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Full Length Research Paper

Germplasm conservation of patchouli (*Pogostemon cablin* Benth.) by encapsulation of *in vitro* derived nodal segments

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Encapsulation of *in vitro* derived nodal segments of patchouli (*Pogostemon cablin* Benth.) was done successfully by employing sodium alginate gel. Among various concentrations of sodium alginate tried to optimize the strength of the bead, 4% sodium alginate produced firm beads and showed the highest percentage of shoot emergence (73.3%). The best storage temperature was found to be 25°C. The encapsulated beads retained regeneration potentiality up to 6 months and later gradually declined. Browning and loss of regeneration was more after 9 months. Various growth regulating factors (6-benzyl adenine, kinetin, coconut water and tomato juice) at different concentrations were tested for their conversion frequency of encapsulated buds. Murashige and Skoog media supplemented with 2.22 µM/l 6- benzyl adenine showed the highest conversion percentage (91.1%) followed by, 10% coconut water (85.4%). Plants retrieved from the encapsulated buds were rooted on half strength Murashige and Skoog basal medium and acclimatized successfully in the soil. This technology can be adopted for *ex situ* germplasm conservation of elite plants of patchouli.

Key words: Conversion frequency, germplasm conservation, growth regulators, encapsulated buds, patchouli.

INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.), belonging to Lamiaceae, yields an aromatic oil and is commercially used in perfumes and cosmetics (Hasegawa et al., 1992; Maheswari et al., 1993). Patchouli is propagated by rooting the vegetative cuttings of stem, and there are reports of *in vitro* propagation and mass production of virus-free patchouli plants (Kukreja et al., 1990). The possible methods of conservation of improved lines of patchouli are maintaining the stock plants in the field or as *in vitro* cultures. The conservation of elite germplasm in the field is prone to diseases, pests and other environmental hazards. Alternatively maintenance of *in vitro* cultures for conservation is associated with somaclonal variations, and not cost effective because of monthly sub cultures and labour intensive processes. In this regard, synthetic seed technology offers an excellent scope for conservation of rare hybrids, elite genotypes and genetically engineered patchouli plants. During the last four decades, synthetic seed technology has gained considerable importance in plant biotechnology as a potential, viable and valuable system for ex situ conservation of commercially important plants (Kavyashree et al., 2004). Encapsulation and storage of the buds at freezing temperatures offers a long-term storage capability, maximal stability of phenotypic and genotypic characteristics, minimum space and maintenance. Low production costs, ease of storage and transport are the additional advantages (Ghosh and Sen, 1994).

Studies on the *in vitro* germplasm conservation by encapsulation of somatic embryos is reported in many plant species, including cereals, vegetables, fruits, ornamentals and medicinal plants (Bapat and Rao, 1988; Ghosh and Sen, 1994; Fowke et al., 1994; Onay et al., 1996; Castillo et al., 1998). In recent years encapsulation of in vitro derived shoot tips or axillary buds has become a suitable alternative in place of somatic embryos (Bapat

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Abbreviations: BA; 6- benzyl adenine, **KN;** kinetin, **MS;** Murashige and Skoog, **NAA;** α-naphthalene acetic acid, **IAA;** 3-indol acetic acid.

et al., 1987; Mathur et al., 1989; Sharma et al., 1994; Maruyama et al., 1997; Sarkar and Naik, 1997; Adriani et al., 2000). This technology is quite promising as developing somatic embryo system is difficult for many plant species. Kageyama et al., (1995) has reported encapsulation and regeneration of patchouli protoplasts, however encapsulation of in vitro derived nodal segments is being reported for the first time in the present study, which is a simpler and easier technique.

The present report describes the encapsulation of nodal segments of micropropagated patchouli in calcium alginate hydrogen. The evaluation of *in vitro* response of encapsulated micropropagules to various concentrations of growth regulators and also the effect of temperature and storage period on conversion rate is also reported in this study Murashige and Skoog, 1962.

MATERIALS AND METHODS

Healthy patchouli plants were selected from the herbal garden of Rishi Herbal Technologies, pvt. Ltd., Bangalore. Nodal segments and shoot tips (2 - 3 cm) of these selected plants were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and washed thoroughly with sterile distilled water. Later, the explants were implanted on MS medium supplemented with 2.22 µM/l BA. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. Cultures were maintained at a temperature of 25 ± 2°C under 16 h light (2000 lux) / 8 h dark photoperiod and sub cultured every 4 weeks. Multiple shoots regenerated on this medium were used for further studies. Nodal segments measuring about 5 mm were explanted carefully and used for immobilization experiments.

The *in vitro* derived explants isolated were immersed for a few seconds in 2 - 6% sodium alginate solution prepared in full strength MS basal medium with 2% sucrose (w/v). Later, the micro-propagules in alginate medium were picked up by tweezers and dropped into a sterile solution of 100mM calcium chloride. The drops, each containing a single micropropagule, were placed in this solution for half an hour to allow polymerization. Calcium alginate beads containing the micropropagule were retrieved from the solution and rinsed twice with autoclaved distilled water to remove the traces of calcium chloride. The beads were then transferred to sterile filter paper in Petri dishes. Blot dried beads were stored in a petri dishes sealed with para film for 1 - 6 months at refrigerator (4°C), incubator (20°C) and plant tissue culture room(25°C).

After storage, the encapsulated buds were cultured in regeneration medium and incubated in a culture room maintained at 25 ± 2°C. Various regeneration media tested were MS medium supplemented with different concentrations of BA (1.11, 2.22 and 4.44 µM/l) or Kinetin (KN) (1.16, 2.32 and 4.65 µM/l). The percentage of shoot emergence from the encapsulated beads was calculated after 3 - 4 weeks of inoculation. To further improve the shoot multiplication and morphology of the plantlet, MS media fortified with 5 - 15% coconut water and tomato juice (5 - 15 g of tomato is added to 100 ml of distilled water and grinded in a mixer to get juice). Similar experiments were also carried out by using filter paper bridges in MS liquid medium in culture tubes to check the regeneration efficiency. For rooting, developed shoots were transferred to half strength and full strength MS basal medium with or without α-naphthalene acetic acid (NAA) at 2.68 and 5.37 µM/l or 3-indol acetic acid (IAA) at 2.85 and 5.71 µM/l. The rooted shoots were planted in net pots containing sterile Soilrite and hardened for 4 weeks in a moisture saturated glass chamber with 80% relative humidity. Hardened plantlets were transferred to pots containing garden soil: manure: sand (1:1:2) under shade conditions. For each treatment 20 replicates were used and the experiments were repeated thrice. The data were subjected to Fisher's method of analysis of variance.

RESULTS AND DISCUSSION

Establishment of micropropagated plants

The surface sterilized explants inoculated on to MS medium fortified with 2.22 µM/l BA resulted in multiple shoot regeneration after 25 days. The development of complete multiple shoots were observed after 3 - 4 weeks of incubation. The nodal segments from these micropropagated plants were used for encapsulation.

Effect of different concentrations of sodium alginate on encapsulation

Sodium alginate is a copolymer composed of D-mannuronic acid and L-glucuronic acid units and has been extensively studied because of its biocompatibility, biodegradability and its capability to form hydro gels in the presence of divalent cations. The rigid structure and large pore size of these gels, which are insoluble in water, make them useful for the encapsulation of live cells of plants. Polymer concentration, degree of viscosity of the alginate used, $CaCl_2$ concentration, and curing time are important parameters determining the permeability, resistance and hardness of the resulting beads and the subsequent success of the encapsulation method (Block, 2003). In the present study, the polymerizing ability of sodium alginate at different concentrations (2 - 6%) varied markedly when used to encapsulate the buds (Table 1). Very firm, clear, isodiametric beads of uniform size and shape, was achieved using 4% sodium alginate solution and 100 mM calcium chloride (Figure 1a). Concentrations of sodium alginate lower than 4% were not suitable as the beads were too soft to handle, while at higher concentrations (5 and 6%), they were too viscous, harder and hindered the emergence of shoot in patchouli. The influence of optimum concentration (4%) of sodium alginate on bead quality and shoot emergence is in agreement with the earlier reports (Mathur et al., 1989; Ghosh and Sen, 1994; Castillo et al., 1998; Jaydip Mandal et al., 2000). The conversion frequency was highest (73.3%) when 4% sodium alginate was used and decreased with increase in sodium alginate concentration to 16.7% at 6%. Different stages of sprouting and regeneration from encapsulated buds are depicted in Figure 1b.

Effect of storage period and temperature on shoot emergence from encapsulated buds

Conversion frequency of shoots is directly dependent on storage period and temperature (Table 2). It was observed

Table 1. Effect of sodium alginate concentration on quality of beads and shoot emergence.

Concentration of sodium alginate (%)	Quality of beads	Conversion frequency (%)
2	Too soft	50 ± 2.51
3	Soft	60 ± 2.56
4	Firm	73.3 ± 2.53
5	Hard	30.0 ± 2.65
6	Hard	16.7 ± 2.62

Data shown are means of ± standard error from 20 beads for each of three replicates per treatments.

Table 2. Effect of storage period and temperature on shoot emergence from encapsulated buds.

Storage period (Months)	Temperature (°C)	Conversion frequency (%)
2	4	53.3 ± 2.86
	20	66.0 ± 2.89
	25	82.3 ± 2.62
4	4	39.3 ± 2.87
	20	46.7 ± 2.68
	25	63.1 ± 2.68
6	4	36.7 ± 2.69
	20	43.3 ± 3.67
	25	52.3 ± 3.68

Data shown are means of ± standard error from 20 beads for each of three replicates per treatments.

observed that 2 months old beads resulted in higher emergence of plants (53.3-73.3%) when compared to the germination response of 4 and 6 months old beads (33.3 - 50.0% and 36.7 - 43.3% respectively). The decline in the germination percentage among the synthetic seeds of shoots stored for a period of 2 - 6 months may be due to inhibited respiration of plant tissues by alginate leading to loss of viability (Redenbaugh et al., 1987). The best storage temperature is 25°C during 2, 4 and 6 months periods. The morphology and growth of regenerated shoots is not affected at 25°C. However, at 4 and 20°C the emerged shoots exhibited slow growth with necrotic and vitrification symptoms. After 6 months of storage, the percent frequency of conversion was reduced, along with death and decay of the encapsulated buds. This might be due to the cracks and dehydration of the bead.

Effect of various growth regulating factors on bud sprouting and regeneration of encapsulated buds

Different growth regulating factors were supplemented to MS media to increase the sprouting and multiplication rate of encapsulated buds (Table 3 and 4). There was a significant difference in percent frequency and nature of response due to different growth regulator supplemented media. The regeneration potentiality of a tissue/organ culture can vary according to type and concentration of growth regulator, the type and age of explant and the species from which it is derived (George, 1993). The percentage of encapsulated buds exhibiting multiple shoots emergence was highest (91.1%) on MS medium supplemented with 2.22 µM/l BA followed by µM/l KN (86.3%). MS basal medium (control) exhibited only single shoot formation. The use of filter paper bridges on the liquid medium was more suitable for regeneration (Figure 1c). This is a useful approach to avoid abnormality and hyperhydricity of the recovered shoots.

Among the natural growth regulating factors supplemented to MS medium, 10% coconut water resulted in good response (Figure 1d). Maximum percentage of multiple shoot emergences with an average shoot length of 2 cm was observed on media supplemented with 10% coconut water and this response is superior to the other treatments (Table 4). Being complex mixtures and inexpensive nature, natural additives provide a good scope for substituting the expensive growth regulators. Coconut water stimulates cell division in other cultured tissues and its use as a supplement is adopted in many laboratories (Morel, 1950; Nickell, 1950; Henderson et al. 1952; Archibald, 1954). Similarly Straus (1960) has shown that tomato juice function by supplying a form of organic nitrogen to *in vitro* cultured explants. Optimizing cost effective protocol by using natural extracts for the

Table 3. Effect of plant growth regulators on shoot emergence from encapsulated buds.

MS + Plant growth Regulator	Concentration (µM/l)	% of beads Exhibiting multiple shoots	% of beads exhibiting shoot length more than 2 cm
BA	0.25	78.3 ± 2.68	86.0 ± 1.14
	0.50	91.1 ± 2.66	93.6 ± 1.16
	1.00	70.0 ± 2.45	58.3 ± 1.15
KN	0.25	71.7 ± 2.66	34.3 ± 1.19
	0.50	86.3 ± 2.66	38.4 ± 1.16
	1.00	70.2 ± 2.71	29.0 ± 1.86
Control	-	0.00	0.00
F-value	-	19.62*	255.31*
SEm ±	-	2.318	2.54
CD at 5% Level	-	5.169	5.96

* Significant at 5% Level. Data shown are from 20 beads for each of three replicates per treatments.

Table 4. Effect of natural extracts on multiplication of shoots emerged from encapsulated buds.

MS + natural extracts	Concentration (%)	% of beads exhibiting multiple shoots	% of beads exhibiting shoot length more than 2 cm
Coconut water	5	76.6 ± 2.67	80.0 ± 1.14
	10	85.4 ± 2.68	91.6 ± 1.16
	15	66.6 ± 2.58	48.3 ± 1.15
Tomato juice	5	13.3 ± 2.66	28.3 ± 1.19
	10	28.3 ± 2.58	38.3 ± 1.16
	15	20.0 ± 2.86	21.0 ± 1.86
Control	-	0.00	0.00
F-value	-	117.55*	255.31*
SEm ±	-	1.10	2.54
CD at 5% Level	-	2.46	5.96

* Significant at 5% Level. Data shown are from 20 beads for each of three replicates per treatments.

Figure 1A. Encapsulated buds of patchouli in calcium alginate.

Figure 1B. Different stages of sprouting and multiplication of encapsulated buds.

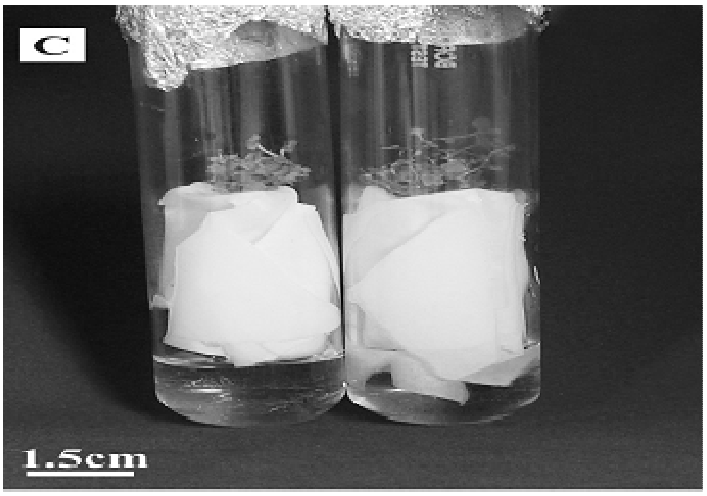

Figure 1c. Multiplication of encapsulated buds in MS liquid medium supported on filter paper bridges.

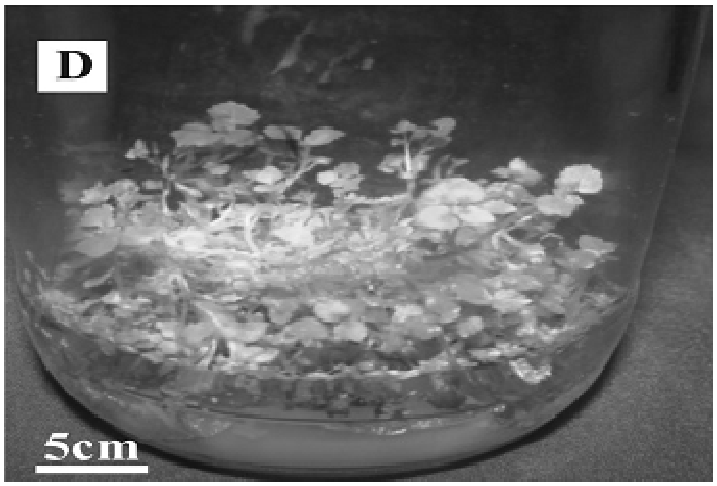

Figure 1d. Multiple shoots regeneration of encapsulated buds on MS + 10% coconut water.

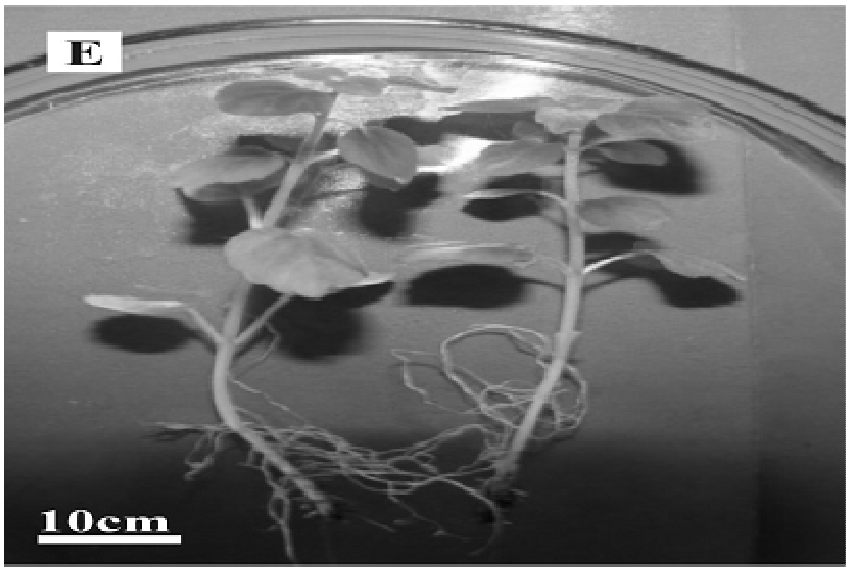

Figure 1E. Rooted plantlets of patchouli regenerated from encapsulated buds.

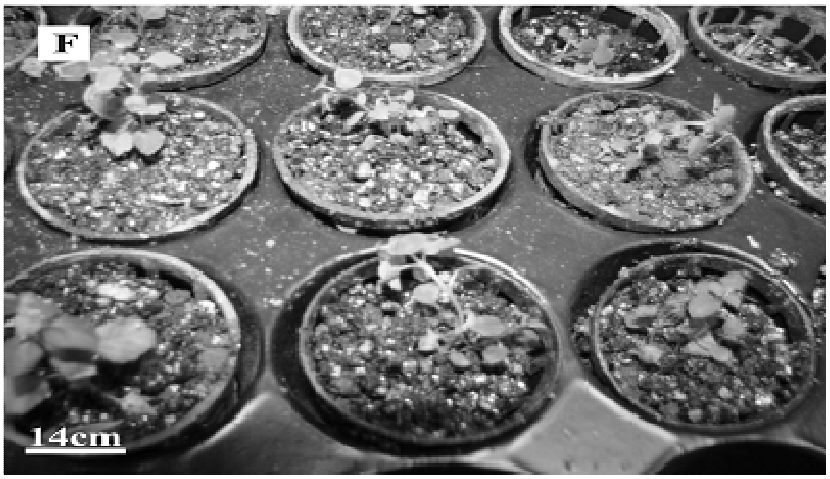

Figure 1f. Acclimatization of regenerated plantlets from encapsulated buds.

using natural extracts for the recovery of plants from encapsulated propagules of patchouli perhaps is a first report made in the present study.

Rooting and acclimatization of plantlets regenerated from encapsulated buds

Individual shoots were rooted on half strength MS medium without hormones to produce whole plants (Figure 1e). The use of NAA and IAA in the media induced rooting with varying degrees with callus formation at the base of the shoot. This suggests that although the addition of auxins is beneficial for rooting, their use is not essential and half strength MS medium is enough to get vigorous rooting. This is in conformity with the results obtained by Bharati (2002). Rooted shoots were successfully hardened off in net pots containing sterile soilrite (Figure 1f). After 4 weeks of hardening, these plantlets were acclimatized well and transferred to green house and planted in the field. Maintenance of high humidity during the early hardening phase in glass chamber was found to be essential for good plantlet survival (90 - 93%). The above results coincides with the findings of Misra (1996) who has reported that the maintenance of 80% relative humidity increase the plant survival rate. To date there are no reports on encapsulation of nodal segments in patchouli. The use of this method can avoid minimum of six subcultures and variations due to prolonged cultures on a growth regulator containing medium.

Conclusions

The present study shows the feasibility of using nodal segments for encapsulation and germplasm conservation of rare hybrids, elite genotype and genetically engineered patchouli plants. Germplasm conservation through tissue culture requires about 5 - 6 subcultures in fresh medium and this can cause variations in the plant morphology. Hence, this encapsulation technology can be adapted to reduce the risk of sub-culturing, the handling cost and risk of contamination during sub culturing. However, further investigations are needed to extend the duration of storage before making the technique applicable to patchouli germplasm storage.

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