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BRIEF COMMUNICATION

Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*

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Abstract

An efficient protocol for encapsulation of nodal segments of *Vitex negundo* L. has been developed for the production of non-embryogenic synthetic seeds. The encapsulations of nodal segments were significantly affected by the concentrations of sodium alginate and calcium chloride. A 3 % Na_2-alginate with 100 mM $CaCl_2$ has been found to be optimum concentration for the production of uniform synthetic seed. For germination, the synseeds were cultured on Murashige and Skoog (MS) basal medium supplemented with kinetin (KIN) and α-naphthalene acetic acid (NAA) either singly or in various combinations. MS medium containing 2.5 μM KIN in combination with 1.0 μM NAA was found to be the optimum for maximum (92.6 ± 3.71 %) plantlet conversion frequency. Well developed regenerated plantlets were hardened, acclimatized and established in field, where they grew well without any detectable variation.

Additional key words: cold storage, growth regulators, micropropagation, synthetic seed.

Recent advances in plant biotechnology have made the synthesis of artificial seeds a reality. The artificial seed is an encapsulated somatic embryo, sown and germinated in the same manner as a conventional seed. Due to low success and high cost of somatic embryo production, buds, shoots, bulbs or other meristematic tissue that can produce a whole plant may also be encapsulated which are also considered as "artificial seeds" (Pond and Cameron 2003). This has opened up a new vista for future plant production programs and can be applied for germplasm conservation, storage or means to reduce the need for transplanting and subculturing during off-season periods. During cold storage, encapsulated nodal segments require no transfer to fresh medium, thus reduces the cost of maintaining germplasm cultures (West *et al.* 2006). Encapsulation of *in vitro* derived vegetative propagule for the production of synthetic seed has been employed as a suitable alternative to the use of somatic embryos (Bapat and Rao 1988, Piccioni and Standardi 1995, Ballester *et al.* 1997, Gonzalez-Benito *et al.* 1997, Adriani *et al.* 2000, Pattnaik and Chand 2000, Chand and Singh 2004, Tsvetkov and Hausman 2005, Faisal *et al.* 2006, Kavyashree *et al.* 2006, Tsvetkov *et al.* 2006, West *et al.* 2006, Faisal and Anis 2007). Na_2-alginate encapsulated vegetative propagule can be used in clonal propagation, conservation and exchange of plant materials between laboratories (Maruyama *et al.* 1997).

As far as we know, no attempt has been made on the development of synseed system in *Vitex negundo*, a valuable medicinal plant for its conservation and mass propagation. However, few reports are available on the regeneration *via* axillary shoot proliferation (Sahoo and Chand 1998, Chandramu *et al.* 2003, Ahmad and Anis 2007).

The purpose of the present study was to optimize the parameters for the production of synseeds, to see their conversion potential under *in vitro* conditions after cold storage and to evaluate the physiological changes during acclimatization.

Nodal segments approximately 3 mm long dissected

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Abbreviations: BA - 6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; NAA - α-naphthalene acetic acid.
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aseptically from *in vitro* established (8-week-old) cultures of *Vitex negundo* were used as explants (Ahmad *et al.* 2008) for encapsulation. Different concentrations of sodium alginate (2, 3, 4 and 5 %, m/v) were prepared using liquid MS medium. For complexation 25, 50, 75, 100 and 200 mM $CaCl_2$ solution was prepared. Both, the gel matrix and complexing agent were sterilized by autoclaving at 1.06 kg cm^{-2} (121 °C) for 15 min after adjusting the pH to 5.8. Encapsulation was accomplished by mixing the nodal segments into the sodium alginate solution and dropping them into calcium chloride solution. The droplets containing the explants were held for at least 30 min to achieve polymerization of sodium alginate. The alginate beads were then collected, rinsed with sterile liquid MS medium and transferred to sterile filter paper in Petri dishes for 5 min under the laminar airflow hood to eliminate the excess of water and thereafter planted into Petri dishes containing MS nutrient medium with various concentrations and combinations of KIN and NAA for germination. The medium was gelled with 0.8 % (m/v) bacteriological grade agar (*Qualigens Fine Chemicals*, Mumbai, India) and sterilized by autoclaving as described above. All the cultures were maintained at 25 ± 2 °C, 16-h photoperiod (irradiance of 50 μmol m^{-2} s^{-1}) and air humidity 55 - 65 % for germination.

Synseeds (encapsulated and non-encapsulated nodal segments) were transferred in Petri dishes containing agar medium and stored in a laboratory refrigerator at 4 °C. Five different low temperature exposure times (0, 1, 2, 4, 6 and 8 weeks) were evaluated for regeneration. After each storage period, encapsulated and non-encapsulated nodal segments were placed on MS medium with or without growth regulators for conversion into plantlets. The percentage of nodal segments forming shoot and root were recorded after 6 weeks of culture.

Plantlets with well developed shoot and roots were removed from the culture medium, washed gently with tap water and transferred to *Thermocol* cups (*Shalimar Thermocol Products*, Mumbai, India) containing sterile *Soilrite*™ (*Keltech Energies*, Bangalore, India), moistened with ½ MS lacking organic supplements and placed under low irradiance (16-h photoperiod). Potted plantlets were covered with a transparent polythene membrane to maintain high humidity and irrigated every 3 d with half strength MS salt solution for 2 weeks. The membranes were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal soil, maintained in a greenhouse and finally transferred to field under full sun.

The net photosynthetic rate (P_N) of *in vitro* regenerated plants was measured during different stages (0, 7, 14, 21 and 28) of acclimatization on fully expanded leaves using portable infrared gas analyzer (*LICOR 6400*, Lincoln, USA). The chlorophyll and carotenoids contents in tissue were estimated by using the methods of Mackinney (1941), and Machlachlan and Zalick (1963), respectively. Activity of carbonic anhydrase (CA) was assayed by the method of Dwivedi and Randhawa (1974).

All the experiments were repeated thrice with 20 nodal segments for each treatment. The data obtained was analyzed using statistical software, *SPSS* version *11* (*SPSS Inc*., Chicago, USA) and means were compared using Duncan's multiple range test (DMRT) at 0.5 % level of significance.

The morphology of encapsulated beads in respect to shape, texture and transparency varied with different concentrations of sodium alginate and $CaCl_2$. A 3 % Na_2-alginate produced clear and uniform beads, while higher concentrations resulted in the production of hard beads and showed considerable delay in germination. On the contrary, Na_2-alginate concentration below 3 % was also not suitable because beads were fragile and difficult to handle.

Of the various concentrations of $CaCl_2$ 100 mM was found to be optimum for the production of uniform synseeds with desired texture. Lower concentration of $CaCl_2$ resulted in either non-formation or very soft beads which may burst during handling (transfer to regeneration medium). Higher concentration of $CaCl_2$ than optimal resulted in the too hard beads.

In previous experiments, BA was supplemented in the medium at various concentrations singly as well as in combination with NAA for optimum *Vitex negundo* shoot multiplication (Ahmad *et al*. 2008). Nodal segments encapsulated in 3 % Na_2-alginate and 100 mM $CaCl_2$ exhibited re-growth within 2 weeks of incubation on MS medium augmented with various concentrations and combinations of KIN and NAA and complete plantlets with well developed root and shoot system were obtained after 4 weeks of incubation. Beside supporting shoot growth, the treatments (KIN + NAA) also supported root growth. Thus special *in vitro* root induction was not necessary prior to the acclimatization. Similar responses have been documented in *Pimpinella pruatjan* (Roostika *et al.* 2006) and *Tylophora indica* (Faisal and Anis 2007). The optimum conversion response (92.6 %) with maximum number of roots (2.8 ± 0.58) per shoot was observed on MS medium supplemented with KIN

Table 1. Effect of different media on conversion of the encapsulated nodal segments of *Vitex negundo* into plantlets and on root formation after 6 weeks of culture. Means ± SE of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

KIN [μM]	NAA [μM]	Conversion [%]	Root number
1.0	-	45.0 ± 1.73^{e}	0.0^{c}
2.5	-	58.0 ± 2.08^{d}	0.0^{c}
5.0	-	69.3 ± 1.76^{c}	0.0^{c}
2.5	0.5	83.3 ± 2.40^{b}	1.4 ± 2.24^{b}
2.5	1.0	92.6 ± 3.71^{a}	2.8 ± 0.58^{a}
2.5	1.5	81.0 ± 2.64^{b}	2.0 ± 0.63^{ab}
2.5	2.0	72.0 ± 2.30^{c}	1.8 ± 0.37^{ab}

(2.5 μM) + NAA (1.0 μM) (Table 1). The average conversion of encapsulated nodal segment was 71 % in comparison to 43.3 % of non-encapsulated nodal segments. The regeneration frequency was clearly influenced by storage time. With an increase in storage time to more than 4 weeks, the conversion frequency decreased considerably (about 50 %) (Fig. 1). Decline in conversion response could be attributed to inhibition of tissue respiration by the alginate matrix, or a loss of moisture due to partial desiccation during storage as reported earlier (Danso and Ford Lloyd 2003, Faisal *et al.* 2006, Faisal and Anis 2007). Encapsulated nodal segments of *V. negundo* were viable (50 %) even after 8 weeks of cold-dark storage, while, only 8 % conversion frequency was observed in non-encapsulated nodal segments. The observations with cold stored encapsulated segments of *Vitex negundo* are in accordance with previous studies on other species (Kinoshita and Saito 1990, Adriani *et al.* 2000, Tsvetkov and Hausman 2005, Faisal *et al.* 2006, Faisal and Anis 2007). According to species, storage needs, and further use of the synthetic seeds, the explants were encapsulated in alginate matrix supplemented with different ingredients, which served as an artificial endosperm (Bapat and Rao 1992, Nieves *et al.* 1998). Germana *et al.* (1998) reported that the synthetic endosperm should contain nutrients and a carbon source for germination and further growth. The synthetic seeds demonstrated high adventitious rooting capacity after sowing (Ganapathi *et al.* 1992, Bapat 1993), while in other cases, they did not perform well (Hasan and Takagi 1995, Piccioni and Standardi 1995, Maruyama *et al.* 1997, Faisal *et al.* 2006) or required an auxin treatment before encapsulation (Chand and Singh 2004).

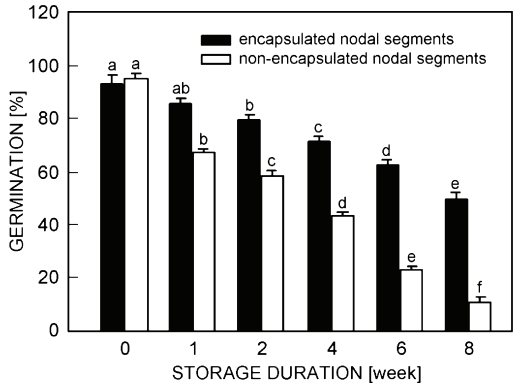

Fig. 1. Effect of storage duration (at 4 °C) on the conversion of encapsulated and non-encapsulated nodal segments of *Vitex negundo* cultured on MS medium augmented with KIN (2.5 μM) + NAA (1.0 μM). The *bars* represent means ± SE. *Bars* denoted by the same letter are not significantly different ($P = 0.05$) according to DMRT.

Acclimatization is the final step in a successful micropropagation system. During this stage plants have to adapt to the new environment of greenhouse or field. The plantlets usually need some weeks of acclimation in shade with the gradual lowering of air humidity (Pospíšilová *et al.* 1998). Fully developed *V. negundo* plantlets with proper shoots and roots were removed from culture medium, transferred to *Thermocol* cups containing sterile *Soilrite* and acclimatized by adopting the standard procedure. Acclimatized plants showed about 90 % survival rate when transferred to field, and grew well.

Considering the importance of this step of micropropagation, various physiological parameters, like chlorophyll and carotenoids contents, net photosynthetic rate and carbonic anhydrase activity were also studied during the acclimatization. During the transfer of tissue culture raised plantlets from *in vitro* to *ex vitro* conditions, the lowest chlorophyll content [0.24 mg g^{-1} (f.m)] was observed at day 0 of acclimation; the value increased up to 0.89 mg g^{-1} after 20 d. Carotenoid content was also increased from 0.08 to 0.33 mg g^{-1} (f.m.) after 28 d (Table 2). A similar observation was also reported earlier (Pospíšilová *et al.* 1999). The increase in chlorophyll content may be attributed to the induction of chlorophyll synthesis (Jeon *et al.* 2005). CO_2 exchange rate was measured 0, 7, 14, 21 and 28 d after *ex vitro* transfer. During the first week, P_N decreased but after one week, it increased again and then remained stable (Table 2). The decline in P_N during the first week after the transfer from *in vitro* to *ex vitro* conditions indicated occurrence of stress in micropropagated plants. Similar results were observed earlier in *Spathiphyllum* (Huylenbroeck and Deberg 1996) and *Rosa hybrida* (Genoud *et al.* 1999). CA activity also increased during acclimatization (Table 2).

Table 2. Changes in various physiological parameters during acclimatization of *in vitro* raised plantlets. Means ± SE. Means followed by the same letter are not significantly different ($P = 0.05$) according to DMRT.

Ex vitro [d]	Chlorophyll content [mg g^{-1}(f.m.)]	Carotenoid content [mg g^{-1}(f.m.)]	P_N [μmol(CO_2) m^{-2} s^{-1}]	CA [mmol(CO_2) g^{-1}(f.m.) s^{-1}]
0	0.24 ± 0.01^{b}	0.08 ± 0.01^{c}	4.25 ± 0.16^{c}	0.63 ± 0.13^{b}
7	0.29 ± 0.03^{b}	0.10 ± 0.01^{c}	3.16 ± 0.11^{d}	1.48 ± 0.25^{a}
14	0.32 ± 0.03^{b}	0.24 ± 0.03^{b}	5.43 ± 0.17^{b}	1.75 ± 0.18^{a}
21	0.61 ± 0.12^{a}	0.31 ± 0.02^{ab}	6.75 ± 0.18^{a}	1.83 ± 0.20^{a}
28	0.80 ± 0.09^{a}	0.32 ± 0.02^{a}	7.08 ± 0.10^{a}	1.92 ± 0.15^{a}

In conclusion, the present study describes a simple, reproducible and efficient protocol for synseed production. This protocol may facilitate conservation, propagation and mass multiplication. Cold storage of encapsulated axillary buds offers possibility for germplasm conservation and exchange between laboratories. Since the plantlets were developed directly from synseeds without an intervening callus phase, somaclonal variation among the regenerants may be avoided.

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