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Dormancy, Storage, and Regrowth of Encapsulated Shoot Tips of Cotton (*Gossypium barbadense* L. cv. 'Termez')

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ABSTRACT

Due to the recalcitrant character of cotton *in vitro* culture as well as the lack of somatic embryogenesis in most cotton cultivars, its shoot tips were used for producing synthetic seeds. Cotton seeds were cultured on Murashige and Skoog (MS) medium containing B5 vitamins and 30 g/l sucrose. Explants were prepared from seedling shoots and placed on three dormant treatment media: 0.6 mg/l abscisic acid (ABA), 150 g/l sucrose and 6 g/l polyethyleneglycol (PEG). Treated and control shoots were encapsulated in calcium alginate and after storage at 4°C for 0, 15, 30, 45 and 60 days, were regrown on MS medium containing B5 vitamins, 30 g/l sucrose, and 0.5 mg/l gibberellic acid (GA_3). After 60 days in storage, PEG-treated shoots showed the highest regrowth rate (88.8%) while non-treated shoots did not show any regrowth. In non-storage conditions, shoots in control and sucrose treatments showed more growth than shoots treated with ABA and PEG. All samples treated for 60 days showed less growth than samples treated for 15 days. The shoots that developed from different treatments as well as control shoots were rooted on MS medium containing B5 vitamins, 0.5 mg/l 1-naphthaleneacetic acid and 30 g/l sucrose, with a 71.2-97.7% rooting percentage. Although the levels of rooting differed in each treatment, in most cases they were not significantly affected by storage period or the type of dormancy treatment.

Keywords: artificial seeds, abscisic acid, polyethyleneglycole, microcutting preparation, storage
Abbreviations: ABA, abscisic acid; **B5**, Gamborg *et al*. (1968) medium; **BA**, 6-benzyladenine; **GA_3**, gibberellic acid; **KT**, kinetin; **NAA**, 1-napthalenecetic acid; **PEG**, polyethyleneglycole; **SUC**, sucrose

INTRODUCTION

Cotton as an important natural source of textile fiber and food is cultivated in a number of countries. However, its cultivation needs to be improved by conventional and modern plant breeding methods (Agrawal *et al*. 1997). A major achievement in plant biotechnology in recent years has been artificial seed production used for regenerating plants for a variety of purposes such as regeneration of artificial hybrids, preservation and regeneration of plant germplasm, regeneration of plants created by genetic manipulation, regeneration of unstable and less fertile genotypes, production of seeds free from viral, bacterial, and fungal infections, regeneration of varieties resistant against pests, and high productivity (Vicient and Martinez 1998; Sharma *et al*. 2012).

Cotton as a recalcitrant plant is cultivated *in vitro*. On the other hand, somatic embryogenesis has been reported only in some cotton cultivars (Trolider and Xhixion 1989; Ghaemi *et al*. 2011). Reports of artificial production of cotton seeds using somatic embryogenesis exist in the literature (Rajasekaran *et al*. 1996); however, due to the lack of seeds with normal growth and appearance uniformity and also in past studies (Ghaemi *et al*. 2011), *Gossypium barbadense* L. cv. 'Termez' has shown somatic embryogenesis and plant regeneration, but most of these attempts have failed. For this reason, in the present study, apical shoots of 'Termez' were used to produce hydrogel-based artificial seeds.

Recently, some reports have been published regarding the *in vitro* cultivation of shoot tips (Ali *et al*. 2004; Aydin *et al*. 2004; Jin *et al*. 2006; Farahani *et al*. 2010). Nevertheless, production and storage of dormant artificial hydrogel seeds using cotton shoot tips have not been reported thus far. In this study, the effects of various dormancy inducing pre-treatments on the regrowth and rooting of shoots which were encapsulated and then stored at 4°C at different intervals were investigated.

MATERIALS AND METHODS

Seed germination

Cotton seeds (*Gossypium barbadanse* L. cv. 'Termez') were obtained from the Cotton Research Institute of Iran. Firstly, cotton seeds were delinted with sulphuric acid (98%). Then plump and mature seeds were chosen and surface-sterilized by 70% ethanol for 1 min, and also shaken in 30% commercial bleach [5.25% (v/v) NaOCl] by stirring for 10 min. They were then washed four times with sterile distilled water. Next, they were dipped and kept in sterile distilled water for 5 h to soften the seed coats and allow their complete removal. Subsequently, the sterilized seeds whose coats had been removed were placed in test tubes containing MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg *et al*. 1968) and 30 g/l sucrose. Finally, the seeds were germinated at 28 ± 2°C under a 16-h photoperiod conditions with the light intensity of approximately 2000 lx.

Microcutting preparation

At this stage, apical buds were cut from the apex of 10-day old seedlings, and cotyledons were also isolated from their attachment point to apical buds using a surgical blade.

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Original Research Paper

Dormancy inducing treatments

The study was conducted to evaluate the effects of three dormancy-inducing pre-treatments on the storage of encapsulated shoot tips at 4°C at different time intervals. To induce dormancy, shoot explants were placed on the following media for 10 days: MS + B5 vitamins + 150 g/l sucrose, MS + B5 vitamins + 30 g/l sucrose + 0/6 mg/l abscisic acid (ABA), and MS + B5 vitamins + 30 g/l sucrose + 6 g/l polyethylene glycol (PEG).

Encapsulation of shoot tips

Following the pre-treatments, shoot tips were placed in sodium alginate solution (2.5%). Then the alginate-coated shoot tips were pipetted with a 5 mm (internal diameter) pipette containing 150 µl of the sodium alginate solution and dropped into a solution of 2% $CaCl_2$ (w/v). After a 30-min incubation period, the hardened alginate capsules with shoot tips were recovered by decanting the $CaCl_2$ three times with distilled water. Some samples were immediately transferred into the *in vitro* culture while the remaining samples were stored at a low temperature (4°C) for 15, 30, 45, and 60 days. As for the control samples, some of the alginated shoots were stored at 4°C for 15, 30, 45, and 60 days while others were immediately cultivated on regrowth medium.

Regrowth and rooting of encapsulated shoots

In pilot studies, in order to obtain the best regrowth medium, a number of shoots which had been stored for 15 days were cultivated on five different regrowth media: MS + B5 vitamins + 1 mg/l 6-benzyladenine (BA) + 30 g/l sucrose, MS + B5 vitamins + 0.5 mg/l gibberellic acid (GA_3) + 30 g/l sucrose, MS + B5 vitamins + 0.3 mg/l kinetin (KT) + 30 g/l sucrose, 1/2MS + 1/2 B5 vitamins + 15 g/l sucrose, and MS + B5 vitamins + 30 g/l sucrose.

After 30 days, data on the regrowth percentage and the growth rate of shoots were gathered and the best culture medium (MS + B5 vitamins + 0/5 mg/l GA_3 + 30 g/l sucrose) was used for the regrowth of encapsulated shoots. Then the developed shoots having 2 or 4 little leaves were placed on rooting medium (MS + B5 vitamins + 30 g/l sucrose + 0/5 mg/l 1-napthaleneacetic acid (NAA)) for 30 days.

Culture conditions

All media were solidified with 7 g/l agar. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 28 ± 2°C under a light intensity of approximately 2000 lx provided by cool white fluorescent lamps for a 16-h photoperiod. Subculturing was done every 15 days.

Data analysis

All experiments were carried out in a completely randomized design with 15 cultures per experiment. Each experiment was replicated three times. One-way ANOVA was performed on the data from each experiment, and the means were then compared using Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Seed germination

The germination of sterilized 'Termez' cotton seeds whose coating had been removed was observed after 24 h. After 3 days, the root system along with expanded cotyledons developed on the germination medium. In order to develop healthy seedlings, healthy seeds having normal growth, are necessary. Moreover, to have uniform explants, the age and size of explants are important factors. Therefore, in this study 3-5 mm long shoot tips of sterilized 10-day-old seedlings were used. Younger and older shoot tips were not used because younger 5-day old seedling shoot tips are difficult to separate from cotyledons and stem due to being small and thin, and the ones older than 10 day are difficult to work with because of having old, hard, and woody tissues.

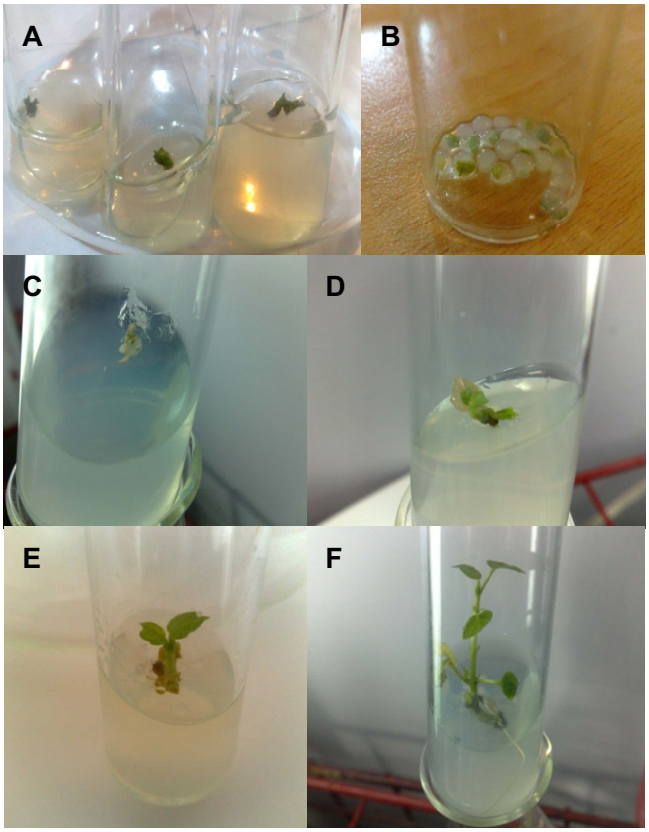

Fig. 1 Synthesis of encapsulated artificial seeds. (**A**) Pre-treatment of PEG. (**B**) Encapsulated shoots by calcium alginate. Regrowth and rooting stages of shoots of PEG treatment after 60 days storage at 4ºC (**C-F**).

Dormancy induction of shoot tips

According to previous studies (Chang and Reed 2001; Fretz and Lorz 1995; Atree *et al*. 1995; Salehi Katouzi *et al.* 2011), the medium that provides dry conditions for shoot tip explants leads to ABA synthesis in explants of shoot tips, which in turn decreases the level of cell water content in shoots. When the shoots under the influence of ABA become dormant, cellular metabolism reaches a trough. On the other hand, storage of encapsulated shoots at 4°C is a suitable method for storage of germplasm for a long time because at low temperatures cellular metabolism decreases. Therefore, in pilot study the suitable dosage and timing for dormancy-inducing pre-treatments were obtained. Shoot tips did not grow after being placed on the dormancy-inducing treatment media. These explants turned dark green in some of their tissue parts. Besides, it seemed that the existing water content in explants of shoot tips has decreased. In the sucrose and PEG treatment media, because of high osmotic pressure, the surface of the media cracked in some parts, and collapsed in other parts (**Fig. 1A**).

Similarly, Frez and Lorz (1995) managed to induce freezing tolerance in barley and wheat cultures with 2 µM ABA. Blakesly *et al*. (1996) induced tolerance to freezing in sweet potato and oil palm somatic embryos using sucrose (0.4 to 0.75 M). Chang and Reed (2001) used ABA and sucrose at different concentrations for pre-treatment of *Pyrus kordata* shoots. Attree *et al*. (1995) concluded that the culture mediums with high molecular mass substances like PEG and dextrans are more effective in improving freezing tolerance than are carbohydrates. Davies *et al*. (1991) and Salehi Katouzi *et al.* (2011) showed that ABA pre-treatment causes decrease in water content and dormancy of shoot tips. Furthermore, Rodriguez *et al*. (1991) proved that like other plant hormones, the adjusting effect of ABA depends on its concentration in the tissue. According to the results of the above-mentioned studies and the pilot phase of the present study, the optimum concentration rate of ABA, PEG and sucrose for pre-treatment of shoots were 0.6 mg/l, 6 g/l, and 150 g/l, respectively for storage at 4°C.

Regrowth of the encapsulated shoot tips

The key to synthetic seed technology is the assessment of the effects of suitable concentrations of sodium alginate and calcium chloride on the texture, shape, and size of the bead. An optimal ion exchange between Na^+ and Ca^{2+} producing firm clear isodiametric beads was achieved by combining 2.5% sodium alginate solution with 2% calcium chloride for 30 min, thereby forming an insoluble gel matrix of calcium alginate (**Fig. 1B**). This is in agreement with many other reports (Wang *et al*. 2007; Salehi Katouzi *et al.* 2011; Shatnawi 2011; Kanchanapoom and Promsorn *et al.* 2012; Thiruvengadam *et al.* 2012).

Calcium alginate capsules placed on shoots acted as an inhibitor of respiration of plant tissues. Therefore, seeds encapsulated with calcium alginate were placed in a one-capacity cation solution like KNO_3 for 90 min before being placed on regrowth medium. As a result, calcium alginate capsules containing shoots collapsed easily in the humid regrowth medium (**Fig. 1C**). Then, in 30 days, shoots started to grow gradually (**Fig. 1C-E**). This is in agreement with the reports of Redenbaugh *et al*. (1987), Kaviani (2010), Salehi Katouzi *et al*. (2011) and Kanchanapoom and Promsorn *et al.* (2012). In this study, the optimum regrowth medium was MS + B5 vitamins + 0.5 mg/l GA_3 + 30 g/l sucrose

The influence of different pre-treatments and storage intervals on the regrowth potential of encapsulated shoots

Based on the results obtained from this study, in without-storage conditions the most regrowth percentage (97.7%) was observed in control shoots, while the least regrowth percentage (68.8%) was recorded for ABA-treated shoots (**Table 1**). Despite this fact, the regrowth percentage of encapsulated-control shoots decreased significantly as the storage days (15 to 60 days) increased so that for the 60-day storage samples, the regrowth percentage reached 0% (**Table 1**). Similarly, the regrowth percentage of encapsulated shoots treated by sucrose, ABA and PEG decreased as the storage days increased from 15 days to 60 days. This regrowth decrease rate was 13.3, 11.1, and 8.8% for sucrose-, ABA-, and PEG-treated shoots, respectively, which was not significant in comparison with the regrowth decrease rate for control shoots (97.7%). The sucrose- and ABA-treated shoots with storage periods of 15, 30, 45, and 60 days displayed more regrowth than their without-storage counterparts. These results suggest that storage at low temperatures might have had positive effects on the regrowth of the treated shoots. After 60 days' storage, the highest regrowth rate was observed in PEG-treated shoots (88.8%), while no regrowth was observed in control shoots (**Table 1**).

The results of this study suggest that PEG, sucrose, and ABA pre-treatments could cause dormancy induction in shoot tips. Among the three pre-treated samples with 60-day storage, the PEG pre-treated shoots had the most regrowth suggesting that PEG-pretreatment can cause maximum storability in seeds (**Table 1**).

In control conditions without storage, sucrose-treated shoots displayed more growth than ABA- and PEG-treated shoots. The shoots stored for 15 days showed higher growth than shoots stored for 30, 45, and 60 days.

The growth rate of regrowing control shoots reduced as the storage period increased from 0 to 45 days (**Table 1**).

Rooting of regrowing shoots

A restricting factor in germination of seeds derived from shoot tips is their monopolarity. In contrast to somatic embryos which have a dipolar structure, shoot tips are monopolar and without a root system. Some studies (Ganapathi *et al*. 1992; Gradi *et al*. 1999) showed that shoot tips existing in the artificial seeds of such species as banana, cardamom, mulberry and raspberry have the potential for rooting. On the other hand, other studies (Hasan and Takagi 1995; Piccioni and Standardi 1995; Maruyama *et al*. 1997; Salehi Katouzi *et al.* 2011; Kanchanapoom and Promsorn 2012) showed that regrowing shoot tips in some species like cotton must be rooted in the rooting medium. Therefore, regrowing shoots of different treatment and control samples (having 2 to 4, i.e., few leaves) were transferred to rooting medium (MS + B5 vitamins + 0.5 mg/l NAA + 30 g/l sucrose) in order to induce rooting.

The results of this study show that these samples displayed rooting levels that varied from 71.2 to 97.7%. On the other hand, the rate of induced root growth was so different (**Table 2**) that in without-storage conditions PEG- and ABA-treated shoots as well as the control shoots had the highest rate of growth, while after 45-day storage, sucrose- and ABA-treated shoots had the highest growth rate. The findings of this study suggest that, in most cases, the

Table 1 Effect of dormancy induction different treatments and storage periods on regrowth percentage and growth rate of developed shoots.

Treatments	Storage days	Number of shoot capsules	Number of germinated shoot capsules	Germinated shoot capsules %	Growth of shoots
Control	0	45	44	97.7 a	+++
	15	45	27	60.0 b	++
	30	45	15	33.3 c	++
	45	45	4	8.8 d	+
	60	45	0	0.0 e	-
ABA	0	45	31	68.8 d	++
	15	45	42	93.3 a	+++
	30	45	41	91.1 ab	+
	45	45	39	86.6 bc	++
	60	45	37	82.2 c	++
Sucrose	0	45	39	86.6 a	+++
	15	45	41	91.1 a	+++
	30	45	40	88.8 a	++
	45	45	40	88.8 a	+++
	60	45	35	77.7 b	++
PEG	0	45	45	100 a	++
	15	45	44	97.7 a	+++
	30	45	43	95.5 ab	++
	45	45	36	80.0 c	++
	60	45	40	88.8 b	+

Data are the means of 3 replicates. Means within columns with the same latter are not statistically different at $P < 0.05$ according the Duncan's multiple rang test (one-way ANOVA). Number of + indicates the growth rate of developed shoots

Table 2 Effect of dormancy induction different treatments and storage periods on rooting percentage and growth rate of developed roots.

Treatments	Storage days	Number of shoots rooting	Rooting of shoots %	Growth of roots
Control	0	38	86.5 a	+++
	15	26	97.1 a	++
	30	13	86.6 a	+
	45	3	83.3 a	+
	60	0	0.0 a	_
ABA	0	26	83.9 a	+++
	15	35	83.3 a	++
	30	31	75.8 a	+
	45	30	76.9 a	+++
	60	28	75.8 a	+
SUC	0	29	74.8 ab	++
	15	36	87.7 a	++
	30	35	87.7 a	+
	45	34	84.7 ab	+++
	60	25	71.2 b	+
PEG	0	28	84.4 b	+++
	15	41	93.1 ab	++
	30	42	97.7 a	++
	45	36	89.2 ab	++
	60	37	92.6 ab	+

Data are the means of 3 replicates. Means within columns with the same latter are not statistically different at $P < 0.05$ according the Duncan's multiple rang test (one-way ANOVA). Number of + indicates the growth rate of developed roots

periods of storage and the type of dormancy treatment had no significant influence on the rooting rate (**Table 2; Fig. 1F**).

CONCLUSIONS

Based on the results of this study, it can be concluded that for artificial seed production, seedling age should be appropriate. Furthermore, shoot dormancy is a major adaptation to low temperatures and long-term storage conditions. PEG and sucrose treatment of apical shoots increased endogenous synthesis of ABA which in turn leads to shoot dormancy. Although exogenous ABA causes shoot dormancy, in this study, PEG-treatment resulted in better shoot storage than ABA treatment.

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