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Encapsulation of shoot tips in alginate beads containing salicylic acid for cold preservation and plant regeneration in sunflower (*Helianthus annuus* L.)

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Abstract

We report encapsulation of *in vitro*-derived shoot tips in alginate medium containing SA for cold preservation and plantlet regeneration in sunflower (*Helianthus annuus* L.) as one of the most important oil-crops in the world. In the present study, four kinds of alginate matrix each containing a certain concentration of either sucrose (0.08 M / 0.75 M) or salicylic acid (25 µM / 50 µM) were used for encapsulation of the shoot tips. Encapsulated and non-encapsulated shoot tips were then stored at low temperature (4˚C) for different storage periods: 15, 30, 60 and 90 days. Encapsulated shoot tips containing sucrose 0.08 M proved to be viable (33%) even after 90 days of cold storage, while non-encapsulated ones lost their viability completely after 60 days. With the increase in sucrose concentration of alginate matrix to 0.75M, the conversion percent of encapsulated shoot tips dropped to 37% and 18% after 60 and 90 days, indicating that it had an inhibiting effect on plant regeneration. Supplementing the alginate matrix with two concentrations of salicylic acid (25µM / 50µM) on the other hand was found to have pronounced effect on the survival frequency. The viability rate of encapsulated shoot tips with salicylic acid 25 µM and 50 µM increased to 48% and 59% respectively after 90 days. Salicylic acid 50µM proved to enhance the percentage of viability of encapsulated shoot tips significantly.

Keywords: Alginate matrix; Cold storage; Encapsulation; Micropropagation; Synthetic seed.
Abbreviations: AM-Alginate matrix; BAP- benzylaminopurine; MS-Murashige&Skoog; SA-Salicylic acid.

Introduction

Over the last 20 years, various techniques including organogenesis and somatic embryogenesis have been offered for plant regeneration in sunflower (Bolandi et al., 2000; Petitprez et al., 2005; Huang et al., 2007). In all of these approaches, a low rate of plant regeneration, abnormal morphogenesis, the non-ability to synchronize embryo maturity and premature flowering are among the most important difficulties (Fiore et al., 1997; Gurel et al., 1998). Moreover, culture systems successful for plant regeneration in different species are usually difficult or impossible to adapt to sunflower since the regeneration frequency depends on genotype and most genotypes are reported to be recalcitrant (Bolandi et al., 2000, Huang et al. 2007). In this context, synthetic seed technology nowadays could be a valuable aid to large scale clonal propagation of plant species (Singh et al., 2006a; Late et al., 2009; Singh et al, 2009a, b).
Encapsulation of vegetative propagules to develop synthetic seeds is a technique that can be used in conjunction with micropropagation for *in vitro* conservation of germplasm, which is particularly important for preservation of elite plant species (Sing et al., 2006b; Singh et al, 2009a). *In vitro* conservation of germplasm is often realized by conditions which reduce the rate of tissue growth to a minimum. Encapsulation of tissues within alginate beads restricts their respiration and hence reduces their growth, and while protecting them allows their stockage under this condition (Bernard et al., 2002).

Alginate encapsulation of shoot tips along with cold preservation offers a strong possibility for germplasm storage and plant regeneration. It can provide a source of axenic plant materials (Hasan and Takagi, 1995; Singh et al., 2006b) that can be used if stock plants or proliferation cultures become infested with bacteria, fungi, or arthropods (West et al., 2006). It also provides a possibility to exchange plant materials between laboratories and a space-saving option for storage at low temperatures above 0˚C (Danso and Ford-Llyod, 2003; Singh et al., 2006a, b; Ray et al., 2008). Furthermore, encapsulated shoot tips can be used as means to reduce the need for subculturing during cold storage since they require no transfer to fresh medium (West et al, 2006). Cold storage is possible to reduce the cost of maintaining germplasm cultures because it reduces the manual labor as a result of less frequent subculturing. It also lessens the possibility of genetic instability from frequent subculturing and adventitious regeneration (West et al., 2006). Despite these advantages, there are but a few reports on encapsulation of vegetative propagules (Nyende et al., 2003; Singh et al., 2006a, b; Ray and Bhattacharya, 2008; Lata et al., 2009 Singh et al., 2009a, b; Ahmad and Anis, 2010). Some ingredients of the encapsulation matrix such as nutrient medium salts, sugars, and growth regulators affect the initial development of microcutting. Sucrose as a central resource in plants is considered to have wide-ranging regulatory effects and appears to play an important role in the starting stage of

Table1. Effect of different storage durations on conversion of encapsulated and non-encapsulated shoot tips of sunflower into plantlets.

Storage duration (days)	Frequency of plantlet conversion (%)	
	Encapsulated shoot tips (50)	Non-encapsulated shoot tips (50)
0	97 ± 1	97 ± 1
15	90 ± 2	66 ± 3
30	80 ± 1	41 ± 3
60	59 ± 3	0
90	33 ± 7	0

Values are expressed as the mean ± standard error (SE). Five replicates, each containing 5 synthetic seeds were used in each treatment. Each experiment was repeated twice.

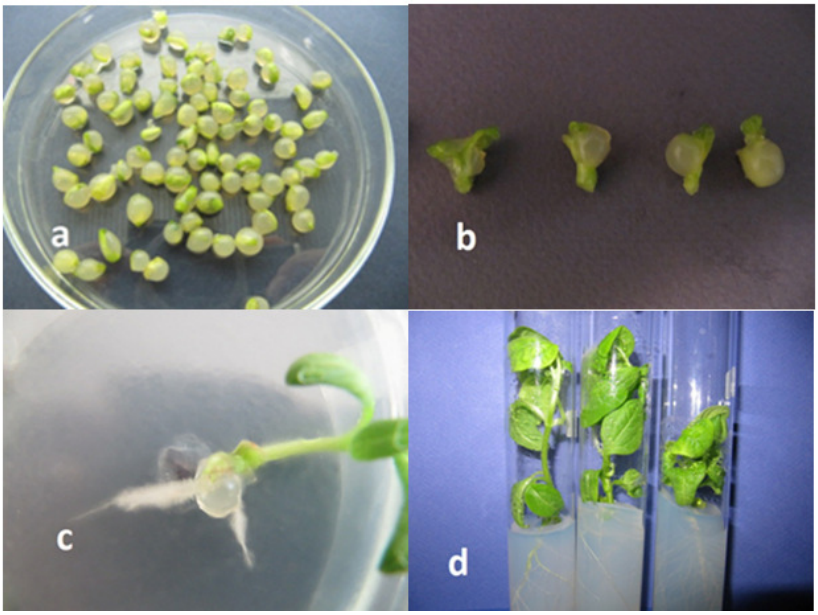

Fig 1. Plantlet regeneration from encapsulated shoot tips of sunflower**. (a)** Shoot tips encapsulated in calcium alginate beads. (**b)** Shoot emergence from encapsulated shoot tips. **(c) I**nitiation of rooting. (**d)** Conversion of encapsulated shoot tips into plantlet on full-strength MS medium.

the regrowth event (Tsvetkov et al., 2006). Salicylic acid has been found to plays a role during the plant response to abiotic stresses such as drought, chilling, heavy metal toxicity, heat, and osmotic stress (Janda et al., 2007). It is an important signal molecule in plant defense after pathogen attack as well (Shah and Klessig, 1999). To the best of our knowledge, there has been no report on synthetic seed production using either somatic embryos or vegetative propagules in sunflower so far. In this study, we report a successful attempt of the utilization of synthetic seed technology for plantlet regeneration in sunflower by employing encapsulated shoot tips. We also report the effect of different periods of cold storage and the presence of some materials such as SA and sucrose in encapsulation matrix on viability of encapsulated shoot tips.

Results and discussion

To achieve synthetic seeds, shoot tips excised from *in vitro* proliferated shoots were encapsulated in alginate matrices (Fig.1a) and then were cultured in MS medium. Irrespective of encapsulation matrix composition, shoots emerged first, from encapsulated shoot tips approximately one week after the culture and roots appeared later, after about 2 weeks (Fig.1b, c). However, conversion into complete plantlets occurred after 4 weeks following the culture (Fig.1d).The response percentage for conversion of encapsulated and non-encapsulated shoot tips before storage was the same (97%) indicating that encapsulation matrix has no inhibiting effect on the regrowth (Table 1). Encapsulated shoot tips showed a relatively higher viability of 90% and 80% after 15 and 30 days respectively, in comparison to non-encapsulated shoot tips with the viability 66% and 41% (Table 1). The regeneration frequency was clearly influenced by storage time. After 60 days of storage, conversion percent of encapsulated shoot tips was about 59%, whereas non-encapsulated shoot tips lost their viability completely (Table 1). Encapsulated shoot tips were viable (33%) even after 90 days of storage. The findings compare with those of Soneji et al. (2002) who reported that 45-75% of encapsulated shoot tips of pineapple stored at 4˚C could germinate after 30 days. In contrast, Ballester et al. (1997) reported only 10% of encapsulated shoot tips of *Camellia japonica* survived after 75 days of storage at 2-4˚C. Ahmad and Anis (2010) also reported that encapsulated nodal segments of *Vitex negundo*

Table2. Effect of sucrose concentration in alginate matrix (AM) of encapsulated shoot tips of sunflower on the conversion percentage in the duration of storage

	Percentage of plantlet conversion (%)	
Storage duration (days)	Alginate matrix 1 (AM1)	Alginate matrix 2 (AM2)
0	97 ±1	97 ± 1
15	90 ± 2	86 ± 1
30	80 ± 1	69 ± 2
60	59 ± 3	37 ± 5
90	33 ± 7	18 ± 5

AM1and AM2 refers to alginate matrix supplemented with 0.08M (30g/l) and0.75M sucrose respectively. Values are expressed as the mean ± SE.

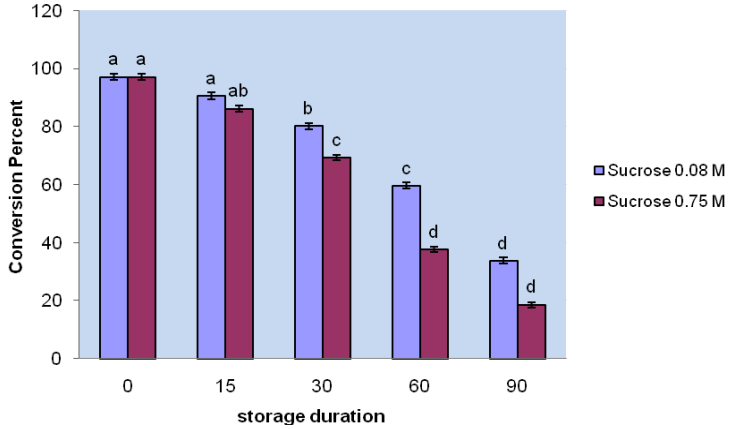

Fig 2. The effect of sucrose concentration of alginate matrix and duration of storage on plant recovery from encapsulated shoot tips of sunflower. The bars represent mean ± SE. Bars denoted by the same latter are not significantly different (P=0.05) according to DMRT.

were vital (50%) after 8 weeks of storage. Therefore, encapsulated shoot tips of sunflower survive cold storage better than encapsulated shoot tips of *Camellia japonica* and encapsulated nodal segments of *Vitex negundo*. However, West et al. (2006) reported 100% viability of encapsulated nodal segments of *Hibiscus moscheutos* even after 6 months of cold dark storage. Although encapsulated shoot tips showed significantly higher resistance to storage at 4˚C than non-encapsulated ones, conversion percent of both encapsulated and non-encapsulated shoot tips decreased gradually as the duration of storage increased. The results were similar to the findings of Singh et al. (2009a, b) whose study also showed a marked decline in the conversion of encapsulated shoot tips of *Eclipta alba* L. and *Spilanthes acmella* L. following storage at low temperature. It is assumed that decline in the conversion of encapsulated shoot tips as a result of prolonged storage time could be attributable to inhibited respiration of tissues or a loss of moisture due to partial desiccation during storage as reported earlier (Danso and Ford- Lloyd, 2003; Faisal et al., 2006; Ray and Bhattacharya, 2008).

The conversion percentage of encapsulated shoot tips in AM2 supplemented with 0.75M sucrose was 86% after 15 days of storage. It was not significantly different from that of the AM1 (control) with 0,08M sucrose (Table 2). However, after prolonged storage for 30, 60 and 90 days, the conversion frequency decreased significantly to 69%, 37%, and 18% respectively (Table 2). Higher concentration of sucrose proved to have deterring effect on viability of the encapsulated shoot tips (Fig 2). The results correspond to the findings Hasan and Takagi (1995) who reported that a polymerization medium supplemented with more than 0.3M sucrose was lethal to shoot development in yam. Danso and Ford-Lloyd (2003) also reported that supplementing the alginate matrix with high levels of sucrose is not ideal for plant development although it imposes a delay on shoot regrowth which provide extra time for the transportation. Nevertheless, as reported by Bernard et al., (2002) and Kaviani (2010), an alginate matrix that contains a high level of sucrose is necessary for cryogenic storage using encapsulation dehydration; sucrose increases the tolerance to dehydration and therefore contributes to maintaining the tissues viability. Conversion percent of encapsulated shoot tips with AM3 and AM4 supplemented with SA (25 µM, 50µM) was almost the same (about 92%) after 15 days storage (Table 3). After 30 days, the conversion frequency was 80% and 90% for synthetic seeds in AM3 and AM4 respectively. It changed to 72% and 78% after 60 days, which indicates a significant increase in comparison to the control (59%). After 90 days of storage, response percent was 33%, 48% and 59% for 0, 25 and 50µM SA concentrations respectively (Table 3). Salicylic acid 50µM proved to be significantly effective on viability of encapsulated shoot tips (Fig. 3). The results showed that SA plays a crucial role in the resistance of these tissues to cold storage. Salicylic acid is a natural substance which is often associated with a role in plant responses to physical and biological aggressions (Raskin, 1992; Bernard et al., 2002). Most papers, on this subject, have reported on the protective effect of exogenous SA against abiotic stress such as low temperature (Janda et

Table3. Effect of salicylic acid in alginate matrix on the conversion percentage of sunflower encapsulated shoot tips

Storage duration (days)	Frequency of plantlet conversion (%)		
	control	SA (25)	SA (50)
0	97 ± 1	96 ± 1	96 ± 1
15	90 ± 2	92 ± 2	92 ± 1
30	80 ± 1	84 ± 2	90 ± 1
60	59 ± 3	72 ± 2	78 ± 3
90	33 ± 7	48 ± 2	59 ± 2

Control treatment has no salicylic acid. SA (25 µM, 50 µM) refers to the alginate matrix supplemented with 25 and 50µM salicylic acid. Values are expressed as the mean ± SE.

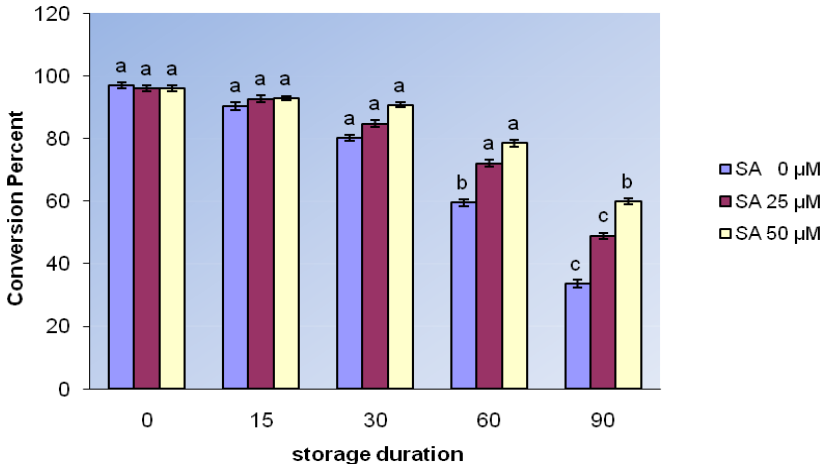

Fig 3. The effect of salicylic acid (SA) in the alginate matrix of encapsulation on the regrowth of the cold preserved encapsulated shoot tips of sunflower. The bars represent mean ± SE. Bars denoted by the same latter are not significantly different (P=0.05) according to DMRT.

al., 2007). In the current study, it puts an apparent role on display in shoot tips submitted to the stress of cold storage. Exogenous application of salicylic acid may lead to an enhancement of endogen SA (Seo et al., 1995), which may also play an active role in the process of resistance to desiccation (Bernard et al., 2002) and low temperature. Endogenous SA is an induction signal for specific defense responses of plants (Shah and Klessig, 1999) and it has been shown that it acts by causing water stress in the tissues (Bernard et al., 2002). It is worth mentioning that water stress is also very high in cold preservation since it is enhanced by our conditions of experimentation i.e. with the stockage of encapsulated shoot tips in empty petri dishes without water support. The presence of SA (50µM) in the medium of encapsulation matrix in the current study and the significant increase in the percentage of viability of shoot tips ($P \leq 0.05$) within the conditions of storage (Fig 3) may offer a better explanation of its effect on the water stress and cold preservation.

Materials and methods

Plant materials and explants preparation

The seeds were obtained from Seed and Plant Improvement Institute of Iran. They were washed with detergents and then immersed in 70% ethanol for 1 minute. They were, then, rinsed and sterilized by 1% sodium hypochlorite (w/v) for 20 minutes and washed 4-5 times with sterilized distilled water. To raise seedlings, seeds were aseptically cultured in MS (Murashige and Skoog, 1962) medium. Shoot tips from *in vitro* grown seedlings were excised and cultured on MS medium supplemented with 0.5mg/l BAP for proliferation. The pH of the medium was adjusted to 5.7 prior to adding 0.8% agar-agar. All cultures were incubated at 25˚C under 16h/8h light/dark cycle.

Preparation of encapsulation matrix:

Four kinds of alginate matrices, namely AM1, AM2, AM3, and AM4 were used in this experiment. To prepare each AM, an alginate solution as well as a polymerization solution was used. To make sodium alginate solution AM1, sodium alginate 3% (w/v) was added to full strength MS medium with 0.08M sucrose. Polymerization solution was prepared with 100mM calcium chloride in liquid MS medium and 0.08M sucrose. In order to study the effect of high concentration of sucrose, AM2 was prepared with sodium alginate 3% (w/v), liquid MS medium supplemented with 0.75M sucrose; calcium chloride (100mM) and 0.75M sucrose were added to liquid MS medium. To study the effect of SA, AM3 and AM4 were prepared with sodium alginate 3% (w/v), liquid MS medium, 0.08M sucrose supplemented with SA (25µM, 50µM respectively). Complexing agent

($CaCl_2$ $2H_2O$) contained the same adjuvents as the sodium alginate solution. The solutions were autoclaved separately for 15 min at a pressure of 1.1 kg cm^{-2} and temperature of 121˚C after adjusting the pH to 5.8.

Encapsulation procedure

Shoot tips excised from *in vitro* proliferated shoots were used for encapsulation. Encapsulation was accomplished by mixing the shoot tips into the sodium alginate solutions explained above and dropping these individually into the calcium chloride solutions. 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 encapsulated shoot tips were later washed with sterilized distilled water.

Regeneration evaluation of encapsulated shoot tips

After encapsulation, some synthetic seeds of each alginate matrix were cultured to hormone-free MS medium for regrowth into complete plantlets as well as for regeneration evaluation. All cultures were maintained as previously described.

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 15, 30, 60 and 90. After each storage period, encapsulated and non-encapsulated shoot tips were cultured on MS medium for regeneration evaluation.

Statistical analysis

The percentage of stored and non-stored encapsulated shoot tips that developed shoots and roots was recorded weekly for 4 consecutive weeks. The conversion of encapsulated shoot tips was determined on the basis of developed shoots with apparent leaves and roots formation after 4 weeks. In every experiment, 25 synthetic seeds in 5 culture vessels were treated. Each culture vessel containing 5 synthetic seeds was considered as one replicate. Each experiment was repeated twice. The data were analyzed statistically using one-way analysis of variance and the significant differences between means were assessed by Duncan's multiple range tests at a 5% probability level using the statistical package for social science (SPSS ver.18).

Conclusion

Plant regeneration from encapsulated shoot tips following storage at low tempeture (4˚C) indicates that the procedure described in this study could be potentially used to preserve the germplasm of sunflower over a short period. Since the plantlets were developed directly from synthetic seeds without an intervening callus phases, somaclonal variation among the regenerants can be avoided. The addition of SA in the encapsulation matrix possibly reinforces the tolerance of the tissues to the cold storage.

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References

Ahmad N and Anis A (2010) Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. Biol Plantarum 54 (4):748-752.

Ballester A, Janeiro LV, Vieitez AM (1997) Cold storage of shoot cultures and alginate encapsulation of shoot tips of *Camellia japonica* L. and *Camellia reticulate* Lindly. Sci Hortic-Amesterdam 7:67-78.

Bernard F, Shaker-Bazarnov H, kaviani B (2002) Effects of salicylic acid on cold preservation and cryopreservation of encapsulated embryonic axes of Persian lilac (*Melia azedarach* L.). Euphytica 123:85-88.

Bolandi AR, Branchard M, Alibert G, Genzbitel L, Berville A, Sarrafi A (2000) Combining-ability analysis of somatic embryogenesis from epidermal layers in the sunflower (*Helianthus annuua* L.). Theor Appl Gener 100:621-624.

Danso KE, Ford-Lloyd BV (2003) Encapsulation of nodal cuttings and shoot tips for storage and exchange of cassava germplasm. Plant Cell Rep 21(8):718-25.

Faisal M, Ahmad N, Anis M (2006) *In vitro* plant regeneration from alginate-encapsulated microcuttings of *Rauvolfia tetraphylla* L. American-Eurasian J Agric Environ Sci 1(1):1-6

Fiore MC, Tabacc T, Sunseri F (1997) High frequency of plant regeneration in sunflower from cotyledons via somatic embryogenesis. Plant Cell Rep 16:295-298.

Gurel E, Kazan K (1998) Development of an efficient plant regeneration system in sunflower (*Helianthus annuus* L.). Turk J Bot 22:381-387

Hasan SMZ, Takagi H (1995) Alginate-coated nodal segments of yam (*Dioscorea* spp) for germplasm exchange and distribution. Plant Genet Resource Newsl 103:32-35.

Huang X, Nabipour AR, Gentzbittel L, Sarrafi A (2007) Somatic embryogenesis from thin epidermal layer in sunflower and chromosomal regions controlling the response. Plant Sci 173:247-252.

Janda T, Horvath E, Szalai G, Paldi E (2007) Role of salicylic acid in the induction of abiotic stress tolerance. 91-150. In: Hayat S and Ahmad A (Eds) Salicylic acid- A plant hormone, springer

Kaviani B (2010) Cryopreservation by encapsulation-dehydration for long-term storage of some important germplasm: seed of lily [*Lilium ledebourii* (Baker) Bioss.], embryonic axe of Persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.). Plant Omics journal 3(6):177-182.

Lata H, Ehandra S, Khan IA, Elsohly M.A (2009) Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L., an important medicinal Plant. Physiol Mol Biol Plants 15(1):79-86.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol. 15:473-497.

Nyende AB, Schittenelm S, Mix-Wagner G, Greef JM (2003) Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.) encapsulated in calcium alginate hollow beads. In Vitro Cell Dev-Pl 39:540-544.

Petitprez M, Sarrafi A, Flores-Berrios E, Xuhan X, Briere C, Gentzbittel L (2005) Somatic embryogenesis by liquid culture of epidermal layers in sunflower: from genetic control to cell development. Plant Cell Tiss Org 81:331-337.

Raskin I (1992) Role of salicylic acid in plants. Annu Rev Plant Phys 43:439-463.

Ray A, Bhattacharya S (2008) Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentine*-An effective way of conservation and mass propagation. S Afr Bot 74:776-779.

Seo S, Ishizuka K, Ohashi Y (1995) Induction of salicylic acid beta-glucosidase in tobacco leaves by exogenous salicylic acid. Plant Cell Physiol 36 (3):447- 453.

Shah J and Klessig DF (1999) Salicylic acid: signal perception and transduction PJJHooykaas, MAHall, K.R.Libbenga (Eds), Biochemistry and Molecular biology of Plant Hormones, Elsevier Science Pp: 513-541.

Singh AK, Sharma M, Varshney R, Agarwal SS, Bansal KC (2006a) Plant regeneration from alginate- encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, a medicinally important plant species. In Vitro Cell Dev-Pl 42:109-115.

Singh AK, Varshney R, Sharma M, Agarwal SS, Bansal KC (2006b) Regeneration of plants from alginate- encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. J Plant Physiol 163(2):220-223.

Singh SK, Rai MK, Asthana P, Pandey S, Jaiswal VS, Jaiswal U (2009a) Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* (L.) Murr. A medicinally important and herbal pesticidal plant species. Acta Physiol Plant 31(3):649-653.

Singh SK, Rai MK, Asthana P, Sahoo L (2009b) Alginate-encapsulation of nodal segments for propagation, short term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). Acta Physiol Plant 32(3):607-610.

Soneji JR, Rao PS, Mhatre M (2002) Germination of synthetic seeds of pineapple (*Ananas comosus* L. Merr.). Plant Cell Rep 20:891-894.

Tsvetkov I, Jouve L, Hausman Jf (2006) Effect of alginate matrix composition on regrowth of *in vitro*-derived encapsulated apical microcutting of hybrid aspen. Biol Plantarum 50(4):722-724.

West TP, Ravindra MB, Preece JE (2006) Encapsulation, cold storage, and growth of *Hibiscus moscheutos* nodal segments. Plant Cell Tiss Org 87(3):223-231.