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Micropropagation of *Pogostemon cablin* Benth. through Direct Regeneration for Production of True to Type Plants

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Abstract

Multiple shoots from nodal explants of *Pogostemon cablin* Benth. MS supplemented with 0.5 mg/l BAP initiated a mean of 45.66 shoots/nodal explant. Within four weeks of initiation, regenerated multiple shoots attained a height of 3.6 cm. Subsequent transfer of these *in vitro* derived nodal segments onto MS supplemented with BAP and Kn (0.5 mg/l), induced a mean of 62.45 shoots. Higher concentration of either BAP or Kn more than 0.5 mg/l resulted in callus proliferation and showed hyperhydric shoots with morphological abnormalities. Rooting was readily achieved upon transfer of shoots on half strength MS supplemented with 100 mg/l activated charcoal. Rooted shoots, following acclimatization in greenhouse, were successfully transferred to soil with 91% survival. Also shoots regenerated *in vitro* were directly transplanted to soil and acclimatized. Tissue cultured plants were analyzed for oil content by employing gas chromatography and found that the patterns were similar to mother plants. This confirms the true to type nature of micropropagated plants.

Introduction

Patchouli (*Pogostemon cablin* Benth.), belongs to Lamiaceae. It is an aromatic plant, native to tropical Asia and widely grown in India, Malaysia, Philippines, Indonesia and Singapore. The patchouli oil, obtained by steam distillation of shade-dried leaves is commercially used in perfumes and cosmetics (Hasegawa et al. 1992, Maheswari et al. 1993). It also possesses anti-insecticidal activities, antifungal and bacteriostatic properties (Kukreja et al. 1990, Yang 1996, Pattnaik et al. 1996). In aromatherapy, it is used to calm nerves, relieve depression and stress. Fibrinolytic and antithrombotic (Sumi 2003, Park et al. 2002) activity of this essential oil is also been reported.

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The plant never flowers in India and hence vegetative propagation through stem cuttings is in practice. However, the feasibility of mass production of patchouli through conventional methods has been limited due to recurrence of mosaic virus (Sastri and Vasanth Kumar 1981), root knot nematodes and insect pests. Apart from this, propagation through vegetative cuttings is slow and insufficient for large scale cultivation. Natural variations occurring in this plant may result in yield fluctuations. The rapidness of tissue culture techniques can be advantageous for the continuous provision of plantlet stock for field cultivation (Reddy et al. 2001) and may further complement breeding programmes.

Many authors have envisaged feasibility of mass propagation of high yielding and disease/pathogen resistant patchouli through tissue culture. Patchouli plants regeneration from stem tip, leaf and nodal callus (Meena 1996, Padmanabhan et al. 1981), plant regeneration from protoplasts encapsulated in alginate beads (Kageyama et al. 1995), mass production of virus free plants by *in vitro* culture and somatic embryogenesis (Kukreja et al. 1990, Rajan et al. 1997) have been reported. Hembrom et al. (2006) has reported the production of true to type plants of *Pogostemon heyneanus* through dedifferentiated axillary buds. However, there are limited efforts to study direct organogenesis, which supports cultivation by providing true to type plants in large numbers. Hence present study is aimed at establishing suitable protocols for rapid regeneration of patchouli by direct organogenesis using nodal explants. This method can mitigate the problem of non-availability of planting materials to meet the global demand.

Materials and Methods

Nodal segments were procured from elite *Pogostemon cablin* Benth. mother plants, maintained at Rishi Herbal Garden, Bangalore, India. All expanded leaves and petioles were removed and the explants were cut into 1 - 2 cm length. The explants were washed three - four times in the tap water and treated with liquid soap, teepol for 15 min followed by thorough washing under tap water. These were then surface sterilized with 0.5% $HgCl_2$ for 10 min. Rinsing was done five times with sterile distilled water to remove traces of $HgCl_2$ completely. Under aseptic conditions, explants were inoculated on MS, containing 2% (w/v) sucrose, supplemented with different concentrations and combinations of BAP (0.25, 0.5, 1.0, 2.0 mg/l) and Kn (0.25, 0.5, 1.0, 2.0 mg/l) for shoot proliferation and multiplication. The pH of the medium was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved at 121ºC, 15 lb pressure for 15 min. All the cultures were incubated at 25 ± 2ºC under a 16 hr light and 8 hr dark regimes with a light intensity of 3000 lux provided by cool-white fluorescent tubes. Weekly observations were recorded. *In vitro* derived shoots from both the explants were

excised after 30 days and subcultured on to fresh medium with the same concentrations of growth regulators unless otherwise mentioned.

For rooting, 5 - 6 cm long regenerated shoots bearing at least four - five internodes were excised and cultured on freshly prepared rooting medium containing MS or half strength MS supplemented with different concentrations of activated charcoal (100, 200 mg/l), IAA and NAA (0.5, 1.0 mg/l). Rooted plantlets were transferred to sterile soilrite in net pots. For direct acclimatization, the *in vitro* derived shoots with thick stems were cut off and directly transferred to sterile soilrite in net pots. Plantlets were hardened for four weeks in a moisture saturated chamber with 80% relative humidity and were transferred to pots containing soil : manure : sand (1 : 1 : 2) under shade condition. The experiments were set up in completely randomized design with different treatments replicated thrice. Twenty cultures were raised for each treatment. Data recorded after 30 days of culture were statistically analyzed and results were presented in Table 1.

Fresh leaves of *P. cablin* were hydrodistilled by a Clevenger-type apparatus. The essential oil was collected and stored at 4ºC until being analysed for its chemical constituents by gas chromatography (GC).

GC analysis of the oil was performed on Varian 3400 (Varian, Les Ulis, France) with an FID and an electronic integrator. The instrument was fitted with a 30 m × 0.25 mm non-polar CP-Sil-5-CB-MS column, film thickness 0.25 mm. Oven temperature was programmed from 50 - 220ºC at 5ºC/min, held at 120ºC for 10 min. Injector and detector temperatures were 250 and 280ºC, respectively. Carrier gas was helium at 16 psi. One ml of oil dissolved in acetone was introduced into the gas chromatograph with a split mode ratio of 1 : 100. The constituents of the oil were identified by running the reference sample under similar condition.

Results and Discussion

The nodal explants underwent direct organogenesis when cultured on MS using various concentrations of BAP and Kn (0.25, 0.5 and 1.0 mg/l) separately or in combinations. Comparatively BAP showed the strongest effect than Kn in terms of shoot induction. It also increased mean shoot length and shoot weight (Table 1). According to George et al. (2008), BAP is most effective in enhancing shoot multiplication and triggering shoot elongation. The use of MS supplemented with 0.5 mg/l BAP was best suitable for bud break (Fig. 1a) and resulted maximum number (45.66) of shoots/explant; higher shoot length (3.6 cm) and fresh weight (2.94 g) of the shoot without callus induction. The result is in agreement with the findings of Bharati (2002) but contradictory to the findings of Kukreja et al. (1990) and Hembrom et al. (2006), who reported the higher

requirement of cytokinin (2 mg/l BAP) for maximum multiple shoot regeneration in patchouli.

Table 1. Effect of on shoot proliferation from nodal segments of patchouli grown on MS after 30 days of first subculture.

MS + BAP and Kn (mg/l)	No. of shoots/ explant	Length of the shoots (cm)	Fresh wt. of the shoots (g)	Callus formation
MS (Control)	13.62	1.23	1.06	_
MS + 0.25 BAP	36.66	1.73	1.42	_
MS + 0.5 BAP	45.66	3.60	2.94	_
MS + 1.0 BAP	20.00	1.65	1.97	+
MS + 2.0 BAP	00.00	0.00	0.00	+
MS + 0.25 Kn	23.33	1.93	1.08	_
MS + 0.5 Kn	34.56	2.43	2.87	_
MS + 1.0 Kn	21.53	2.23	2.44	_
MS + 2.0 Kn	00.00	0.00	0.00	+
F- value	49.72*	5251.03*	6239.80*	
SEM	2.84	0.01	0.01	
CD	8.46	0.04	0.04	

*Significant at 5% level. + : Callus induction, – : No callus formation.

However, BAP at 0.5 mg/l when used for subsequent subcultures resulted in callus, indicating the elevation of endogenous hormonal levels. Hence though initiation was made on medium supplemented with 0.5 mg/l BAP, multiplication was better evidenced on 0.25 mg/l BAP. As the concentration of cytokinins was increased beyond 0.5 mg/l, it resulted in decrease in number of shoot buds coupled with callus proliferation.

The decrease in shoot production at higher concentration of BAP may be due to the inhibition of organogenesis and induction of callus proliferation. Patchouli is a very sensitive plant and it expresses its morphogenetic potentiality even at very low concentrations of cytokinins. Shoot proliferation was satisfactory on MS supplemented with 0.25 mg/l BAP and 0.5 mg/l Kn separately. However, Kn has no significant effect on multiple shoot regeneration but played a role in increasing the length and strength of shoots. The combination treatment (0.5 mg/l BAP along with 0.5 mg/l Kn) was found to exhibit highest frequency of shoot multiplication (62.45%). The highest mean shoot length (5.2 cm) and mean fresh weight of the shoot (5.07 g) was also evidenced in the same treatment (Table 2). The efficacy of BAP over Kn, when used singly, and in combination has been demonstrated for the axillary bud proliferation in many medicinal plants of Lamiaceae like *Mentha spicata* and *Lavendula viridis* (Hirata and Kukreja 1990, Dias and Nickell 2002). Higher effect of the combination of BAP and Kn may be

due to the synergy of cytokinins as reported in *Rollinia mucosa* and *Solanum surrattense* (Figueiredo 2001, Pawar 2002). The above result clearly indicates that combination of BAP and Kn is a better choice for patchouli as it significantly exhibited better morphogenetic response in terms of multiple shoot regeneration, length of the shoots and production of biomass (Fig. 1b).

Table 2. Effect of combination of cytokinins on elongation of shoots regenerated from primary node cultures of patchouli grown on MS after 30 days.

Concentrations (mg/l)		Shoot length (cm)	Number of multiple shoots	Fresh weight of the shoots (g)	Callus formation
BAP	KN				
0.0	0.0	0.21	13.14	1.10	_
0.25	0.25	1.95	62.66	2.46	_
0.5	0.25	4.65	61.33	4.67	_
1.0	0.25	1.66	22.66	1.86	+
0.25	0.5	3.31	41.66	3.06	_
0.5	0.5	5.20	62.45	5.07	_
1.0	0.5	2.05	21.37	2.35	+
0.25	1.0	2.32	23.00	2.96	_
0.5	1.0	2.67	42.10	4.14	_
1.0`	1.0	1.92	21.33	2.35	+
F value		1531.7*	354.1*	8141.7*	
SEM		0.01	0.98	0.01	
CD		0.03	2.93	0.03	

*Significant at 5% level. + : Callus induction, – : No callus formation.

The effect of strength of MS with and without activated charcoal, IAA and NAA at different concentrations on rhizogenesis was studied (Table 3). Among the treatments, half strength MS was found enough for better rooting. This is in conformity with the results obtained by Bharati (2002) in patchouli. However, it was not possible to induce high frequency of rooting (93.33%) when shoots implanted on half strength MS with 100 mg/l activated charcoal. Mean number of roots/shoot (15.23) and root length (6.23 cm) was found to be superior among all other treatments (Fig. 1c). This is the first report of its kind in patchouli. Activated charcoal is an antioxidant and known to induce rhizogenesis in *Decalepis hamiltonii* (Obul et al. 2001) and *Annona cherimoya* (Padilla and Encina 2004). This is because it provides darkness in the medium, which is essential for rooting. The result obtained by using half strength MS and activated charcoal is superior to the results obtained by using auxins. Both IAA and NAA were shown to induce rooting with varying degrees, however not suitable for patchouli as both the auxins invariably triggered callus proliferation. The results of Meena

(1996) support the usage of auxins for rhizogenesis in patchouli which is in contrary to the present observation. This suggests that although the addition of auxins is beneficial for rooting, their use is not essential in patchouli. The similar result is reported in *Ulmus* species (Paula et al. 2008).

Table 3. Effect of various concentrations IAA, NAA and AC on rooting of proliferated shoots of patchouli cultured on MS.

MS (strength) + auxin (mg/l)	Root induction (%)	Mean number of roots/shoots (cm)	Mean root length (cm)
MS (½)	91.01	13.00	5.40
MS (½) + IAA (0.5)	71.31	12.10	5.13
MS (½) + IAA (1.0)	64.33	13.66	5.16
MS (½) + NAA (0.5)	65.66	14.06	5.43
MS (½) + NAA (1.0)	61.00	13.78	5.66
MS (½) + AC (100)	93.33	15.23	6.23
MS (½) + AC (200)	92.66	15.00	6.10
MS	82.12	11.20	5.10
MS + IAA (0.5)	51.00	11.66	5.14
MS + IAA (1.0)	67.66	12.12	5.23
MS + NAA (0.5)	63.33	12.00	5.45
MS + NAA (1.0)	55.33	12.01	5.50
MS + AC (100)	90.66	13.21	5.76
MS + AC (200)	85.00	13.00	5.80
F value	664.43*	56.51*	60.23*
SEM	0.34	0.35	0.04
CD	1.00	1.13	0.12

* Significant at 5% level.

This will add to the cost effectiveness of the protocol used for micro-propagation of patchouli. After four weeks, 89% of *in vitro* derived plants were directly acclimated (Fig. 1e). This is comparable with the results of *in vitro* rooting. Present results lead to the conclusion that formation of *in vitro* roots prior to acclimatization is not needed. The same conclusion is reported by Paula et al. (2008) and Zong and Nian (1995) that *in vitro* rooting could be eliminated to reduce time and cost.

In vitro raised plantlets were transplanted to soilrite in net pots (Fig. 1d). During the early hardening phase, maintenance of 80% relative humidity in the chamber showed 91% plantlets survival. After four weeks of hardening, the plantlets were transferred to pots filled with sand : soil : manure (2 : 1 : 1) under shade. Gradual transfer of the established plants to the sunlight was ideal for tissue culture derived patchouli plants in the field (Fig. 1f) rather than a direct

transfer to sunlight, which caused wilting of plants and charring of leaves. Similar observations are recorded by Meena (1996).

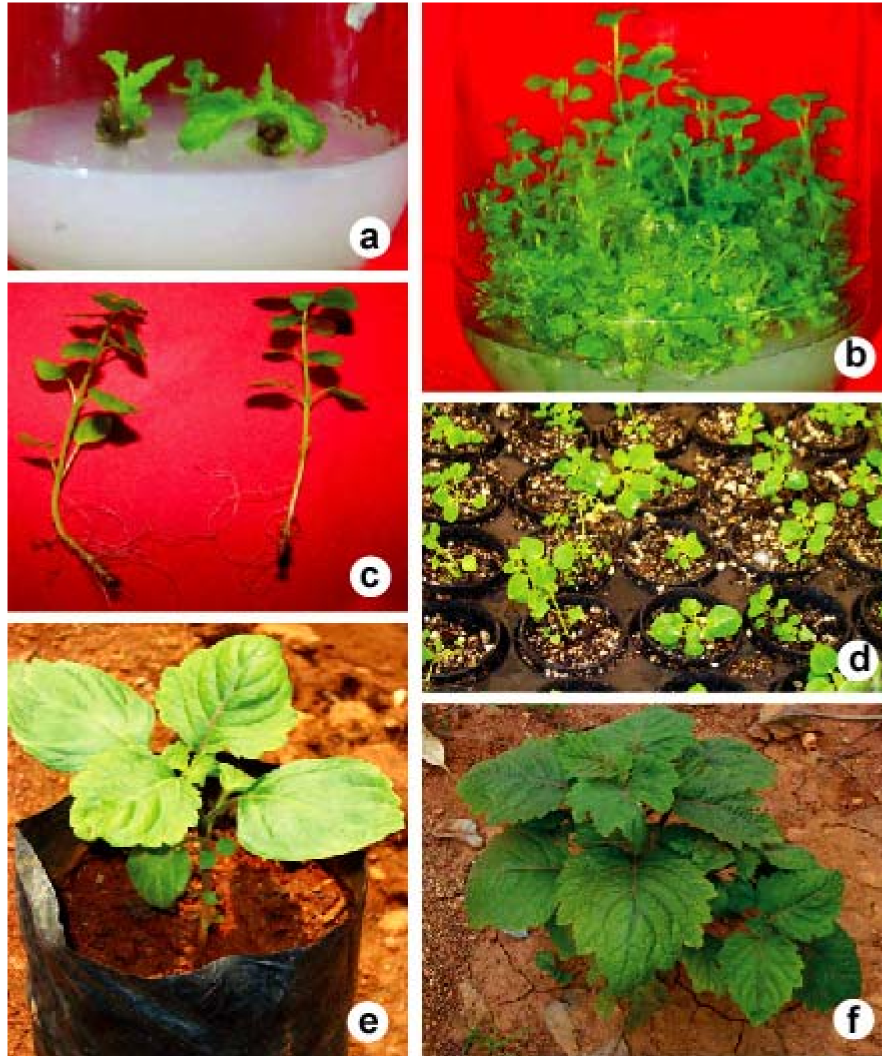

Fig. 1. *In vitro* regeneration of *Pogostemon cablin* from nodal explants. a. Induction of shoots in four weeks of culture on MS + 0.5 mg/l BAP. b. Multiple shoot regeneration from nodal segments on MS + 0.5 mg/l BAP and 0.5 mg/l Kn. c. Rooting of shoots on MS (half) + activated charcoal (100 mg/l). d. *In vitro* raised plantlets transplanted to soilrite in net pots. e. Directly acclimated plantlet in the soil. f. *In vitro* regenerated plant in the field.

All the regenerated plants grown for four months were similar in leaf morphology, plant height and number of branches per plant. The patchouli oils were extracted from the leaves of *in vitro* grown plants and their GC patterns were compared. There were no differences in the chemical composition among

the regenerants with all sequiterpenes present in the oil. The oil yield was found to be 0.30% (v/w) of fresh weight. Patchouli alcohol at 30.31% was found to be the predominant component in the oil. Plantlets were also compared with those of the mother plants. No differences were observed in the oil composition. This confirms the true to type nature of the micropropagated plants.

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