Viability of encapsulated shoot tips of *Capparis orientalis* Duh.

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Abstract: A method was developed for plant regeneration from alginate-encapsulated shoot tips of *Capparis orientalis* Duh. Shoot tips excised from *in vitro* proliferated shoots were encapsulated in calcium alginate beads. The best gel composition was achieved using 3% sodium alginate and 100 mM calcium chloride ($CaCl_2.2H_2O$). The regrowth ability of encapsulated shoot tips on Murashige and Skoog (MS) medium was affected by the storage duration at 4˚C, and the presence or absence of MS nutrients and sucrose in calcium alginate beads. Both high viability and regrowth performance of explants were registered in all encapsulation mixtures tested. Nutrient medium and sugar significantly affected the initial development of the shoot tips. Sucrose appeared to play an important role in the starting stage of the regrowth event. The present synthetic seed technology could be useful in short-term preservation and germplasm distribution and exchange of *C. orientalis*.
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Key words: *Capparis orientalis*, encapsulation, *in vitro* preservation, shoot tips.

Abbreviations: BA, 6-benzylaminopurine; $CaCl_2.2H_2O$, calcium chloride; MS, Murashige and Skoog; Na-alginate, sodium alginate; NaOCl, sodium hypochlorite.

Introduction

Capparis orientalis Duh. is a rare species belongs to family Capparaceae. It is an economically important shrub. Immature flower buds are pickled in vinegar or preserved in granular salt. Semi-mature fruits and young shoots with small leaves may also be pickled for use as a condiment or eaten as vegetables (Alkire, 2001). They have a piquant flavor and add pungency, a peculiar aroma and saltiness to comestibles such as pasta sauces, pizza, fish, meats and salads. The strong flavor comes from mustard oil: methyl isothiocyanate (released from glucocapparin molecules) arising from crushed plant tissues (Mithen *et al.*, 2000). Said to reduce flatulence and to be anti-rheumatic in effect. In medicine, they are recorded as hepatic stimulants and protectors, improving liver function. They are used for arteriosclerosis, as diuretics, kidney disinfectants, vermifuges and tonics (Ahmed *et al.*, 1972). Infusions and decoctions from caper root bark have been traditionally used for dropsy, anemia, arthritis and gout. Capers contain considerable amounts of the anti-oxidant bioflavinoid rutin. Extracts and pulps have been used in cosmetics.

The conventional propagation of *C. orientalis* is restricted due to poor seed germination and cuttings gave low rooting percentages. An efficient protocol for micropropagation was developed by Hegazi *et al.* (2011) to conserve *C. orientalis*, using stem node sections and shoot tips. So far, synthetic seed production has not yet been reported in *C. orientalis* using vegetative propagules or somatic embryos.

Synthetic seed technology could be useful in germplasm conservation of elite, endangered and commercially important plants by using appropriate storage technique as well as exchange of axenic plant material between laboratories (Hasan and Takagi, 1995; Ara *et al.*, 2000; Rai *et al.*, 2008a&b). Furthermore, they can be used for cryopreservation *via* encapsulation dehydration and encapsulation-vitrification (Pennycooke and Towil, 2001; Wang *et al.*, 2002). Synthetic seed technology using encapsulation of vegetative propagules of woody plant species has become a potentially cost effective clonal propagation system. Encapsulation of shoot tips in calcium alginate offers a space-saving option for storage at low temperature but above 0˚C (Lisek and Olikowska, 2004). The main advantages of using vegetative propagules for the preparation of synthetic seeds would be in those cases where somatic embryogenesis is not well established or somatic embryos do not germinate into complete plantlets. In such cases, synthetic seed production from shoot tips can be used for cost-effective mass clonal propagation, germplasm storage, and delivery of tissue-cultured plants (Singh *et al.*, 2006a). Among several non-embryogenic propagules, shoot tip explants are more responsive than other non-embryogenic propagules because of greater mitotic activity in the meristem (Ballester *et*

al., 1997). Despite these advantages, there are few reports on encapsulation of shoot tips obtained from *in vitro*-raised plants such as (Ganapathi *et al.*, 1992; Sicurani *et al.*, 2001; Danso and Ford-Lloyd, 2003; Nyende *et al.*, 2003; Lisek and Orlikowska, 2004; Singh *et al.*, 2006a&b; Rai *et al.*, 2008a&b; Singh *et al.*, 2009).

In the present paper, the first attempt of the encapsulation of shoot tips of *C. orientalis* in calcium alginate beads is reported. Efforts were also made to test the ability of the encapsulated shoot tips to retain viability following storage at a low temperature (4˚C) for different durations.

Materials and Methods

Plant material and in vitro culture

Juvenile shoots were collected from mature shrubs of *C. orientalis* growing at Agyba's beach in Marsa Matrouh, North-West Coast, Egypt (Figure 1). Cultures were established using mature nodal explants and shoot tips, then produced axillay shoots were multiplied following the procedure as described in the earlier work (Hegazi *et al.*, 2011). The initial explants were washed under running tap water for 2-3 h. Surface sterilization was carried out, using commercial bleach (Clorox) containing 5.25% sodium hypochlorite (NaOCl), under complete aseptic conditions in the Laminar Air Flow Hood. Stem node sections of *C. orientalis* needed higher concentrations and durations of NaOCl application (1.5% NaOCl for 12 min) comparing to shoot tips which needed only 0.5% NaOCl for a short duration (7 min) to give 100% of survived explants. For shoot regeneration, explants were cultured routinely on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa, Haarlem, the Netherlands) containing 3% sucrose and supplemented with 3 mg/L 6-benzyladenine (BA) (Sigma Cell Culture, min. 90%, USA), solidified with 2.7 g/L phytagel (Duchefa, Haarlem, the Netherlands). The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at a pressure of 1.06 Kg cm^{-2} and 121°C for 20 min. Cultures were maintained at 24 ± 2°C with a 16 h photoperiod under white cool fluorescent tubes (F140t9d/38, Toshiba). Subcultures were performed every 4 weeks.

Encapsulation of shoot tips and storage conditions

Shoot tips (about 3-5 mm long) were dissected aseptically from *in vitro* proliferated shoots for encapsulation. Calcium alginate beads were produced by the encapsulation method after Kinoshita and Saito (1992). Sodium alginate (Na-alginate) (CDH, India) solution was prepared in the range of 1, 1.5, 2.0, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 % and the pH was adjusted at 5.7-5.8. Whereas 25, 50, 75, 100, 125, 150 and 200 mM calcium chloride ($CaCl_2.2H_2O$) (Merck, Germany) solutions were prepared in distilled water. Encapsulation was accomplished by mixing the shoot tips into the Na-alginate solution, in order to allow the explants to be covered by the gel. The alginate-covered shoot tips were then dropped in the $CaCl_2.2H_2O$ solution. The beads (each usually containing one or two shoot tips) were left for 20-30 min in the calcium chloride solution to ensure polymerization of calcium alginate. After hardening, the encapsulated beads were rinsed 3 times with sterilized distilled water. The capsules had an average diameter of 5-6 mm (Figure 2), and could be easily handled under sterile conditions, preventing dehydration of the explants.

In order to study the effects of alginate matrix composition on plantlet conversion, shoot tips were immersed in sterilized encapsulation mixtures with different composition: a) 3% Na-alginate + distilled water, b) 3% Na-alginate + full-strength MS medium, c) 3% Na-alginate + full-strength MS medium + 3% sucrose. Encapsulated shoot tips were stored for 0, 15 and 30 days at 4°C (in the dark) on water agar medium (0.7% w/v agar). Non-encapsulated shoot tips were act as control.

Plant retrieval from encapsulated shoot tips

Encapsulated and non-encapsulated shoot tips were placed directly on plant growth regulators-free MS medium for the recovery of plantlets. After 4 weeks, viability, regrowth and the average shoot length (cm) were recorded. The capsules were considered alive if the shoot tips were still green, with no necrosis or yellowing. Regrowth rate was evaluated as percentage of capsules that had shown any visual growth activity, *i.e.* an increase in size, with breakage of the capsule and extrusion of at least one small shoot.

Data analysis

The experiments were subjected to the completely randomized design. Variance analysis of data was carried out using Anova program for statistical analysis. The treatment means were compared by using Duncan´s multiple range test (Duncan, 1955). Means followed by the same letter are not significantly different at $P \leq 0.05$.

Results and Discussion

Alginate encapsulation

Sodium alginate and calcium chloride play an important role in gel matrix formation and gel complexation, and capsule hardiness depends upon optimal ion exchange of Na^+ and Ca^{2+} (Singh *et al.*,

2006a). A key factor for synthetic seed technology is the assessment of the effects of various concentrations of Na-alginate and calcium chloride on the texture, shape and size of the beads. The beads differ morphologically with respect to texture, shape, diameter and transparency with different combinations of Na-alginate and calcium chloride. An optimal ion exchange between Na^+ and Ca^{2+} producing firm clear, isodiametric beads was achieved by complexation of a 3% solution of Na-alginate with 100 mM $CaCl_2.2H_2O$ for 20-30 min, thereby forming an insoluble gel matrix of calcium alginate. Lower concentrations of Na-alginate (1-2.5%) became unsuitable for encapsulation perhaps because of a reduction in its gelling ability following exposure to a high temperature during autoclaving (Larkin *et al.*, 1988), they not only prolonged the polymerization duration, but also resulted in fragile beads, which were difficult to handle. Whereas, at higher concentrations of Na-alginate (3.5-6%), beads were isodiametric but too hard that may suppressed the shoot emergence. Higher concentrations of $CaCl_2.2H_2O$ (125-200 mM) inhibited the conversion of shoot tips while lower concentrations (25-75 mM) not only prolonged the ion exchange (polymerization) duration but also resulted in fragile beads which were difficult to handle. This different response may be due to a synergistic effect of alginate and calcium concentration. Hence, 3% Na-alginate and 100 mM $CaCl_2.2H_2O$ was used for the encapsulation of shoot tips of *C. orientalis* in further experiments. Similar observations were also made in *Malus* sp., *Pyrus* sp. and *Morus bombysis* (Niino and Sakai, 1992), *Camellia* sp. (Janeiro *et al.*, 1997), *Punica granatum* L. (Naik and Chand, 2006), *Phyllanthis amarus* (Singh *et al.*, 2006a), *Withania samnifera* (Singh *et al.*, 2006b), *Psidium guajava* L. (Rai *et al.*, 2008a&b) and *Spilanthes acmella* (L.) Murr. (Singh *et al.*, 2009).

Plant retrieval from alginate encapsulated shoot tips

Non-encapsulated and encapsulated shoot tips of *C. orientalis* in 3% Na-alginate and 100 mM $CaCl_2.2H_2O$ exhibited shoot formation within one week of culture. At all alginate matrix compositions, the encapsulated and non-encapsulated shoot tips demonstrated high values of viability and regrowth percentages (Table 1). It was shown that encapsulation did not affect viability and regrowth of shoot tips, since viability rates were always 100%, and regrowth rates were very high for both encapsulated and non-encapsulated control shoot tips. However, the growth rate was lower in coated shoot tips compared to uncoated shoot tips (data not shown). Hence, the uncoated (non-encapsulated) shoot tips are directly in contact with the medium as opposed to encapsulated shoot tips.

The sodium alginate combined with full-strength MS nutrients demonstrated significant superiority over the distilled water with respect to plantlet conversion and shoot length. This finding suggests that the MS nutrients are essential ingredients of sodium alginate matrix for plantlet conversion. Gelling matrix supplemented with nutrient ingredients served as “artificial endosperm”, which provides nutrients to the encapsulated explants for plant regrowth (Bapat and Rao, 1992). Also, Singh *et al.* (2006a) found that retrieval of plantlets from stored encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn was feasible only when gelling matrix was prepared in MS nutrients.

Table 1. Effect of composition of Na-alginate matrix on the regrowth performance of encapsulated shoot tips of *C. orientalis*

Alginate matrix composition	Viability %	Regrowth %	Average Shoot length (cm)
Non-encapsulated shoot tips	100^a	100^a	1.61^b
Distilled water	100^a	83.3^b	0.42^d
Full-strength MS medium	100^a	100^a	0.59^c
Full-strength MS medium + 3% sucrose	100^a	100^a	2.18^a

Na-alginate (3%) and 100 mM $CaCl_2.2H_2O$ were added to distilled water or MS medium with or without 3% sucrose. Regrowth was evaluated after 4 weeks of culture.

Viability, regrowth and average shoot length gave their maximum values when sucrose was augmented in the medium (Figure 3). These findings suggest that sucrose is an essential ingredient of the sodium alginate matrix. In realizing the idea of providing an “artificial endosperm”, the nutrient ingredients and sugar of the alginate beads are of key importance for both the storage and conversion efficiency of the propagules encapsulated (Tsvetkov *et al.*, 2006). According to earlier reports, the presence of sugar or a complete medium in the beads enables better survival of stored explants (Pattnaik *et al.*, 1995; Ballester *et al.*, 1997; Watt *et al.*, 2000; Rout *et al.*, 2001; Lisek and Orlikowska, 2004).

Sucrose is frequently used as a carbohydrate and energy source in plant tissue culture (Bhojwani and Razdan, 1996) and complete inhibition of shoot emergence from alginate beads of *Psidium guajava* L. in sucrose lacking medium confirm the essentiality of a carbohydrate source in the medium (Rai *et al.*, 2008a&b). Since, Sucrose is known to provide a carbon source for *in vitro* propagules, and its inclusion in the alginate matrix enhanced plant recovery (Danso and Ford-Lloyd, 2003). It is obvious that a proper optimization of the alginate matrix composition may enhance the regrowth performance of encapsulated explants and directly impact the efficiency and practical applicability of the technique.

Effect of different storage durations on conversion of encapsulated shoot tips

A desirable feature of the synthetic seeds is their ability to retain viability and regrowth potential even after storage. Low temperature and high humidity were essential conditions for retention of viability and thus for storage of encapsulated shoot tips. Percent response for the conversion of encapsulated shoot tips decreased gradually with increasing storage duration at 4˚C (Table 2). Similarly, the conversion frequency of encapsulated shoot tips of many other plants also declined markedly following storage at low temperature (Singh *et al.*, 2006a&b, Rai *et al.*, 2008a&b; Singh *et al.*, 2009). After storage for 30 days, the percentage response for conversion of encapsulated shoot tips reached 69.1%. It is assumed that any rate of decline in the regrowth frequency observed among encapsulated propagules stored at low temperatures may have resulted because of inhibited respiration of plant tissues perhaps due to alginate cover (Redenbaugh *et al.*, 1987; Naik & Chand, 2006). Also, Redenbaugh *et al.* (1991) 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.* (1992) 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 (2003) reported that the 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.

Table 2. Effect of different storage durations on conversion of encapsulated shoot tips of *C. orientalis*

Storage duration (days)	Percentage response for conversion of encapsulated shoot tips
0	100[a]
15	81.8[b]
30	69.1[c]

Na-alginate (3%) and 100 mM $CaCl_2.2H_2O$ were added to MS medium incorporated with 3% sucrose. Encapsulated shoot tips were stored in Petri plates containing water agar medium at 4˚C. For conversion, stored encapsulated shoot tips cultured on MS medium were evaluated after 4 weeks.

Successful plant retrieval from encapsulated shoot tips following the cold storage (4˚C) makes encapsulated shoot tips very attractive for germplasm exchange as growth can resume immediately after culture.

This study reports, for the first time, the production of synthetic seeds in *C. orientalis* using shoot tips. Successful plant retrieval from encapsulated shoot tips following storage at low temperature (4˚C) indicates that the method described in this article could be potentially used to preserve the germplasm of this rare economically important plant species over a short period. This could also facilitate the transport of encapsulated shoot tips to laboratories of distant places while maintaining their viability. Generally, synthetic seeds consisting of *in vitro* regenerated vegetative propagules encapsulated in a protective coating have many advantages over organogenesis for propagation including case of handling, storage, high scale-up production and low cost of production. In addition, storage on agar plates at 4˚C may be cost effective (Chand and Singh, 2004). However, further investigations are needed to extend the duration of storage of encapsulated shoot tips of *C. orientalis* and for large scale application of the process.

Figure 1 **Figure 2** **Figure 3**

Figure 1. A branch of *C. orientalis* growing at Agyba's beach in Marsa Matrouh, North West Coast, Egypt.

Figure 2. Calcium alginate beads formed by encapsulation of shoot tips of *C. orientalis* using 3% Na-alginate and 100 mM $CaCl_2.2H_2O$.

Figure 3. Regrowth of encapsulated shoot tips of *C. orientalis* one week after placing the beads on full strength MS medium.

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