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RESEARCH ARTICLE

In vitro Plant Regeneration of *Helianthus Annuus* (Hyb. Azargol) From Alginate-Encapsulated Shoot Tips for Short Term Storage, Germplasm Exchange and Distribution

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Abstract: The present study demonstrates the potential of nutrient-alginate encapsulation of shoot tips of sunflower, *Helianthus annuus* (hyb. Azargol) for synthetic seed technology, which could be useful in germplasm distribution and exchange. Shoot tips from *in vitro* shoot cultures derived from mature seed explants were encapsulated in 3% sodium alginate and 100 mM $CaCl_2$. $2H_2O$ are supplemented with three different matrices (include distilled water, liquid MS medium and plant growth regulators) and they are stored for several periods (15, 30, 45 and 60 days) at 4°C. After each storage period for regeneration and regrowth evaluation, encapsulated and non-encapsulated shoot tips were cultured on hormone-free MS medium. The regrowth ability of encapsulated shoot tips affected by the storage duration and the presence or absence of MS nutrients in calcium alginate beads. Percentage response for the conversion of encapsulated and non-encapsulated shoot tips decreased gradually after storage at 4°C by increasing storage durations. Indeed, encapsulated vegetative propagules showed a higher resistance to storage at 4°C than non-encapsulated. Addition of MS nutrients in calcium alginate beads significantly improved encapsulated explants regrowth after storage periods.

Keywords: Encapsulation, Germplasm, *Helianthus annuus*, Shoot tips, Synthetic seeds

Abbreviations: PGRs: Plant growth regulators, NAA: α-naphthalene acetic acid, 2,4-D: 2,4-dichlorophenoxy acetic acid, BAP: 6-benzyl amino purine, MS: Murashige and Skoog

INTRODUCTION

Synthetic seed technology is an efficient means for mass propagation of plant species irrespective of season, space, environmental factors and other inhibitory conditions. Encapsulation of axillary buds, shoot tips, nodal segments can be used for clonal propagation system as a vegetative propagules. Also, they have a potentially cost-effective impact as an alternative to synthetic seeds derived from somatic embryos [25, 6]. Indeed, these encapsulated vegetative propagules can be used for germplasm conservation of elite plant species and exchange of axenic plant materials among laboratories for the relative ease of handling these structures and also small bead size [17, 20, 21]. Alginate encapsulation of shoot tips with cold preservation can be used for plant regeneration and germplasm storage. It can be provided as a source of axenic plant material [27] and can be used if stock plants or proliferation cultures become polluted with bacteria, fungi, or arthropods [34]. Also, this is a new mean for

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plant production programs and can be applied for germplasm conservation, and storage. Further, it reduces the transplanting and sub-culturing during off-season periods. During cold storage, encapsulated nodal segments didn't require transfer to fresh medium, thus reduces the cost of maintaining germplasm cultures [34]. synthetic seed production and plantlet regeneration successfully have been reported for cereals, vegetables, fruits, ornamentals, medicinal plants and forest trees [23, 5, 1, 15, 22, 28]. Shoot tip explant is non-embryogenic propagules, and it is more responsive than other explants because of greater mitotic activity in the meristem [3]. Despite these advantages, the encapsulation of non-embryogenic *in vitro*-derived micropropagules for germplasm storage and exchange has been explored in only few plant species [26, 32, 30, 31]. It is essential for preparation of an artificial endosperm to provide carbon sources and plant growth regulators for plantlet development from encapsulated micropropagules [18].

The sunflower is one of the most important oil-producing crops being on the second place in Europe after rapeseed and after palm tree, soybean and rapeseed on the fourth place in the world [13, 9]. Also, it is an important plant for biotechnological research such as interspecific fusion of the protoplasts and gene transfer techniques which represents new ways for transferring valuable agronomic traits in sunflower genomes. The improving of the *in vitro* regeneration capacity of the sunflower is a first step for implementing such methods [19, 10].

Sunflower had poor *in vitro* regeneration among economically important plants [11]. Regeneration frequency depends on genotype and most genotypes are reported to be recalcitrant [8]. In this context, synthetic seed technology nowadays could be a valuable aid to large scale clonal propagation of plant species [28, 14, 30, 29]. The aim of the present study is to verify the possibility to store the encapsulated shoot tips for a period sufficient for germplasm exchanges and distribution. Also, effects of various compositions of sodium alginate matrix have been examined on regrowth of encapsulated shoot tips of sunflower hyb. Azargol.

MATERIALS AND METHODS

Plant materials and explants preparation

The seeds were obtained from Seed and Plant Improvement Institute of Iran (Karaj, Iran). They were sterilized by Benomyl 0.3% (w/v) for 10 minutes, 70% ethanol for 1 minute and 50% sodium hypochlorite (v/v) for 20 minutes and washed thoroughly with sterilized distilled water. For raising seedlings under *in vitro* conditions, seeds were aseptically cultured on MS [16] medium. Shoot tips from *in vitro* grown seven-days-old seedlings were excised and used as explants for proliferation of shoots. These were cultured on MS medium supplemented with 0.5 mg/l BA for shoot regeneration. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl prior to adding 0.8% agar–agar. The medium was autoclaved at 121˚C at 1.2 kg cm^{-2} for 15 min. All cultures were incubated at 25˚C under a 16 h/8 h light/dark cycle, with the light intensity of 50 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent tubes.

Encapsulation procedure

Shoot tips excised from *in vitro* proliferated shoots were used for encapsulation. For encapsulation of shoot tips, sodium alginate (Sigma Chemicals™, USA) at the concentration of 3% (w/v) was prepared in different matrixes including distilled water, liquid MS medium and, liquid MS medium containing 2 mg l^{-1} BAP and 2 mg l^{-1} IAA, and 100 mM of calcium chloride (Merck™, Germany), that was prepared in double distilled water. Encapsulation was accomplished by mixing the shoot tips into the sodium alginate solutions and dropping into the calcium chloride solutions individually. The droplets, each containing one shoot tip, were then maintained in this medium for 20 minutes with slow agitation to achieve polymerization of the sodium alginate. To remove the traces of calcium chloride, the encapsulated shoot tips were washed with sterilized distilled water.

Cold storage of encapsulated shoot tips

For cold preservation, encapsulated shoot tips of each alginate matrix and non-encapsulated shoot tips were placed in a number of empty sterilized petri dishes. Petri dishes were afterwards sealed with Parafilm and stored in dark at 4°C for 0, 15, 30, 45 and 60 days. At the end of each storage period, stored encapsulated shoot tips were immediately transferred to fresh sowing medium (0.8% agar solidified full-strength MS medium) and placed under standard conditions.

Experimental design and data analysis

The percentage of conversion of stored and non-stored encapsulated shoot tips into plantlets was recorded after four weeks of culture. The conversion of encapsulated

shoot tips was determined on the basis of differentiation of shoots and roots with apparent leaves. Five replicates were used for treatments. The mean standard error and analysis of variance were calculated using the MSTAT-C software. The mean separations were carried out using Duncan's multiple range tests (Duncan, 1955) and the significance was determined at (α=0.05). Data expressed as percentage and were transformed using arc sine prior to ANOVA and converted back to the original scale.

RESULTS

The results of this study revealed that the regrowth ability of shoot tips was significantly influenced by storage duration and alginate matrix composition. Statistical analysis showed that there was a significant difference (α=0.01) between storage duration and alginate matrix composition for regeneration (Table 1). The percentage of conversion of encapsulated and non-encapsulated shoot tips to plantlets, decreased gradually after storage at 4°C when the duration of storage increased. In the present study, encapsulated shoot tips showed significantly (P=0.05) higher resistance to storage at 4 °C than non-encapsulated shoot tips (Figure 1 and 2).

The orthogonal group comparison between treatments showed a very significant difference—between encapsulated and non-encapsulated shoot tips that were kept in the different cold storage durations as well as presence or absence of MS nutrients in alginate matrix. Also, the use of growth regulators in alginate matrix affected on percentage of root and other traits (Table 2). A significant improvement in plantlet conversion was observed when MS nutrients and plant growth regulators were added in alginate gel matrix (Figure 3). These findings suggest that the MS nutrients are essential ingredients of sodium alginate matrix for plantlet significant superiority over the double distilled water with respect to shoot growth.

DISCUSSION

After 60 days of cold storage of encapsulated, shoot tips significantly reduced in plantlet recovery (P=0.05).

The highest percentage of shoot tips germination was obtained non-stored shoot tips (100%) whereas the lowest (30%) was observed after 60 days of cold storage. It is assumed that any rate of decline in the regrowth frequency observed among encapsulated propagules stored

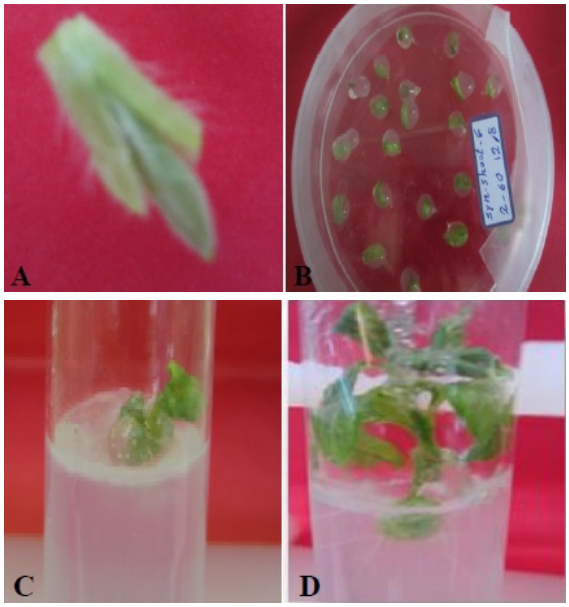

Figure 1. Plantlet regeneration from encapsulated shoot tips of sunflower. **A**, Shoot tips excised from *in vitro* proliferated shoots. **B**, Shoot tips encapsulated in calcium alginate beads. **C**, Shoot emergence from encapsulated shoot tips. **D**, Conversion of encapsulated shoot tips into plantlet on full-strength MS medium.

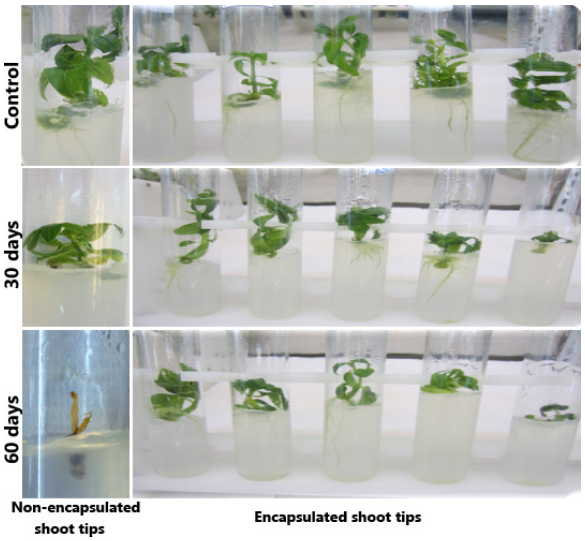

Figure 2. Conversion percent of encapsulated and non-encapsulated shoot tips of sunflower decreased gradually after storage at 4°C when the duration of storage increased.

Table 1. The results of the analysis of variance according to the analyzed effect of different storage durations and alginate matrix composition on germination percent, rooting percent, stem length, root length and number of leaf of encapsulated and non-encapsulated shoot tips of sunflower (F value).

		Mean Square				
Source	**DF**	**Germination Percent**	**Rooting Percent**	**Stem Length**	**Root Length**	**Number of Leaf**
Factor A	4	7896.87500**	11084.37500**	3672.07299**	2862.62595**	245.4756250**
Factor B	3	1739.58333**	3422.91667**	2239.73560**	3613.83416**	260.5406250**
AB	12	359.37500**	334.37500ns	200.67088**	59.19350**	8.9906250**
Error	80	128.12500	209.37500	1.77195	4.66705	1.683125
C.V		14.65273	21.35763	3.000224	6.702956	10.17731

Factor A: Storage duration B: Alginate matrix composition ; ** Significant at = 0.01; *Significant at = 0.05; ns: Non significant.

Table 2. ANOVAs for germination percent, rooting percent, stem length, root length and number of leaf based on orthogonal group comparison in alginate matrix composition

		Mean Square				
Comparisons	**DF**	**Germination Percent**	**Rooting Percent**	**Stem Length**	**Root Length**	**Number of Leaf**
1	1	0.00024300**	3502.083333**	1844.822412**	3458.486533**	414.7752083**
2	1	0.00060000**	1666.666667**	4549.718214**	6345.432481**	322.6666667**
3	1	0.00039200**	50.000000ns	324.666162**	1037.583458**	44.1800000**

** Significant at = 0.01; *Significant at = 0.05; ns: Non significant.

1: Control (non-encapsulated shoot tips) vs. encapsulated shoot tips.

2: Presence of MS nutrients vs. absence of MS nutrients in alginate matrix.

3: Presence of plant growth regulators vs. absence of plant growth regulators in alginate matrix.

at low temperatures may have resulted because of inhibited respiration of plant tissues perhaps due to alginate cover [24, 17]. Also, Redenbaugh et al. [23] stated that the decline in plant recovery from stored encapsulated vegetative propagules may be due to both oxygen deficiency in the calcium alginate bead and its rapid drying. Bazinet et al. [4] found in *Daucus carota* that plant regeneration rate after storage was reduced by loss of viability caused by mechanical constraints or diffusional limitation. However, Danso and Ford-Lloyd [7] reported that decline in morphogenesis could be attributable to an inhibited respiration of the tissues by the alginate matrix or a loss of moisture due to partial desiccation which was observed during storage. It is not known whether the explanted tissue or the alginate matrix caused this moisture loss. Positive effect of encapsulation for cold storage plants has been reported by other researchers [6, 27, 31, 12, 2, 35]. Gelling matrix supplemented with nutrient ingredients served as 'artificial endosperm' which provides nutrients to the encapsulated propagules for plant regrowth [28].

To realize the idea of providing an 'artificial endosperm,' the nutritive ingredients (nutrient medium salts, sugars, growth regulators) of the alginate beads are of key importance for both the storage and conversion efficiencies of the propagules encapsulated [33]. Tsvetkov et al. [33] also found that the addition of adjuvant components in the gelling matrix improved the plant growth of hybrid aspen. Also, Singh et al. [28] found that retrieval of plantlets from stored encapsulated shoot tips of *Phyllanthus amarus* was feasible only when gelling matrix was prepared in MS nutrients. Alginate encapsulation is a technique that can be used for germplasm storage or for reducing the need for transferring and sub-culturing out of season. Cold storage has the potential to reduce the cost of maintaining germplasm cultures because of the reduced need for manual labor due to less frequent sub-culturing [34]. An important feature of the encapsulated vegetative propagules is their capability to retain viability after storage for a sufficient period required for exchange of germplasm between laboratories and extension centers [22].

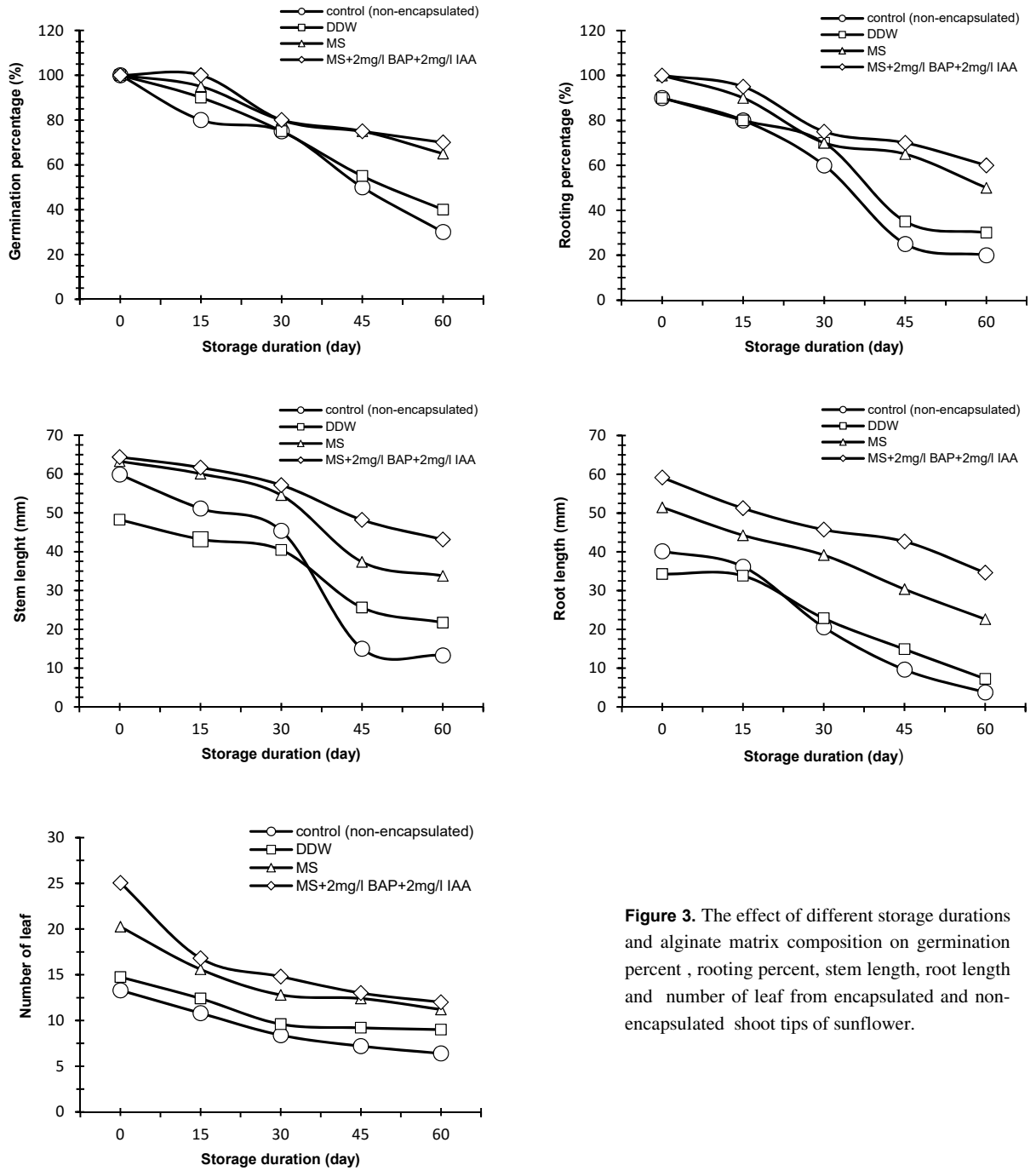

Figure 3. The effect of different storage durations and alginate matrix composition on germination percent , rooting percent, stem length, root length and number of leaf from encapsulated and non-encapsulated shoot tips of sunflower.

Successful plant retrieval from encapsulated shoot tips following low temperature indicates that the method described in this paper could be potentially used to preserve desirable elite genotype of *H. annuus* over a short period. This could also facilitate transport of encapsulated shoot tips to laboratories and extension centers of distant places. For large-scale application of process, however, further experiments are needed to achieve a higher percentage of conversion after the storage of encapsulated shoot tips of *H. annuus*.

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باززایی گیاه آفتابگردان *Helianthus annuus* (هیبرید آذرگل) از نوک شاخه‌های کپسوله شده جهت نگهداری، توزیع و تبادل ژرم پلاسم

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چکیده

در مطالعه حاضر پتانسیل استفاده از نوک شاخه‌های کپسوله شده آفتابگردان *Helianthus annuus* (هیبرید آذرگل) در آلژینات حاوی مواد غذایی جهت تولید بذر مصنوعی مورد بررسی قرار گرفت که تکنولوژی مناسبی برای توزیع و تبادل ژم‌پلاسم می‌باشد. نوک شاخه‌ها حاصل از کشت بذرهای بالغ در آلژینات سدیم ۳ درصد و کلرید کلسیم ۱۰۰ میلی‌مولار تهیه شده با سه بستر مختلف (شامل آب مقطر، محیط MS مایع و تنظیم کننده‌های رشد گیاهی) کپسوله و سپس در دوره‌های مختلف زمانی (۱۵، ۳۰، ۴۵ و ۶۰ روز) نگهداری شدند. بعد از هر دوره زمانی نوک شاخه‌های کپسوله شده و کپسوله نشده جهت ارزیابی میزان رشد و باززایی روی محیط MS فاقد تنظیم کننده‌های رشد کشت شدند. توانایی رشد نوک شاخه‌های کپسوله شده توسط مدت زمان نگهداری و حضور مواد غذایی در دانه‌های آلژینات کلسیم تحت تأثیر قرار گرفت. درصد تبدیل نوک شاخه‌های کپسوله شده و کپسوله نشده بعد از نگهداری در دمای ۴ درجه سانتی‌گراد به تدریج کاهش یافت که ریزنمونه‌های رویشی کپسوله شده نسبت به ریزنمونه‌های کپسوله نشده مقاومت بیشتری به نگهداری در دمای ۴ درجه سانتی‌گراد نشان دادند. افزودن مواد غذایی MS به دانه‌های آلژینات کلسیم به طور معنی‌داری رشد ریزنمونه‌های کپسوله شده را پس از دوره‌های نگهداری بهبود بخشید.

کلمات کلیدی: بذر مصنوعی، ژرم‌پلاسم، کپسوله کردن، نوک شاخه‌ها، آفتابگردان