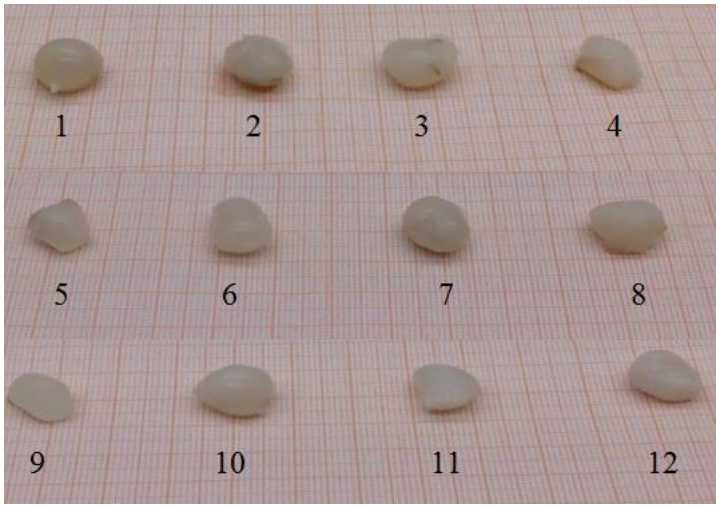


Plate (1): Encapsulated somatic embryos in calcium-alginate capsules (4,5 and 6% alginic acid-sodium salt (Sigma) gel (w/v) supplemented in basal MS medium free of calcium salt + 3% sucrose + 100 mg l^{-1} KH2P04.2H20, 100 mg l^{-1} NaH2P04.2H20, 200 mg l^{-1} glutamine, 100 mg l^{-1} myo-inositol, and Abscisic acid (ABA) concentration at 0.0, 0.5, 1.5 and 2.0 mg l^{-1} as follow:
1- 4% sodium alginate and 0.0 mg l^{-1} of ABA. 2- 4% sodium alginate and 0.5 mg l^{-1} of ABA.
3- 4% sodium alginate and 1.5 mg l^{-1} of ABA. 4- 4% sodium alginate and 2.0 mg l^{-1} of ABA.
5- 5% sodium alginate and 0.0 mg l^{-1} of ABA. 6- 5% sodium alginate and 0.5 mg l^{-1} of ABA.
7- 5% sodium alginate and 1.5 mg l^{-1} of ABA. 8- 5% sodium alginate and 2.0 mg l^{-1} of ABA.
9- 6% sodium alginate and 0.0 mg l^{-1} of ABA. 10- 6% sodium alginate and 0.5mg l^{-1} of ABA. 11- 6% sodium alginate and 1.5mg l^{-1} of ABA. 12-6% sodium alginate and 2.0mg l^{-1} of ABA.

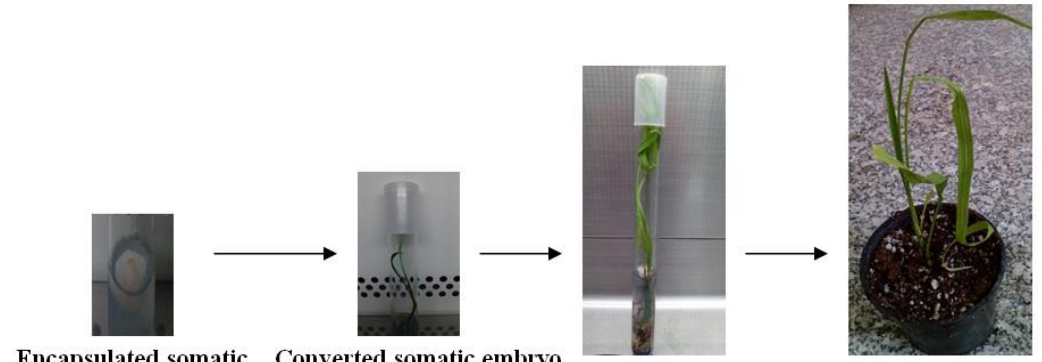


Plate (2): Micro-propagation of date palm through encapsulated somatic embryos

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الحفظ المعملى وإنبات الأجنة الجسدية المكبسلة للصنف البرحي لنخيل البلح

رحاب أحمد صدقى*وإبراهيم محمد شمس الدين على*

***** المعمل المركزي لأبحاث وتطوير نخيل البلح ، مركز البحوث الزراعية ، الجيزة.

تعتبر البذور الصناعية أداة قوية للإكثار المعملى للأنواع النباتية المنتخبة ذات القيمة التجارية العالية. تم إستخدام الأجنة الجسمية لنخيل البلح صنف بارحي بطول 1،5 سم فى تجربتين الأولى لدراسة تأثير تداخل مادة الصوديوم الجينات بتركيزات 4% و5% و6% مع حامض الأبسيسك بتركيزات 0 و 0،5 و 1،5 و 2،0 ملليجرام/لتر على حفظ وإنبات البذور الصناعية لنخيل البلح صنف بارحي بعد أربعة أشهر من الحفظ. أما التجربة الثانية والتى تهدف إلى الحصول على أعلى نسبة إنبات للبذور الصناعية المحفوظة لمدة أربعة أشهر الناتجة من أحسن معاملة من التجربة السابقة فلقد تم دراسة ثلاثة تداخلات من منظمات النمو كالتالى: المجموعة الأولى (0,1 ملليجرام/لتر نفثالين حمض الخليك + 0,05 ملليجرام/لتر بنزيل أدنين) والمجموعة الثانية (0,1 ملليجرام/لتر نفثالين حمض الخليك + 0,02 ملليجرام/لتر بنزيل أدنين + 0,03 ملليجرام/لتر كينيتين) أما المجموعة الثالثة فهى (0,1 ملليجرام/لتر نفثالين حمض الخليك + 0,02 ملليجرام/لتر بنزيل أدنين + 0,03 ملليجرام/لتر كينيتين + 0,5 ملليجرام/لتر حامض الجبريلك). ولقد أشارت النتائج إلى أن أحسن معاملة أعطت نسبة إنبات (71،6 %) و أعلي درجة لنمو الأجنة (=4) هى 5% من مادة الصوديوم الجينات + 1،5 ملليجرام/لتر حامض الأبسيسك. كما أشارت نتائج التجربة الثانية إلى إن إستخدام التداخلين الثانى والثالث كان لهما تأثير معنوى على نسبة إنبات للبذور الصناعية حيث بلغت نسبة الإنبات (80،1 %) و (80،6 %) على التوالى.

