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In Vitro Propagation of Date Palm (*Phoenix dactylifera* L.) Embryos Using Synthetic Seeds

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Abstract: *Synthetic seed technology offers tremendous potential in micropropagation and germplasm conservation. Somatic embryos at the torpedo stage of Fraihy and Bartamuda date palm cultivars were used as synthetic seeds. They were encapsulated in sodium alginate matrix provided with Murashige and Skoog (MS) nutrient medium, antibiotic protectant (Cefotaxime) and without plant growth regulators (PGRs) as artificial endosperm to enhance germination and conversion percentage. The highest percentages of survival and germination were noticed with 3% sodium alginate, which gave 80.0% survival percentage for both cultivars, with germination percentage of 66.7 and75.5% for cvs Fraihy and Bartamuda, respectively. The application of self-breaking gel beads technology (treatment with potassium nitrate) sustained and increased the germination (86.7%) and sprouting took 20-21 days for both cultivars. Moreover, conversion percentage was enhanced and recorded 73.3 and 80.0% for cvs Fraihy and Bartamuda, respectively. The encapsulated embryos of cvs Fraihy and Bartamuda were sown directly under aseptic conditions on MS medium supplemented with 0.1 mg/l NAA and different substrate media. The highest germination percentage was 94.4% for both cultivars. with conversion percentages of 83.3 and 88.9% for cvs Fraihy and Bartamuda, respectively when cultured on MS medium supplemented with 0.1mg/l NAA, followed by growth substrate medium containing peat moss and vermiculite, which gave germination and conversion percentages of 83.3 and 55.6%, respectively for cv Fraihy and 88.9 and 66.7%,respectively for cv Bartamuda of synthetic seeds. The encapsulated embryos of both cultivars those were sown directly under aseptic conditions, show germination and conversion. So, by applying this protocol date palm embryos synthetic seeds could be produced and converted into whole plants, when sown in vitro on different media. While, those were sown directly under ex vitro conditions (greenhouse) on different substrate growth media, were contaminated and did not show any germination. Plantlets regenerated from encapsulated embryos in vitro were acclimatized successfully.*

Keywords: *date palm, somatic embryos, synthetic seeds, self- breaking gel beads*

I. Introduction

Date palm (*Phoenix dactylifera* L.) is a monocotyledonous, dioecious and economically important fruit tree (Alshuaibi, 2011). Its cultivation is established with selected ecotypes clonally propagated *via* offshoots. However, this method is relatively slow, since a limited number of offshoots are produced by a date palm tree during its lifetime. So, *in vitro* propagation is increasing becoming an attractive alternative for large - scale propagation of date palm. *In vitro* plant regeneration of date palm occurs through organogenesis and somatic embryogenesis depending on genotypes and hormonal manipulations (Al-Khayri, 2003). Somatic embryogenesis is a powerful tool for the improvement of date palm, as well as for mass propagation (Diab, 2008 and Abo Elfadl, 2008) and in synthetic seeds production of many crop varieties (Kumar and Thomas, 2012).

Synthetic seeds "Synseed" technology using encapsulation of *in vitro* single somatic embryo has become an important asset to micropropagation (Naik and Chand, 2006). Therefore, it has been developed based on the use of somatic embryos as real seeds and posses the ability to convert into whole plant under *in vitro* and *in vivo* conditions (Gray, 1987 and Sharma *et al*., 2013). The advantages of using synthetic seeds include easy handling, germplasm conservation of elite, endangered and commercially important plants by using storage, exchange of axenic plant material between laboratories, reduction in costs and direct delivery to the field (Rai *et al*., 2009 and Sandoval- yugar *et al*., 2009).

Recently, synthetic seed technology has been widely studied and is found to be applicable to several plant species including *Phoenix dactylifera* L. (Bekheet *et al*., 2002 and 2005), *Carica papaya* L. (Rady, 2004), *Allium sativa* (Bekheet, 2006), *Olea europeaea* L. (Ikhlaq *et al*., 2010) and *Rhinacanthus nasutus* L. (Meena *et al*., 2012). Alginate coat protects the embryos, but the composition of the gel matrix is an important factor that significantly affects the conversion performance of encapsulated tissue. The low germination and conversion capacity of synthetic seed is due to absence of a nutritive tissue like the endosperm of the natural seed (Sanada *et al*., 1993) and the physical hindrance of the root and shoot emergence caused by the gel capsules. This can be overcome by adopting self-breaking alginate gel beads technology in which synthetic seeds are pretreated with potassium nitrate (KNO_3), the K^+ ions replace the Ca^{++} ions of the calcium alginate capsule thus allowing the synthetic seed to soften and allow the subsequent conversion to plantlets (Onishi *et al*., 1994). A self-breaking

treatment was applied to *Oryza sativa* synseeds with an artificial endosperm by immersing them in 200 mM KNO_3 solution for 60 min and rinsing in sterile tap water for 40 min or more until the beads became swollen (Arun Kumar *et al*., 2005). While, *Stevia rebaudiana* synseeds were dipped in 100 mM and 200 mM KNO_3 solution for 20 and 5 min, respectively (Ali *et al*., 2012).

For germination and field planting, the synthetic seed have the possibility of being an alternative planting material meant for forestry sector (Asmah *et al*., 2011). Synthetic seeds would allow direct planting of plant propagules into the greenhouse or field, thus bypassing many of intermediate steps. Fujii *et al*. (1989) found that maturation of alfalfa somatic embryos with ABA showed high soil conversion of 48 to 64%. Encapsulation provides a convenient and reliable means of producing plants *in vitro*. Synthetic seeds can be efficiently planted *in vitro* either on semi-solid culture medium or planting substrates (Perlite, vermiculite, vermicompost, soil and sand) for conversion into complete plantlets (Mandal *et al*., 2000 and Pinker and Abdel-Rahman, 2005). Generally, nutrient - rich media are more effective than nutrient - deficient substrates for successful recovery of plantlets (Mandal *et al*., 2000).

This study was conducted with the aim of establishing an *in vitro* propagation protocol for date palm Fraihy and Bartamuda cultivars *via* synthetic seeds.

II. Materials And Methods

Culture establishment

Explants preparation and embryos induction of Fraihy and Bartamuda date palm cultivars were used according to procedures described by (Diab, 2008 and Abo El-Fadl, 2008), respectively. Shoot tips of cv Fraihy (Egyptian dry date palm grown at Siwa Oasis, Matroh Governorate, Egypt) and cv Bartamuda (Egyptian dry date palm grown at Aswan Governorate, Egypt) were excied from 3-4 years old offshoots. Shoot tips of cv Fraihy were surface sterilized with commercial Clorox [5.25% sodium hypochlorite (NaOCl)] contained two drops of Tween-20 per 100 ml solution (double surface sterilization), firstly by 3.2% NaOCl for 5 min and secondly by 2.1% NaOCl for 20 min and thoroughly washed with sterilized distilled water for two times. After that one leaf was carefully removed from the shoot tips followed by immersion in mercuric chloride ($HgCl_2$) at 0.05% for 7 min. Then, they were rinsed with sterilized water for five times. Whereas, shoot tips of cv Bartamuda were surface sterilized with 3% NaOCl for 30 min followed by 0.1 mg/l $HgCl_2$ for 10 min followed by 2% NaOCl for 15 min. Then, they were rinsed with sterilized water for three times. All sterilized shoot tips were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 10 mg/l dichlorophenoxy acetic acid (2,4-D), 3 mg/l 2-isopentenyladenine (2iP), 40 mg/l adenine sulfate, 170 mg/l NaH_2PO_4, 30 g/l sucrose, 3 g/l activated charcoal, 100 mg/l ascorbic acid, 150 mg/l citric acid and 2 g/l gelrite. Cultures were incubated at 27 $\pm$ 2°C under complete darkness for eight months with regular transfer to fresh medium of the same composition every four weeks. After this period, embryogenic callus was formed and then transferred to MS medium without plant growth regulators (PGRs) to obtain somatic embryos.

Encapsulation of somatic embryos

The obtained somatic embryos (Torpedo stage) were used as explants for the production of synthetic seeds. Sodium alginate (Na-alginate) solution (SAS chemicals, Mumbai-India) at the concentration of 2, 3 or 4% (w/v) and 100 mM calcium chloride ($CaCl_2$) solution (MERCK, U.S.A) were prepared in the MS nutrients medium (except $CaCl_2.2H_2O$), vitamins and 30g/l sucrose supplemented with 250mg/l cefotaxime and without any PGRs (as artificial endosperm). Both the gel matrix and complexing agent were autoclaved at 121°C and 1.2 Kg cm^{-2} pressure for 15 min. Encapsulation was accomplished by mixing the somatic embryos into the Na-alginate solution and dropping these into the $CaCl_2$ solution. Each drop contained one somatic retrieved from the solution rinsed three times with sterilized distilled water to remove traces of $CaCl_2$, then cultured on MS medium. To investigate the effect of gel concentration on survival and germination of encapsulated somatic embryos; 2, 3 and 4% of Na-alginate as gel matrix were tested. Synthetic seeds with artificial endosperm were given self-breaking treatment by dipping the synthetic seeds in 100 mM potassium nitrate (KNO_3) solution for 20 min, then rising in sterile double distilled water for 40 min or more till the beads became swollen according to (Onishi *et al*., 1994). Its performance was then compared with the synthetic seeds with endosperm (non-treated with KNO_3).

Immediately after this procedure, treated and non treated synthetic seeds with KNO_3 were sown on MS medium to evaluate the effect of self-breaking coat on germination period and percentage, in addition to conversion percentage. Therefore, treated synthetic seeds with KNO_3 were sown on different substrate growth sterilized media (peat moss, vermiculite and perlite, alone or in mixture) at equal volume and distributed into large jars (200 ml) filled with 50 g of substrate medium, in addition to MS medium supplemented with 0.1mg/l naphthaleneacetic acid (NAA) as a control. No nutrients and PGRs were added to substrate, except the distilled water then, cultures were incubated in growth room under a 16-h photoperiod with a light intensity of 40.5 µmol m^{-2} sec^{-1} provided by cool white fluorescent lamps. The same previous treatments were used again in

greenhouse. The germination and conversion percentages of synthetic seeds were recorded. Data were statistically analyzed and subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan's multiple range tests (Duncan, 1955) as described by Snedecor and Cochran (1990).

Acclimatization of plantlets

The plantlets with well developed roots and shoots were taken and washed with running tap water and then disinfected by immersion in Benlate solution (0.1%) for 5 min. Then, they were transplanted in plastic pots contained mixture of peat moss, vermiculite and washed sand (at equal volume). The plantlets were covered with transparent polyethylene bags for one month and were sprayed with water to maintain a high moisture around the plantlets. Small holes were poked into bags for air circulation. Gradually, the humidity was reduced and the covers were completely removed after six weeks of transplaning. The plants were sprayed with the copper oxychloride, fungicide solution at 2 g/l and irrigated with $^1/_{10}$ strength MS inorganic salts once a week.

III. Results And Discussion

Encapsulation of somatic embryos

Calcium alginate beads with entrapped embryos were differed morphologically in respect to texture, shape and transparency with different concentrations of Na-alginate.

The results presented in Table (1) show the effect of 2, 3 and 4% Na-alginate as a gel matrix on the survival and germination percentages of encapsulated somatic embryos of Fraihy and Bartamuda date palm cultivars. The gelling matrix of 3% Na-alginate and 100 mM $CaCl_2$ was found most suitable for formation of ideal beads. This concentration gave the highest survival percentage of 80.0 % for both cultivars and the germination of 66.7 and 75.0 % for cvs Fraihy and Bartamuda, respectively.

Table (1): Effect of gel concentration on survival and germination of encapsulated somatic embryos of Fraihy and Bartamuda date palm cultivars.

Gel concentration %	cv Fraihy		cv Bartamuda	
	Survival %	Germination %	Survival %	Germination %
2	60.0 b	44.4 b	60.0 b	55.6 b
3	80.0 a	66.7 a	80.0 a	75.0 a
4	26.7 c	25.0 c	40.0 c	33.3 c

Each treatment is the average of 15 replicates

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

The beads formed by 2% Na-alginate were fragile and irregularly shaped and too soft to handle and the embryos turned brown, whereas at the high concentration of Na-alginate (4%), the beads were hard enough to cause considerable delay in sprouting and the embryos dried. Hence, 3% Na-alginate was used for encapsulation of embryos in further experiments. Similar observations were also recorded in *Phoenix dactylifera* L. (Bekheet *et al*., 2002), and *Hyoscyamus muticus* L. (Pandey and Chand, 2005). Encapsulation of vegetative propagules and formation of isodiametric beads is influenced by Na-alginate and $CaCl_2$ concentrations (Singh *et al*., 2006) and gel complexation and capsule hardness depends upon optimal ion exchange of Na^+ and Ca^{++} (Redenbaugh *et al*., 1993). In most of the reports, 3% (w/v) Na-alginate and 100 mM CaCl for 20-30 min has proved to be the best combination for the formation of an ideal synseed (Ahmad and Anis, 2001; Rai *et al*., 2008 and Huge and Trueman, 2012).

In vitro germination and conversion

In vitro germination period and percentage in addition to conversion percentage of the capsules were recorded to evaluate the effects of presence or absence of KNO_3 at 100 mM for 20 min as a self-breaking treatment. Encapsulated somatic embryos sprouted within 32-35 days for both cultivars on MS medium without PGRs and self-breaking treatment (Table 2).

Table (2): Effect of self-breaking with or without KNO3 on germination and conversion of synthetic seeds of Fraihy and Bartamuda date palm cultivars

Self-breaking coat	cv Fraihy			cv Bartamuda		
	Germination		Conversion	Germination		Conversion
	Period (days)	%	%	Period (days)	%	%
Without	32-35	60.0 b	53.6 b	32-35	73.3 b	66.7 b
With	20-21	86.7 a	73.3 a	20-21	86.7 a	80.0 a

Each treatment is the average of 15 replicates

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

The percentage of germination was 60.0 and 73.3% for cvs Fraihy and Bartamuda, respectively. Whereas, the percentage of conversion was 53.6 and 66.7% for cvs Fraihy and Bartamuda, respectively. In the present study, adoption of self-breaking synthetic seed technology significantly enhanced germination (86.7%) and sprouting took 20-21 days for both cultivars and conversion percentage reached 73.3% and 80% for cvs Fraihy and Bartamuda, respectively (Fig. 1). These results show that capsule weakening through KNO_3 treatment.

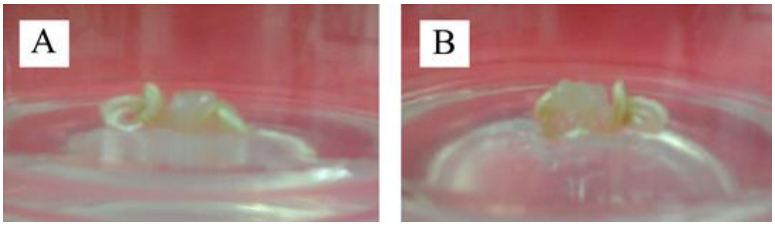

Fig. (1). Effect of self-breaking coat on germination, conversion and sprouting of synthetic seeds of date palm; (A) Fraihy and (B) Bartamuda cultivars after three months.

The results are in line with those reported by Sandoval-Yugar *et al*., (2009). They reported that the capsules with MS salts formation treated with 100 mM KNO_3 showed 76% conversion of *Musa* sp cv Grand Naine microshoots. Also, in this respect, Arun Kumar *et al*., (2005) reported that the application of self-breaking gel beads technology increased the germination (52%) and conversion (47%) of synthetic seeds in hybrid rice.

Inclusion of self- breaking coat has enhanced the germination and conversion to the maximum extent by increasing the diffusion of gases, diffusion of nutrients and respiration of alfalfa embryoids (Zhong and Wang, 1987). Data in Table (3) show the germination and conversion of synthetic seeds of Fraihy and Bartamuda date palm cultivars on different substrate growth media *in vitro*.

The highest percentage of germination was 94.4% for both cultivars and (83.3 and 88.9%) conversion percentages for cvs Fraihy and Bartamuda, respectively on MS medium supplemented with 0.1 mg/l NAA for both cultivars (Fig. 2).

Table (3): Growth of synthetic seeds with self-breaking coat of Fraihy and Bartamuda date palm cultivars on different substrate growth media *in vitro*

Substrate growth media (At equal volume)	cv Fraihy		cv Bartamuda	
	Germination %	Conversion %	Germination %	Conversion %
Peat moss (Peat)	50.0 f	27.8 d	50.0 f	33.3 f
Vermiculite	55.6 d	27.8 d	61.1 d	27.8 d
Perlite	55.6 d	27.8 d	61.1 d	27.8 d
Peat+Vermiculite	83.3 b	55.6 b	88.9 b	66.7 b
Peat+Perlite	55.6 d	27.8 d	61.a d	27.8 d
Peat+Vermiculite+ Perlite	72.2 c	50.0 c	77.7 c	55.6 c
MS basal + 0.1mg/l NAA	94.4 a	83.3 a	94.4 a	88.9 a

Each treatment is the average of 18 replicates

Means within columns followed by the same letters are not significantly ($P < 0.05$) different

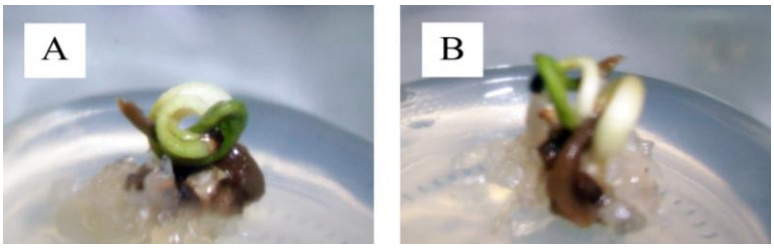

Fig. (2). Germination of synthetic seeds of date palm; (A) Fraihy and (B) Bartamuda cultivars on MS medium supplemented with 0.1 mg/l NAA.

Moreover, the synthetic seeds germinated on all sterilized substrate media had given the satisfying results and that its highest percentages of germination (83.3 and 88.9%) and conversion (55.6 and 66.7%) for cvs Fraihy and Bartamuda, respectively were observed on growth substrate medium containing peat moss and vermiculite (Fig. 3), followed by growth medium containing peat moss, vermiculite and

Perlite, which gave germination and conversion percentages 72.2 and 50.0%, respectively for cv Fraihy and 77.7 and 55.6%, respectively for cv Bartamuda (Fig. 4).

Whereas, the lowest percentage of germination (50.0% for both cultivars) and conversion percentages (27.8 and 33.3% for cvs Fraihy and Bartamuda, respectively) were observed on peat moss growth medium . However, more than 55.0 and 61.0% of synthetic seeds for cvs Fraihy and Bartamuda, respectively were germinated on vermiculite or perlite only or peat moss and perlite media.

So, the encapsulated embryos of date palm cultivars, which were sown directly under aseptic conditions on MS or different substrate growth media gave positive results. Thus, the MS nutrients and PGRs enhanced and helped for subsequent growth and development of encapsulated embryos. On the other hand, those were sown directly under greenhouse conditions did not show any germination and got contaminated (data not shown). Because during direct sowing of the capsules, contamination by microorganisms is one of the major hurdles for the commercialization of encapsulation t echnology. Moreover, nutrients and especially organic, released by the beads are responsible for serving contamination.

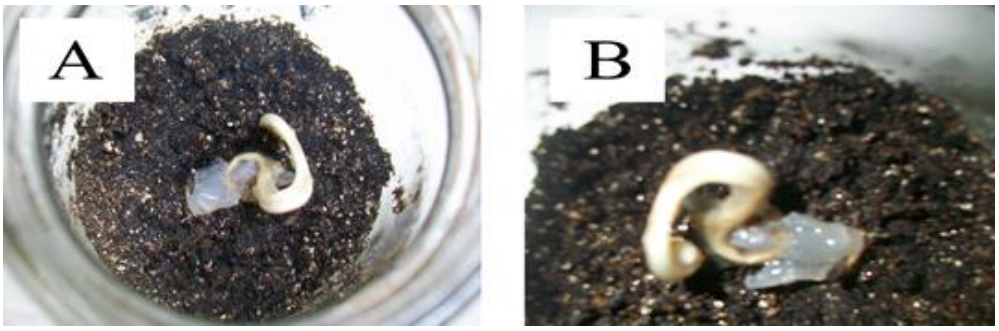

Fig. (3). Germination of synthetic seeds of date palm on sterilized substrate growth medium containing peat moss and vermiculite of (A) Fraihy and (B) Bartamuda cultivars.

Fig (4). Germination of synthetic seeds of date palm on sterilized substrate growth medium containing peat moss, vermiculite and perlite of (A) Fraihy and (B) Bartatmuda cultivars.

The results are in line with that reported by Rady (2004). He found that the highest rates of germination and shoot growth of stored encapsulated shoot tips of papaya were observed on MS medium supplemented with 0.5 mg/l BA and 0.5 mg/l IAA. While, the non-stored synthetic seeds were observed on growth medium containing peat moss and vermiculite. Although, the use of different strength of MS medium for plantlets formation(conversion percentage) and increasing fresh weight from encapsulated embryos of Zaghloul date palm were reported by Bekheet *et al.* (2002). They found that the highest conversion (75%) and highest fresh weight (1.09 g) were observed when full strength MS-salts was used. On the other hand, encapsulated somatic embryos of alfalfa (*Medicago sativa*) were planted directly into the field to demonstrate the feasibility of using synthetic seeds for direct sowing (Fujii *et al*., 1992) and reported successful field planting of alfalfa synthetic seeds derived from embryoids encapsulated in calcium alginate, with 23% plant conversion. However, the synthetic seeds of *Citrus reticulate*, which were sowing on soil mix medium did not result in satisfactory conversion (Antonietta *et al*., 2007).

Acclimatization of plantlets

Well-developed plantlets (Fig. 5) regenerated from encapsulated somatic embryos of date palm cvs Fraihy and Bartamuda were hardened and successfully transplanted to greenhouse conditions (*ex vitro*) using pots contained mixture of peat moss, vermiculite and washed sand (at equal volume) and covered with transparent polyethylene bags (Fig. 6).

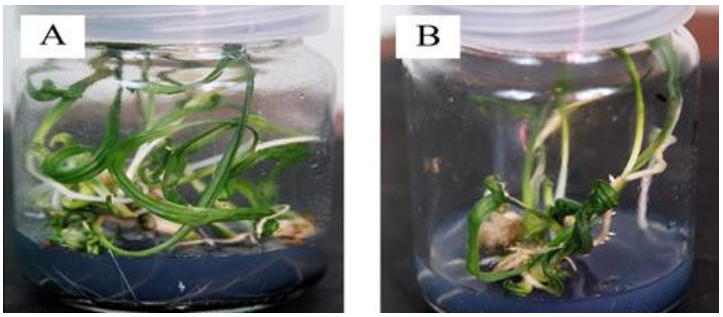

Fig (5). Plantlets development from encapsulated somatic embryos of date palm (A) Fraihy and (B) Bartatmuda cultivars on MS medium after 10 weeks.

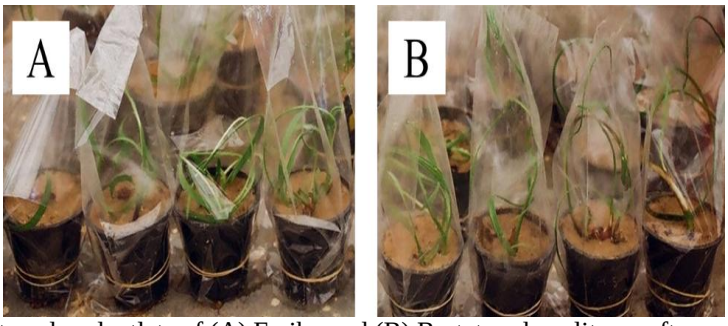

Fig (6). Adapted date palm plantlets of (A) Fraihy and (B) Bartatmuda culitvars after ex vitro transfer to the greenhouse.

In conclusion, a procedure for encapsulation of somatic embryos in cvs Fraihy and Bartamuda date palm were optimized and viability of somatic embryos was tested on different substrate media. These encapsulated somatic embryos could be used for germplasm exchange, *in vitro* storage and micropropagation, easy in handling due to small size of capsules and direct delivery to the field. Although, more studies are needed on the composition of artificial endosperm and substrate growth media to enhance the germination and conversion frequencies of synthetic seeds and subsequent plantlet growth in soil when their sowing directly *in vivo* (greenhouse or field) conditions.

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