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Artificial seeds for In Vitro production of Arabidopsis thaliana Nossen (NO-0) plants

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Keywords: Arabidopsis thaliana Nossen (NO-0), Artificial seeds, Somatic, Sodium alginate.

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Abstract

Somatic embryos are regarded the primary plant material for producing artificial seeds, and these seeds provide for agricultural institutions genetically uniform seeds, unlike the traditional seeds that often produce plants with different traits from the parent plant; in addition to the possibility of storing it for a long time Arabidopsis thaliana, locally known as mouse ears, is a promising model because it is self-fertile, which makes it conserved, and can produce thousands of seeds, which makes it ideal for mutation experiments. It is diploid (10=2n), and the identification of selectable traits is more straightforward because it has a relatively small genome (125 Mpb). The genome organization in Arabidopsis thaliana is simple and particularly suitable for genetic and molecular biology experiments.. This study produced Arabidopsis thaliana Nossen (NO-0) plants from the differentiation of callus tissues from its growing seedlings on MS medium (roots, stems, leaves). The B5 medium free of growth regulators successfully induced root callus, while the highest response for callus induction from stem segments was observed on B5 medium containing 0.05 mgL-1 kinetin (Kin) and 0.5 mgL -1 2-4, Dichlorophenoxy acetic acid (2,4-D). The B5 medium containing 0.05 mgL-1 (Kin) kinetin and 1.0 mg/L-1 (2,4-D) 2,4-Dichlorophenxy acetic acid (2,4-D) also affected leaf callus induction. The results of the statistical program SPSS version 16 also showed a significant difference at a probability level of 1% in the percentage of calyx creation for the plant parts used .The MS medium containing 0.5 mgL-1 Thidiazuron (TDZ) and 1.0 mgL-1 6-Benzyl adenine (BA) was effective in producing embryonic phases from transferred root callus. Meanwhile, the embryonic phases from stem and leaf callus were produced on MS medium supplemented with 0.5 mgL-1 Thidiazuron (TDZ), resulting in a group of 290 shoots. A 2% sodium alginate concentration was used in experiments to encapsulate somatic embryos, yielding the best form of artificial seeds in spherical structures (Beads) and achieving a high conversion rate of 80% when stored for 20 days at 4°C, and 60% when stored for 20 days at 25°C. There are significant differences at the 5% level in the germination of seeds stored for 20 days at a temperature of 25°C. The number of plants resulting from the artificial seeds was 36 green shoots. The experiments were carried out over a period of six months in the Tissue Culture and Genetic Applications Laboratories at the College of Education for Pure Sciences/University of Mosul.

Keywords: Arabidopsis thaliana Nossen (NO-0), Artificial seeds, Somatic, Sodium alginate.

البذور صناعية لإنتاج نباتات Arabidopsis thaliana Nossen (NO-0) خارج الجسم الحي

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الخلاصة:

تُعدُّ الأجنة الجسمية المادة النباتية الأساسية في إنتاج البذور الصناعية وأن هذه البذور توفر للمؤسسات الزراعية بذوراً متجانسة وراثياً على عكس البذور التقليدية التي تعرف بإنتاجها نباتات غالباً ماتكون مغايرة لصفات النبات الأمّ فضلاً عن امكانية خزنها لمدة طويلة و يعد Arabidopsis thaliana،المعروف محلياً بأذان الفأر نموذجاً واعداً كونه ذاتي الاخصاب مما يجعله محافظاً على صفاته، ويمكنه انتاج الاف البذور مما يجعله مثالياً لإجراء تجارب الطفرات وهو ثنائي المجموعة الكروموسومية 2n=10 إن تحديد الصفات المنتخبة يكون اكثر وضوحاً لامتلاكه جينوم صغير نسبيا (125 Mpb) كما ان تنظيم الجينوم في Arabidopsis thaliana بسيط ومناسب بشكل خاص في تجارب الوراثة والبيولوجي الجزيئي،انتجت هذه الدراسة نباتات Arabidopsis thaliana Nossen(NO-0) من تمايزكالس اجزاء بادراتها النامية على وسط ميوراشيك وسكوك (الجذور،السيقان ،الاوراق)، وقد نجح وسط B5 الخالي من منظمات النمو في استحثاث كالس الجذور في حين كانت اعلى استجابة لاستحثاث الكالس من قطع السيقان على الوسط B5 المحتوي على ٠,٠٥ملغم لتر-١ kinetin (Kin) و 0.5ملغم لتر-١ 2,4-Dichlorophenxy acetic acid (2,4-D) وكان للوسط الزرعي B5 المحتوي على ٠,٠٥ملغم لتر-١ kinetin (Kin) و ١,٠ ملغم لتر-١ 2,4-Dichlorophenxy acetic acid (2,4-D) اثر في استحثاث كالس الاوراق كما بينت نتائج البرنامج الاحصائي spss version 16 عن وجود فرق معنوي عند مستوى احتمال ١٪ في نسبة استحداث الكاس للاجزاء النباتية المستخدمة وتألق الوسط MSالمحتوي على (٠,٥) ملغم لتر-١ Thidiazuron (TDZ) و ١,٠ ملغم لتر-١ 6-Benzyl adenine (BA) في تكوين الاطوار الجنينية من كالس الجذور المنقول اليه في حين تكونت الاطوار الجنينية لكالس السيقان والاوراق على وسط MSالمدعم بـ(٠,٥) ملغم لتر-١ Thidiazuron (TDZ)

ليكون مجموعة الافرع الخضرية المتكونة 290 فرعاً وقد اعتمد التركيز ٢٪ الجينات الصوديوم في التجارب لتغليف الأجنة الجسمية اذ اعطى افضل شكل للبذور الصناعية بهيئة تراكيب كروية (Beads) وملائماً جداً في اعطاء أعلى نسبة تحولconversion بلغت ٨٠٪ عند خزنها مدة ٢٠ يوم في ٤°م مئوية و ٦٠٪ عند خزنها مدة ٢٠يوم في ٢٥°م مئوية وبلغت عدد النباتات الناتجة من البذور الصناعية ٣٦ فرعاً خضريا. نفذت التجارب خلال ستة اشهر في مختبرات زراعة الانسجة والتطبيقات الوراثية في كلية التربية للعلوم الصرفة /جامعة الموصل

الكلمات المفتاحية: Arabidopsis thaliana Nossen (NO-0)، البذور الصناعية، الاجنة الجسمية، الجينات الصوديوم، الكالس.

Introduction:

The genus Arabidopsis, from the Brassicaceae strains family, includes nine species and eight subspecies, Arabidopsis thaliana is one of its most important species. It shows a wide range of features and genetic variations among of the wild type strains and provides unmatched information regarding genetic material, that makes it suitable for studying natural variations genetic experiments and molecular [1].

Wild Arabidopsis plants represent nearly homologous genotypes known as strains, which have been studied to describe genetic diversity and their morphological differences [2]. Arabidopsis thaliana plants are considered a promising model for studying plant genomics and expression, attracting botanists due to its high-quality genome sequence, short life cycle, and various phenotypes across different ecology patterns [3]. Controlled conditions and artificial growth mediums have been used to study its morphological traits, which cannot be replicated in natural environments [4].

Arabidopsis thaliana is a good model for elucidating the genome, that aims to correlate genotype and phenotype relationships, and through it, 36 new genes were identified, which likely associated with flowering traits [5].

Using basic methods to plant tissue culture for Arabidopsis thaliana is beneficial for producing large numbers of genetically similar plants in a relatively short growth period; and the production of callus and its cellular suspensions transplants, along with plant regeneration, serves as essential inputs for studying biochemical processes and cellular molecular processes [6]. The application of tissue culture in Arabidopsis thaliana (Col-0, No-0, Ler-0, Ws-0) has taken a significant place in plant regeneration, obtaining sterile plants, overcoming seed dormancy, and forming somatic embryogenesis and comparing the morphological traits of the resulting plants [7].

Artificial seeds represent a distinct technique in tissue culture, it relies on obtaining them from somatic embryos or other plant parts such as growing tips and buds. The storage of artificial seeds is a crucial factor that may influence conversion, preservation of genetic resources, and maintaining viability and germination potential even after storage periods [8]. Mature seeds of Arabidopsis thaliana are not viable, and their small size and light weight induce dormancy that prevents germination. The application of tissue culture in this plant type has taken a significant role in renewing plants and overcoming seed dormancy and forming somatic embryogenesis [9]. Production of good artificial seeds for the storage and propagation of rare plants with desirable genes is also vital to reduce costs associated with certain seeds or overcome seed dormancy or low viability [10].

2. Material and methods:

2.1 Seed Development

Arabidopsis thaliana (NO-0) seeds were sterilized after obtaining them from the Nottingham Arabidopsis Stock Center in the UK, by placing them in 97% alcohol for 3 minutes in a sterilized Eppendorf centrifuge tube (1.5 ml) by three consecutive washes with sterilized water for 2 minutes each to remove the sanitizer [11]. Seeds were dried on sterile filter paper to remove residual water and seeds were planted on solid MSO medium [12] in 100 ml glass bottles, with 2-5 seeds per bottle. The bottles containing the seeds were placed in a culture room under alternating light conditions (16 hours light/8 hours dark) at a temperature of 25 ± 2°C.

2.2 Plant Parts and Their Culture

Thirty-day-old seedlings were used as a source for inducing callus of root, stem, and leaf using the following media:

S1: MSO

S2:B5

S3:B5 + 0.5 mgL^{-1}2,4-D + 0.1 mgL^{-1} Kin

S4:B5 + 1.0 mgL^{-1} 2,4-D + 0.05 mgL^{-1} Kin

S5:B5 + 1.5 mgL^{-1} 2,4-D + 0.05 mgL^{-1} Kin

Callus induction from the plant parts was observed, after 30 days, measuring the fresh weight from samples kept in the culture room.

2.3 Production of Artificial Seeds

The production process of somatic embryogenesis involved culture of segments of callus of root, stem, and leaf with weighing 1g/piece on the following embryo production media:

M1: MSO
M2:MS + 0.5 mgL^{-1}TDZ
M3:MS + 0.5 mgL^{-1} TDZ + 1.0 mgL^{-1} BA
M4:MS + 0.5 mgL^{-1} TDZ + 1.5 mgL^{-1} BA

Samples were kept in the culture room until embryonic phases (spherical, heart, torpedo, and cotyledon) were formed. The torpedo phase was used to produce artificial seeds using a 2% sodium alginate solution mixed with 1L of MS medium free of $CaCl_2·2H_2O$, with stirred continuously, and the pH was adjusted to 5-8. The solution was sterilized in a pressure apparatus for 20 minutes. The embryos were excised at the torpedo phase and then immersed in the 2% sodium alginate solution in a 150 ml beaker for one minute, and the plant parts were removed using a micropipette. Drops of sodium alginate with the plant parts were placed in a calcium chloride ($CaCl_2·2H_2O$) solution, left for 15 minutes to form beads, then washed 3 times with distilled sterilized water to remove the hardening agent. They were dried on sterile filter paper, transferred to Petri dishes, sealed tightly [13], and stored at 4°C and 25°C for 20 days. After the storage period, the beads were planted on solid MS medium for 3 weeks and then transferred to the culture room at 25 ± 2°C under 16 hours light/8 hours dark conditions.

2.4 Acclimatization of Resulting Plants

The resulting plants from the callus were transferred to MS medium free of growth regulators and MS containing 1.0 mg/L^{-1} IAA. The plants with root clusters were washed with distilled sterilized water to remove any residual sodium alginate beads, then cultured in the same medium and kept under culture room conditions.

3. Results and Discussion

The surface sterilization method used showed high efficiency in germinating the seeds of Arabidopsis thaliana (NO-0), achieving a 100% germination rate and producing healthy seedlings within 7 days of planting. Thirty-day-old seedlings were used as a source for obtaining plant parts, as the ecology patterns of A. thaliana differ in their positive response to seed dormancy and variations in leaf shape, stem length, and flower morphology [14]. Root, stem, and leaf segments showed a clear response to callus induction when cultured on B5 medium. The roots planted on B5 medium **Table 1**. It was observed that the roots cultured in a medium free of growth regulators showed a distinctive response for callus induction within

5 days, callus was characterized by a yellowish-white color and friable texture **Figure 1-A**. Meanwhile, the B5 medium containing 2,4-D and Kin had a good effect in inducing callus from stems and leaves, with a friable texture and greenish-yellow color **Figure 1-B,C**. Additionally, it was observed there was an increase in the fresh weight of callus from the three segments successfully after 30 days of culture on the same medium **Figure 1- D,E,F** at a rate of 20 replicates per plant segment.

Table 1: Callus Induction from Plant Segments of Arabidopsis thaliana (NO-0)

Induction Media	Callus Induction Percentage (%)			Duration for Callus Induction (day)			Average Fresh Weight		
	Roots	Stems	Leaves	Roots	Stems	Leaves	Roots	Stems	Leaves
S1	٠	0	٠	0	٠	0	٠	0	٠
S2	100	0	٠	5	0	٠	٣,٨	0	٠
S3	75	٩٥	90	٧	١٤	١٥	٣,٦	٣,٨	٤,٣
S4	٨٥	١٠٠	٩٥	٧	١٣	٩	٣,٤	٤,٠	٤,٥
S5	٦٥	٧٥	٩٠	١٠	١٧	١٢	٣,١	٣,٧	٤,١
Chi^2	٨٩,٢٧٦	٩٢,٨٥٢	٨٧,٣١٨	٧,٣٧٩	١١,٢١٧	١٠,١٢٨	٢,١١٨	٠,٨٥٧	٠,٨٥٧
p-value	**٠,٠٠	**٠,٠٠	**٠,٠٠	n.s٠,١١٧	*٠,٠١١	*٠,٠١٨	n.s٠,٧١٤	٠,٨٣٦ n.s	٠,٨٣٦ n.s

- Number of pieces used: 20 /plant segment

The data in **Table 1** above, derived from visual observations and weight measurements, reflect the variation in the color and structure of the callus, influenced by the type of added growth regulators and their concentrations, as well as the accumulated hormone content in the plant, particularly the high levels of auxins associated with callus production [15,16]. It has been reported that the relative level between 2,4-D and Kin is essential for inducing root callus of Arabidopsis thaliana, and the response of the leaves, due to their possession of active meristematic cells through their divisions, resulted in the production of parenchymal tissue characterized by its continuous vitality in the presence of abundant nutritional requirements and the repeated transfer to new media [17].

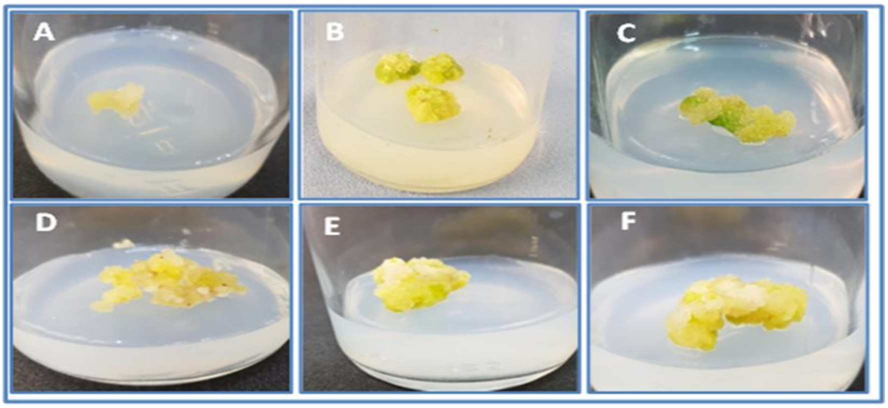

Figure 1: Callus of plant parts of Arabidopsis thaliana (NO-0) in culture media

A: Beginning of root callus induction D: Rate of root callus growth after 30 days

B: Beginning of stem callus induction E: Rate of stem callus growth after 30 days

C: Beginning of leaf callus induction F: Rate of leaf callus growth after 30 days

The callus induced from different segments was transferred to selected media for the production of somatic embryos, which are essential for generating artificial seeds. These embryos are produced from somatic cells of a single plant, unlike natural seeds that result from fertilized embryos from sexual reproduction between two different plants [18]. It was observed that the MS medium supplemented with growth regulators TDZ and BA influences in the production of green shoots **Table 2** from root callus **Figure 2-A**. Whereas, using TDZ at 0.5 mg L^{-1} has an effect in the production of embryonic phases from stem and leaf callus, starting with the spherical phase **Figure 2-B**, then the heart phase **Figure 2-C**, the torpedo phase **Figure 2-D**, and finally the cotyledon phase **Figure 2-E**, and leading to leaf production **Figure 2-F**.

Table 2: Production of green shoots and development of embryonic phases from callus of plant parts of Arabidopsis thaliana (NO-0)

Nutrient Medium (mg L^{-1})	Plant Segment	Rate of Somatic Embryos/ phase				Number of Shoots
		Spherical	Heart	Torpedo	Cotyledon	
M1	Leaves	٣٣	٣٠	١٥	١٠	٧٥
M2		٢٥	١٤	١٠	٩	٤٥
M3		٢٠	١٥	١٠	١٠	٣٠

Chi^2		٣,٣٠٨	٨,١٦٩	١,٤٢٩	١,٨٢٤	٢١,٠٠
p-value		n.s٠,١٩١	*٠,٠١٧	n.s٠,٤٩٠	n.s٠,٤٠٢	**٠,٠٠
M1	Stems	٢٥	٢٠	١٥	١١	٦٠
M2		٢٠	١٥	١٠	٩	٣٠
M3		١٥	١٢	١٠	٩	٣٠
Chi^2		٢,٥٠	٢,٠٨٥	١,٤٢٩	٠,٢٧٦	١٥,٠
p-value		n.s٠,٢٨٧	n.s٠,٣٥٣	n.s٠,٤٩٠	n.s٠,٨٧١	**٠,٠٠١
M1	Roots	١٥	١٠	٩	٩	١٥
M2		١٠	٨	٥	٥	١٠
M3		٠	٠	٠	٠	٠
Chi^2		١٠,٦٩٢	٧,٠٥٣	٦,٤٠	٦,٤٠	١١,٦١٥
p-value		**٠,٠٠٥	*٠,٠٢٩	*٠,٠٤١	*٠,٠٤١	**٠,٠٠٣

- Number of pieces used: 3 callus segments/treatment

The response of the ecology patterns of A.thaliana (Col-0, Ws-0, No-0, HR-10) varied in their germination rates and abilities to form new plants, with different rates, the highest rate recorded in Ws-0 and No-0 at 30.67% for callus induction and new plant production [19]. The cells of A. thaliana are capable of renewing the organs and tissues and even cells; in addition, they are able to produce walls and divisions for forming new plants [20]. Moreover, adding cytokinins is essential for the production of green shoots, and the cells of the wild types of A. thaliana are capable of forming buds on nutrient media containing varying ratios of cytokinins to increased auxins [20,21]. The formation of roots from the new plants posed a major obstacle in the process of developing complete plants, which was overcome by adding low concentrations of auxins [19], it was observed that rooting of resulting plants at 100%.

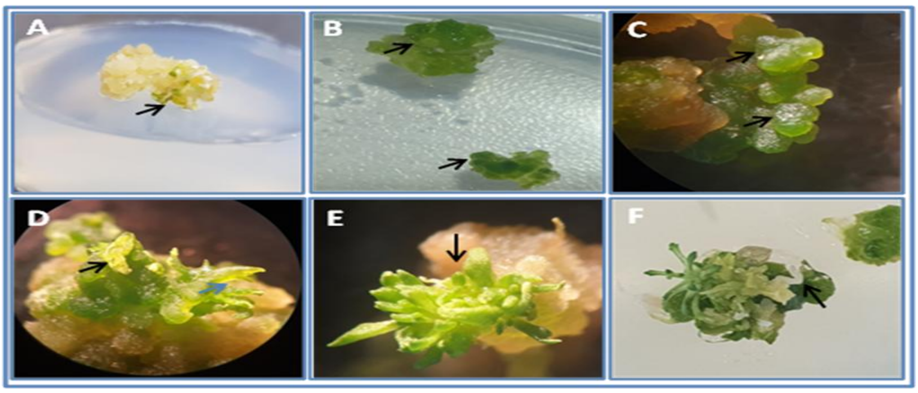

Figure 2: Emergence and Development of Somatic Embryos from Arabidopsis thaliana (NO-0) Callus

A: Formation of shoots from root callus.

B: Development of somatic embryos to the spherical .

C: Development of somatic embryos in (B) to the heart phase using a dissecting microscope.

D: Development of somatic embryos to the torpedo phase (see blue marker) and the cotyledon stage of the somatic embryos in (D) (see black markers) using a dissecting microscope..

E: Emergence of real leaves from the embryonic mass (indicated part) using a dissecting microscope..

F: Elongation of the formed shoots and rooting in medium MS + 0.1 mg L^{-1} IAA

The physiological state of the plant parts used as a source for somatic embryos is a significant factor in their emergence and development. Other factors may also contribute to the successful formation of somatic embryos in this study, such as the polar accumulation of endogenous hormones in the plant part [23].

In this study, the torpedo phase was obtained from the embryonic phases formed from stem and leaf callus using sodium alginate and $CaCl_2.2H_2O$ at 2%; and the interaction between them played a role in forming spherical structures (Beads) for artificial seeds (Figure 3,A). After storing the seeds at 4ºC and 25ºC (Figure 3-B), the highest conversion rate for the artificial seeds containing somatic embryos was 80% for samples stored at 4ºC (Table 3). The presence of sodium alginate at specific concentrations contributed to forming cohesive gel-like coatings around the embryonic stages, protecting them from moisture loss and facilitating ion exchange between Na^{+} and Ca^{++}; and the hardness and softness of seeds are determined by the ionic exchange between sodium ions and calcium [24]. Although the commercial source of sodium genes is a determining factor that may lead to variations in the concentration ability of this substance for producing artificial seeds, several studies have indicated that a concentration of 2% of sodium genes is the optimal concentration, as observed in Piper nigrum L. [25].

Table 3: Effect of storage duration and temperature on the percentage of conversion of artificial seeds from somatic embryos of A. thaliana (No-o) to Complete-grown Plants.

Storage Duration (Day)	Conversion of Artificial Seeds (%)		Chi^2	p-value	Number of Resulting Plants		Chi^2	p-value
	4ºC	25ºC			4ºC	25ºC		
٧	٨٠	٦٠	2.857	$0.091^{n.s}$	٢٠	١٥	0.714	$0.398^{n.s}$
٢٠	٧٢	٤٠	9.143	0.002**	١٠	١٠	0.0	$1.0^{n.s}$
Chi^2	0.421	4.0			3.33	1.0		
p-value	$0.516^{n.s}$	0.046*			$0.068^{n.s}$	$0.317^{n.s}$		

- Number of pieces used: 25 seeds/treatment

The highest conversion rate for artificial seeds stored at 25ºC was 60% during 20 days into plantlets after 4 weeks after germinating them on MSO medium **Figure 3-C**. They started planting after 6 days **Figure 3-D**. The successful germination of artificial seeds of somatic embryos, which resulted in green shoots **Figure 3-F** and roots formation after 4 weeks **Figure 3-E**; and plants continued to grow normally. The high conversion rates for seeds stored at 4º for the same period can be explained by reduced metabolic activity, especially respiration, and water and nutrient absorption at 25ºC, which assists seeds in maintaining their viability during storage [26]. Several studies have indicated the effectiveness of storage at 4ºC for various periods in preserving the viability of stored artificial seeds and their successful germination rates [27]. Increasing the storage duration of seeds at 4ºC reduces the conversion rate of artificial seeds to complete plants, likely due to the process of impaired gas exchange in the plant tissues within the beads, and this leads to a decrease in oxygen levels in cells, which leads to an increase in carbon dioxide, affecting the potential energy of the plant tissue. Consequently, there is a decrease in vital processes such as respiration, protein synthesis, and other functions with increased storage periods due to the occurrence of respiration processes [28].

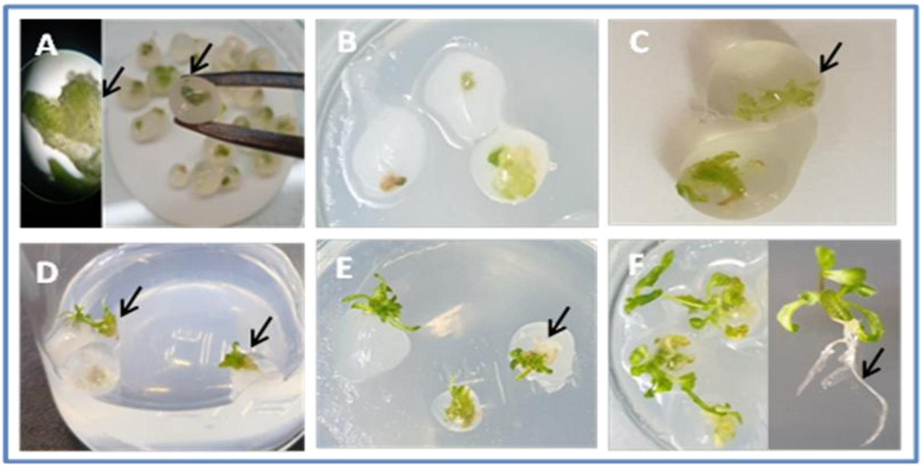

Figure 3: Encapsulation of somatic embryos of A.thaliana (No-0) and formation of artificial seeds, germination, and development of new plants

A: One of the embryonic phases in the artificial seed beads .

B: Artificial seed beads after hardening with $CaCl_2.2H_2O$.

C: Conversion of artificial seeds stored at 4º C after 6 days of planting .

D: Conversion of artificial seeds stored at 25ºC after 6 days of planting .

E: Emergence of rooted shoots from the artificial seeds .

F: Plants produced from artificial seeds after four weeks of planting .

The change in color of the somatic embryos coated with sodium alginate to yellow or brown negatively affects the germination of seeds and the formation of plants; as the color change in nodal buds of Aguilaria malacensis trees coated with sodium alginate after prolonged storage and increasing their size, leading to decomposition due to inhibited respiration [29].

In the present study, the decrease in the conversion rate of seeds stored at room temperature 25° for 15.7 days is attributed to the drying of the seed coat and the retention of the plant part, which resulted in reduced oxygen levels and decreased moisture inside the storage containers [30]. A recent study [27] compared four genetic varieties of Cicer arietinum L. when the growing tips were wrapped and stored at 4° for 5 months. The results showed that the Giza4 and Giza88 varieties excelled, with germination rates of 70-75% for both classes, compared to the other two classes.

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