RESEARCH PAPER

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Germplasm conservation of patchouli (*Pogostemon cablin* Benth.) by encapsulation of micropropagated buds in calcium alginate

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Encapsulation of *in vitro* derived nodal segment explants of patchouli (*Pogostemon cablin* Benth.) was done successfully by using 4 per cent sodium alginate gel for storage and germplasm conservation. Various concentrations of sodium alginate were tried to optimize the strength of the bead, which can give maximum conversion frequency. Various-growth regulators, and natural extracts were tested for their efficiency to regenerate healthy sprouts from encapsulated explants without vitrification. The highest frequency of shoot emergence and maximum number of shoots per bud were recorded on media supplemented with 0.5 mg/l 6- benzylaminopurine (BA). Among the natural extracts tried, 10 per cent coconut water exhibited equally good response with high frequency of shoot multiplication and broader leaves. Regenerated shoots were rooted on half strength Murashige and Skoog (MS) medium devoid of growth regulators. Plants retrieved from the encapsulated buds were hardened and established in soil. The effect of storage period and temperature on the conversion frequency of encapsulated buds was studied. This technology can be adopted for *ex situ* germplasm conservation of high yielding varieties of patchouli

Key words : Essential oil, Growth regulators, Natural extracts, Multiple shoots, Somaclonal variations, Propagation

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INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.), belonging to the family Lamiaceae is an aromatic crop. The essential oil obtained by steam distillation of its shade-dried leaves is commercially used in perfumes and cosmetics (Hasegawa *et al.*, 1992; Maheswari *et al.,* 1993). It also possesses anti insecticidal activities (Sharma *et al*., 1994). The conventional method used for propagating this herb is only through vegetative cuttings. Vegetative propagation under natural conditions is season dependent and is subjected to loss due to diseases, pests and environmental disasters. Apart from being expensive, somaclonal variations are associated with *in vitro* culture maintenance. Under these conditions, synthetic seed technology offers excellent scope for propagation and ex situ conservation of rare hybrids, elite genotypes and genetically engineered patchouli plants. Encapsulation and storage of the buds at freezing temperatures offers long-term storage capability, maximal stability of phenotypic and genotypic characteristics, minimum space and maintenance requirement.

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

RESEARCH METHODOLOGY

Healthy patchouli plants were selected from greenhouse of College of Agricultural Biotechnology, Loni. Nodal segments and shoot tips of these selected plants were surface sterilized with 0.1 per cent (w/v) $HgCl_2$ for 10 min and washed thoroughly with sterile distilled water. Later, the explants were implanted on Murashige and Skoog (MS) medium supplemented with 0.5 mg/l 6-benzylaminopurine (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/8 h dark photoperiod and subcultured 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 explants isolated were immersed for a few seconds in 2-6 per cent sodium alginate solution prepared in full strength MS basal medium with 2 per cent sucrose (w/v). Later, the micropropagules in alginate medium were picked up by tweezers and dropped into a sterile solution of 100 mM calcium chloride. The drops, each containing a single micropropagules, were placed in this solution for half an hour to allow complexation. Calcium alginate beads containing the micropropagules 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 Petridishes. Blot dried beads were stored for 1-6 months at 4, 20, and 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 (0.25 mg/l, 0.5 mg/l and 1.0 mg/l) or Kinetin (KN) (0.25 mg/l, 0.5 mg/l and 1.0 mg/l). The conversion frequency of encapsulated beads into shoots was calculated. MS media fortified with different natural extracts (5-15 per cent of coconut water and tomato juice) were tried. For rooting, developed shoots were transferred to half strength and full strength MS basal medium with or without a-naphthalene acetic acid (NAA) or 3-indol acetic acid (IAA) (0.5 and 1.0 mg/l). The rooted shoots were planted in net pots containing sterile soil rite and hardened for 4 weeks in a moisture saturated glass chamber with 80 per cent 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.

RESULTS AND ANALYSIS

The experimental findings of the present study have been presented in the following sub heads:

Establishment of micropropagated plants:

Nodal segments treated with 0.1 per cent $CaCl_2$ for 10 min. produced clean mother cultures. The explants inoculated on to MS medium fortified with 0.5 mg/l BAP resulted in multiple shoot regeneration after 15 days. The development of complete multiple shoots were observed after 3-4 weeks of incubation. The shoots developed were subcultured to get more multiple shoots. The nodal segments from these micropropagated plants were used for encapsulation.

Effect of different concentrations of sodium alginate on encapsulation:

In the present study, the complexing ability of sodium alginate at different concentrations (2-6 per cent) varied markedly (Table 1). An optimal ion exchange between Na+ and Ca+, producing firm, clear, isodiametric beads of uniform size and shape, was achieved using 4 per cent sodium alginate solution and 100 mM calcium chloride. Concentrations of sodium alginate lower than 4 per cent were not suitable as the beads were too soft to handle, while at higher concentrations (5 and 6 per cent), they were too viscous, harder and hindered the emergence of shoot. The influence of optimum concentration (4 per cent) of sodium alginate on bead quality and shoot emergence is in agreement with the earlier reports (Mathur *et al.*, 1989). The percentage of beads exhibiting shoot emergence was highest (73.3 per cent) when 4 per cent sodium alginate was used and decreased with increase in sodium alginate concentration to 16.7 per cent at 6 per cent.

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

Concentration of sodium alginate C (%)	Quality of beads	% of beads exhibiting shoot emergence
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.

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

Varied emergence of shoots was noticed, when beads were stored for 2, 4 and 6 months at varied temperatures (4, 20, and 25^{0}C) (Table 2). It was observed that up to 2 months old beads resulted in higher emergence of plants (53.3-73.3 per cent) when compared to the germination response of 4 and 6 months old beads (33.3-50.0 per cent and 36.7-43. per cent, respectively). The decline in the germination percentage among the synthetic seeds of shoots stored for a period of 1 to 6 months may be due to inhibited respiration of plant

tissues by alginate leading to loss of viability. Conversion frequency was also influenced by storage period and temperature. The best temperature for storage was 25°C during 2, 4 and 6 months periods. However, with the increase in storage period, the per cent frequency of conversion was reduced invariably.

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

Storage period (Months)	Temperature (^{0}C)	% of beads showing shoot emergence
2	4	53.3±2.86
	20	50.0±2.89
	25	73.3±2.62
4	4	39.3±2.87
	20	46.7±2.68
	25	50.0±2.68
6	4	36.7±2.69
	20	43.3±3.67
	25	43.3±3.68

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

Effect of various growth regulators and natural extracts on bud sprouting and regeneration of encapsulated buds:

Different growth regulators and natural extracts were supplemented in MS media to increase the sprouting and multiplication rate of encapsulated buds (Table 3 and 4). There was a significant difference in per cent frequency and nature of response due to different growth regulator supplemented media. Cytokinins appear to be necessary for cell division and are very effective in promoting direct and indirect shoot regeneration. The percentage of encapsulated buds exhibiting multiple shoot emergences was highest (91.1 per cent) on MS medium supplemented with 0.5 mg/l BA followed by 0.5 mg/l KN

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

Plant growth regulator	Concentration (mg l^{-1})	% of beads exhibiting multiple shoots	%of beads showing single shoot emergence
MS + BA	0.25	78.3±2.68	12.3±5.58
	0.50	91.1±2.66	03.1±4.63
	1.00	70.0±2.45	10.0±2.69
MS + KN	0.25	71.7±2.66	10.7±3.33
	0.50	86.3±2.66	09.3±3.54
	1.00	70.0±2.71	10.0±3.43
MS Basal	-	0.00	42.1±2.30

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

Among the natural extracts supplemented to MS medium, 10 per cent coconut water resulted in good response; the percentage of beads exhibiting multiple shoots (85.4±2.68) and shoot length more than 2 cm (91.6±1.16) was highest and it was superior to tomato juice (10 per cent) supplemented media (Table 4). Research efforts have addressed the problem of using growth regulating compounds and salt mixes, which are expensive, and there seems to be a good scope for substituting these expensive chemicals with low cost natural extracts. 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, yeast extract function by supplying a form of organic nitrogen to *in vitro* cultured explants. Optimizing cost effective protocol by using natural extracts for the recovery of plants from encapsulated propagules of patchouli perhaps is a first report made in the present study.

Table 4 : Effect of natural extracts on multiplication of shoot emerged from encapsulated buds

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±2.56	38.3±1.16
	15	10.0±2.86	20.0±1.86
Control	-	0.00	16.0±1.40

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

Rooting of regenerated plants from encapsulated buds:

The effect of strength of MS basal media and MS media with NAA at different concentrations on rhizogenesis was studied. Roots were not induced during shoot multiplication in the cytokinin regime. Individual shoots when implanted in half or full strength MS medium without hormones resulted in poor and few numbers of roots with low frequency. However, addition of IAA and NAA to MS medium enhanced the rate of rhizogenesis in both frequency as well as number of roots. Of the two auxins tested, 0.1 mg/l NAA induced the highest number of roots per shoot compared to other concentrations of IAA and NAA tested (Table 5).

Table 5 : Effect of strength of MS media, IAA and NAA on rhizogenesis of micropropagated shoot from encapsulated buds of patchouli

Treatment	Concentration of IAA / NAA (mg/l)	% of beads exhibiting multiple roots	Number of roots / explants
MA +IAA	0.25	74	7.0±1.18
	0.50	86	8.0±1.47
	1.00	88	9.0±0.74
MS +NAA	0.25	76	8.0±1.52
	0.50	90	9.0±0.86
	1.00	96	10.0±1.05
Half strength MS	-	73	6.0±1.11
Full strength MS	-	70	7.0±1.04

Acclimatization of plantlets regenerated from encapsulated buds:

The rooted plantlets, retrieved from encapsulated shoot buds on rooting medium, were successfully hardened off in net pots containing sterile soil rite. After 4 weeks of hardening, these plantlets were acclimatized well, transferred to green house, and planted in the field. Maintenance of high humidity (80 per cent RH) during the early hardening phase hi glass chamber was found to be essential for good plantlet survival (90-93 per cent).

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. In the present study, it is evident that the encapsulated buds of patchouli can be successfully stored for six months. The conversion frequency is affected after six months of storage. Encapsulating the regenerated buds and again storing for another six months can solve this problem. In this way, the technology can be used for patchouli germplasm storage.

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