African Journal of Biotechnology Vol. 9(14), pp. 2069-2075, 5 April, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

In vitro multiplication of *Pogostemon cablin* Benth. through direct regeneration

M. Kumara Swamy[1,2], S. Balasubramanya[2] and M. Anuradha[2]*

[1]Padmashree Institute of Management and Sciences, Kommagatta campus, Kengeri, Bangalore- 560060.
[2]Rishi Foundation, #234, 10th C main, 1st Block, Jayanagar, Bangalore- 560011.

Accepted 14 January, 2010

An efficient method was developed to initiate multiple shoots from nodal explants of *Pogostemon cablin* Benth. MS medium supplemented with 0.5 mg/L BA initiated a mean of 45.66 shoots/nodal explant. Within 4 weeks of initiation, regenerated multiple shoots attained a height of 3.6 cm. Subsequent transfer of these *in vitro* derived nodal segments onto MS medium supplemented with BA and KN (0.5 mg/L), induced mean of 62.45 shoots. Higher concentration of either BA 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 medium supplemented with 100mg/L activated charcoal. Rooted shoots, following acclimatization in green house, 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.

Key words: Growth regulators, regeneration, activated charcoal, patchouli, organogenesis, callus, gas chromatography.

INTRODUCTION

Patchouli (*Pogostemon cablin* Benth.), belonging to the family Lamiaceae is an aromatic plant, native to tropical Asia and is 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, anti-fungal 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 (Bowel et al., 2002). Fibrinolytic and anti thrombotic (Sumi, 2003; Eunkyung et al., 2002) activity of this essential oil is also been reported.

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 VasanthKumar, 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 compliment breeding programmes.

Several 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 (Misra, 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

*Corresponding author. Email: swamy.bio@gmail.com.

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

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

Medium + concentration of cytokinins (mg/L)	Mean number of shoots/explants ± SD	Mean length of the shoots (cm) ± SD	Mean fresh weight of the shoots (g) ± SD	Callus formation
MS (CONTROL)	13.62 ± 1.05	1.23 ± 1.52	1.06 ± 1.39	-
MS + 0.25 mg/L BA	36.66 ± 1.81	1.73 ± 1.71	1.42 ± 2.02	-
MS + 0.5 mg/L BA	45.66 ± 0.71	3.60 ± 2.08	2.94 ± 2.01	-
MS + 1.0 mg/L BA	20.00 ± 1.53	1.65 ± 1.72	1.97 ± 1.84	+
MS + 2.0 mg/L BA	00.00	0.00	0.00	+
MS + 0.25 mg/L KN	23.33 ± 0.35	1.93 ± 1.90	1.08 ± 1.98	-
MS + 0.5 mg/L KN	34.56 ± 0.38	2.43 ± 2.10	2.87 ± 1.23	-
MS + 1.0 mg/L KN	21.53 ± 0.98	2.23 ± 1.21	2.44 ± 1.31	-
MS + 2.0 mg/L KN	00.00	0.00	0.00	+
F- value	*	*	*	
CD	8.46	0.04	0.04	

* Significant at 5% level. +: Callus induction. -: No callus.

cultivation by providing true to type plants in large numbers. Hence the present study is aimed to establish suitable protocol for rapid regeneration of patchouli by direct organogenesis using nodal explants. Also the essential oil from mother plant and *in vitro* grown plant was analyzed by using gas chromatography to check the quality of the oil obtained. 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 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 3 - 4 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 (Murashige and Skoog, 1962) medium, containing 2% (w/v) sucrose, supplemented with different concentrations and combinations of BA (0.25, 0.5, 1.0 and 2.0 mg/L) and KN (0.25, 0.5 and 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 121°C, 15 lb pressure for 15 min. All the cultures were incubated at 25 ± 2°C under a 16 h light and 8 h 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 sub cultured 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 4 - 5 internodes were excised and cultured on freshly prepared rooting medium containing half strength or full strength MS medium supplemented with different concentrations of activated charcoal (100 and 200 mg/L), IAA and NAA (0.5 and 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 4 weeks in a moisture saturated chamber with 80% relative humidity. Hardened plants 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. 20 cultures were raised for each treatment. Data recorded after 30 days of culture were subjected to Fisher's method of analysis of variance. Wherever, the 'F' test was significant for comparison of treatment means, C. D value was worked out at probability level of 5%.

Fresh leaves of *P. cablin* after 4 months were hydrodistilled by a Clevenger-type apparatus. The essential oil was collected and stored at 4°C until being analyzed 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 - 220°C at 5°C /min, held at 120°C for 10 min. Injector and detector temperatures were 250 and 280°C. Carrier gas was helium at 16 psi. 1 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 BA and KN (0.25, 0.5 and 1.0 mg/L) separately or in combinations. Comparatively, BA 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), BA is most effective in enhancing shoot multiplication and triggering shoot elongation. The use of MS medium supplemented with 0.5 mg/L BA was best suitable for bud break (Figure 1a) and resulted maximum number of shoots/explant (45.66 ± 0.71); higher shoot length (3.60 ± 2.08 cm) and fresh

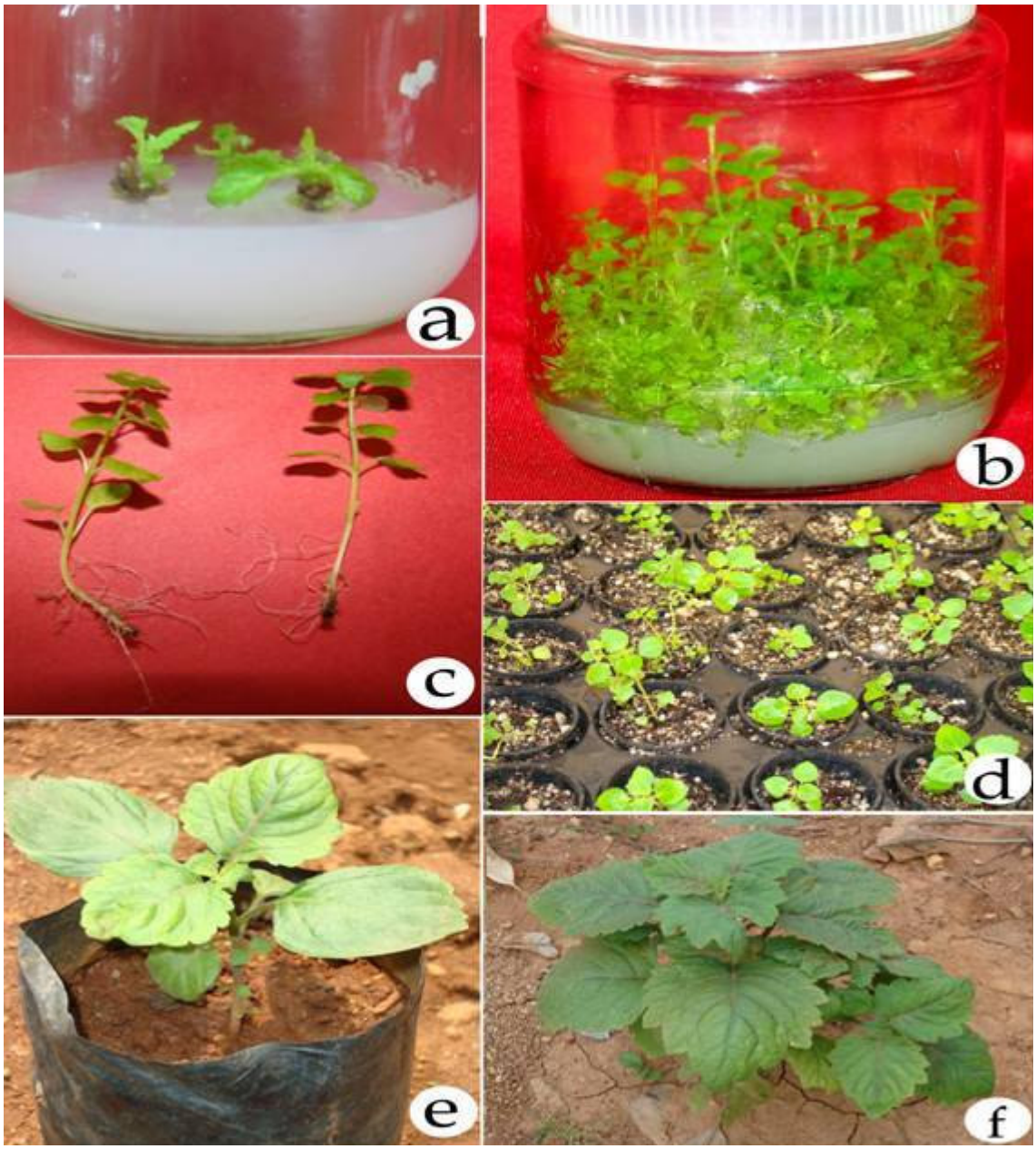

Figure 1. *In vitro* regeneration of *Pogostemon cablin* Benth. from nodal explants. **a.** Induction of shoots in 4 weeks of culture on MS + 0.5 mg/l BA. **b.** Multiple shoot regeneration from nodal segments on MS + 0.5 mg/l BA and 0.5 mg/l KN. **c.** Rooting of shoots on MS (½) + Activated charcoal (100 mg/l). **d.** *In vitro* raised plantlets transplanted to soil rite in net pots. **e.** Directly acclimatized plantlet in the soil. **f.** *In vitro* regenerated plant in the field.

weight of the shoot (2.94 ± 2.01 g) without callus induction. The result is in accordance 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 BA) for maximum multiple shoot regeneration in patchouli. In our study, higher concentrations of cytokinins resulted in callus formation. However, BA at 0.5 mg/L when used for subsequent sub cultures resulted in callus, indicating the elevation of endogenous hormonal levels. Hence though initiation was made on medium supplemented with 0.5 mg/L BA, multiplication is better evidenced on 0.25 mg/L BA (Figure 2). 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 BA 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.

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

Cytokinins (mg/L)		Mean Shoot length (cm) ± SD	Number of multiple shoots ± SD	Fresh weight of the shoots (g) ± SD	Callus formation
BA	KN				
0.0	0.0	0.21 ± 1.3	13.14 ± 0.4	1.10 ± 1.9	-
0.25	0.25	1.95 ± 1.8	62.66 ±0.7	2.46 ± 1.7	-
0.5	0.25	4.65 ± 1.4	61.33 ± 1.0	4.67 ± 1.6	-
1.0	0.25	1.66 ±0.9	22.66 ± 1.1	1.86 ±1.7	+
0.25	0.5	3.31 ± 1.1	41.66 ± 0.8	3.06 ± 1.2	-
0.5	0.5	5.20 ± 1.7	62.45 ± 0.6	5.07 ± 0.9	-
1.0	0.5	2.05 ± 1.7	21.37 ± 0.4	2.35 ± 1.4	+
0.25	1.0	2.32 ± 0.8	23.00 ± 1.2	2.96 ± 1.4	-
0.5	1.0	2.67 ± 1.4	42.10 ± 1.5	4.14 ± 1.3	-
1.0	1.0	1.92 ± 1.2	21.33 ± 0.8	2.35 ± 1.1	+
F- value		*	*	*	
CD		0.03	2.93	0.03	

* Significant at 5% level. +: Callus induction. -: No callus.

Shoot proliferation was satisfactory on MS medium supplemented with 0.25 mg/L BA and 0.5 mg/L KN separately. However, kinetin 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 BA along with 0.5 mg/L KN) was found to exhibit highest frequency of shoot multiplication (62.45 ± 0.6%). The highest mean shoot length (5.20 ± 1.7 cm) and mean fresh weight of the shoot (5.07 ± 0.9 g) was also evidenced in the same treatment (Table 2). The efficacy of BA over KN, when used singly and in combinations 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). Superior effect of the combination of BA 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 BA 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 biomass (Figure 1b).

The effect of the strength of MS basal media, MS media with activated charcoal, IAA and NAA at different concentrations on rhizogenesis was studied (Table 3). Among the treatments tried, ½ strength MS medium is enough to get better rooting. This is in conformity with the results obtained by Bharati (2002) in patchouli. However, we could able to induce high frequency of rooting (93.33 ± 0.9%) when shoots implanted on ½ strength MS media with activated charcoal with 100 mg/l activated charcoal. Mean number of roots/shoot (15.23 ± 0.6) and root length (6.23 ± 1.8 cm) was found to be superior among all other treatments (Figure 1c). This is the first report of its kind in patchouli. Activated charcoal is an anti-oxidant 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 medium 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 Misra (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 report is published in *Ulmus* species (Conde et al., 2008). After 4 weeks, 89% of *in vitro* derived plants were directly acclimatized (Figure 1e) suggesting that the formation of *in vitro* roots prior to acclimatization is not needed and this can reduce time and cost. The similar observations are reported by Conde et al. (2008) and Cheng and Shi (1995).

In vitro raised plantlets were transplanted to the soil in net pots (Figure 1d). During the early hardening phase, maintenance of 80% relative humidity in the chamber showed 91% plantlet survival. After 4 weeks of hardening, the plantlets were transferred to pots filled with sand: soil: manure (2:1:1) under shade (Figure 1e). Gradual transfer of the established plants to the sunlight was ideal for tissue culture derived patchouli plants in the field (Figure 1f) rather than a direct transfer to sunlight, which caused wilting of plants and charring of leaves. Similar observations are recorded by Misra (1996).

All the regenerated plants grown for 4 months were similar in leaf morphology, plant height and number of branches per plant. The essential oils were extracted

Table 3. Effect of various concentrations IAA, NAA and activated charcoal on rooting of proliferated shoots of patchouli.

Medium (strength) + Auxin (mg/L)	Root induction (%) ± SD	Mean number of roots/shoot (cm) ± SD	Mean root length (cm) ±SD
MS (½)	91.01 ± 1.0	13.00 ± 1.2	5.40 ± 1.9
MS (½) + IAA (0.5)	71.31 ± 1.0	12.10 ± 0.8	5.13 ±1.8
MS (½) + IAA (1.0)	64.33 ± 0.9	13.66 ± 0.5	5.16 ± 1.4
MS (½) + NAA (0.5)	65.66 ± 1.6	14.06 ± 0.3	5.43 ± 0.9
MS (½) + NAA (1.0)	61.00 ± 2.1	13.78 ± 1.3	5.66 ± 1.5
MS (½) + Activated charcoal (100)	93.33 ± 0.9	15.23± 0.6	6.23 ± 1.8
MS (½) + Activated charcoal (200)	92.66 ± 1.0	15.00 ± 0.7	6.10 ± 1.5
MS	82.12 ± 0.8	11.20 ± 0.8	5.10 ± 1.4
MS + IAA (0.5)	51.00 ± 1.0	11.66 ± 1.1	5.14 ± 1.3
MS + IAA (1.0)	67.66 ±1.7	12.12 ±1.4	5.23 ± 1.8
MS + NAA (0.5)	63.33 ± 0.9	12.00 ± 1.6	5.45 ± 0.9
MS + NAA (1.0)	55.33 ± 1.2	12.01 ± 0.8	5.50 ± 1.5
MS + Activated charcoal (100)	90.66 ± 1.2	13.21 ± 0.6	5.76 ±0.9
MS + Activated charcoal (200)	85.00 ± 1.0	13.00 ± 0.5	5.80 ± 0.8
F-value	*	*	*
CD	1.00	1.13	0.12

* Significant at 5% level.

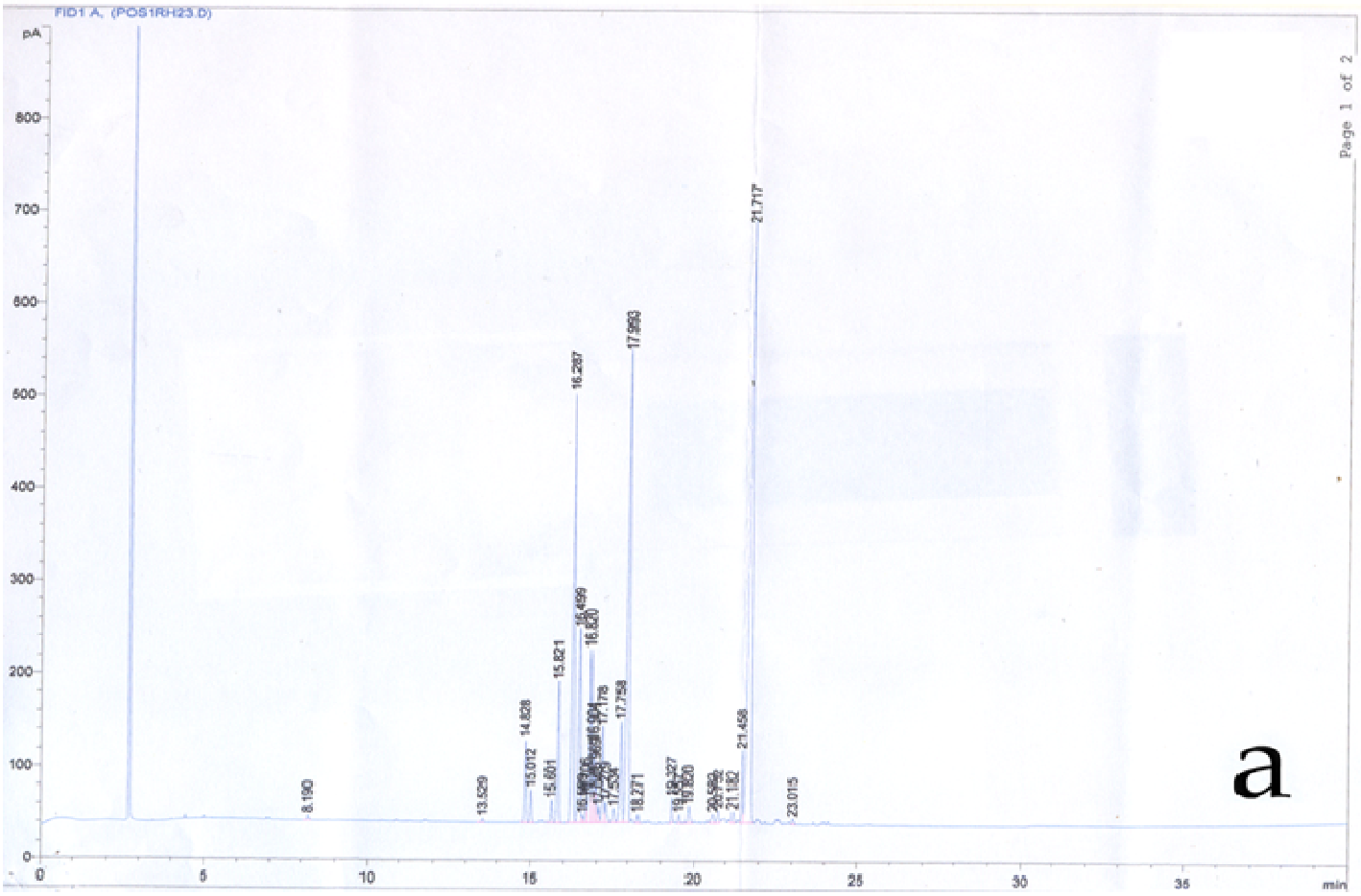

Figure 2. Gas chromatographic profiles of essential oils extracted from **(a)** the leaves of mother plant and **(b)** *in vitro* derived regenerants.

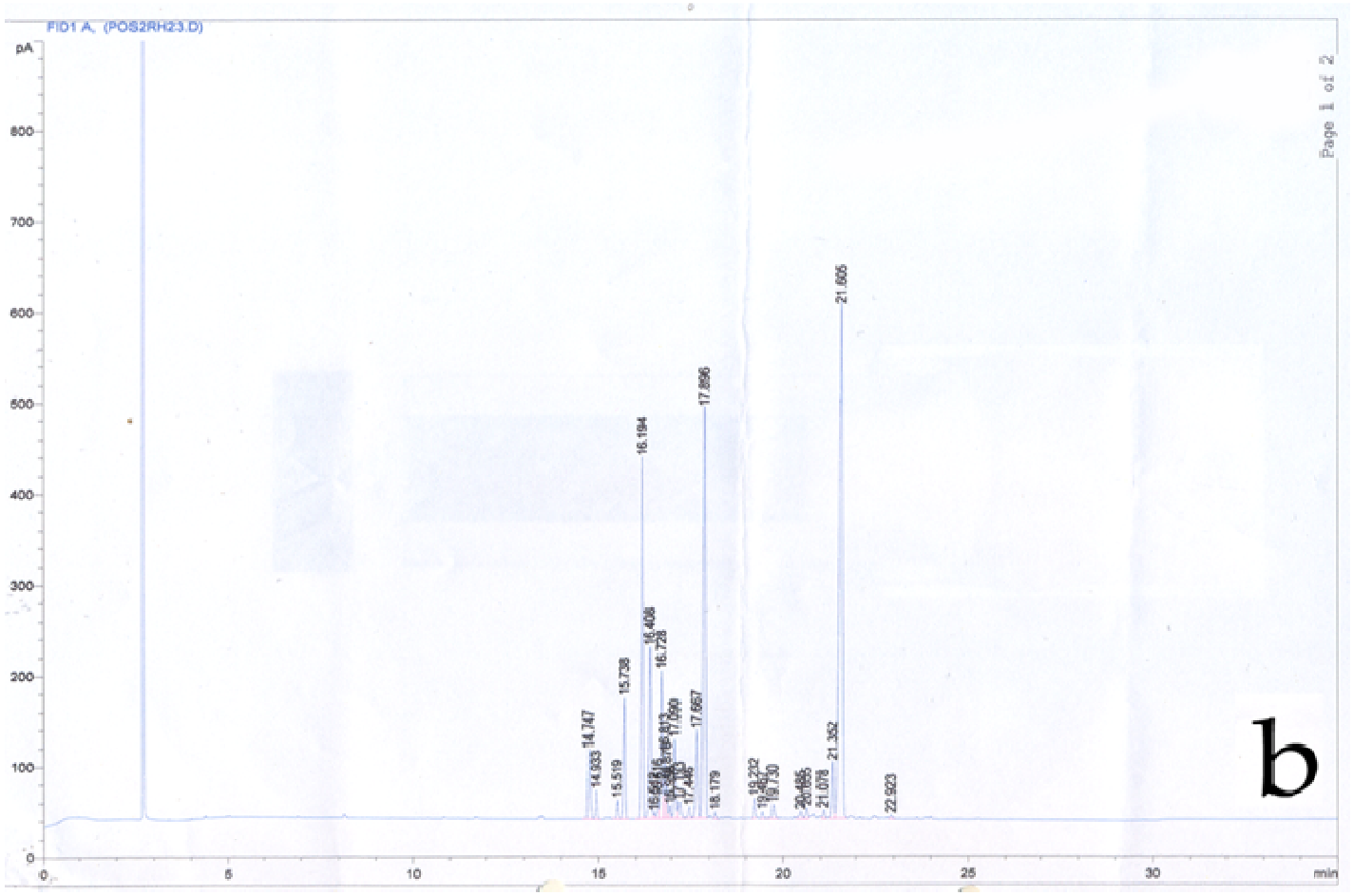

Figure 2. Continued.

from the leaves of *in vitro* grown plants and mother plant. The oil yield was found to be 0.30% (v/w) of fresh weight. The essential oils were analyzed by using GC and their patterns were compared. Patchouli alcohol at 30.31% was found to be the predominant component in the oil. The uniform pattern of essential oils in the GC profile of the regenerants and the mother plant (Figures 2a and b) suggests the feasibility of the protocol.

In conclusion, the above protocol describes an efficient protocol for rapid multiplication of patchouli by direct regeneration, which is preferred for generating true-to-type plants over callus regeneration. The present protocol can ensure a stable supply of this commercial crop irrespective of seasonal variations and thus meet the global demand for its essential oil.

REFERENCES

Bharati N (2002). Biotechnology in commercial production of patchouli in North Eastern Region. In: Patchouli; National Workshop on Commercialization of patchouli in North Eastern region held at Guwahati, Assam. NEDFC and NHB, pp. 46 -51.

Bowel EJ, Griffiths DM, Quirk L, Brownriggs A, Croot K (2002). Effect of essential oils and touch on resistance to nursing care procedures and other dementia-related behaviours in a residential care facility. Int. J. Aromather. 12: 22-29.

Cheng ZM, Shi NQ (1995). Micropropagation of mature Siberian elm in two steps. Plant Cell Tissue Organ Cult. 41(2): 197-199.

Conde P, Sousa A, Costa A, Santos C (2008). A protocol for *Ulmus minor* Mill. Micropropagation and acclimatization. Plant Cell Tissue Organ. Cult. 92(1): 113-119.

Dias MC, Nickell GL (2002). Rapid multiplication of *Lavandula viridis* L through *in vitro* axillary shoots proliferation. Plant Cell Tissue Organ Cult. 68: 99-102.

Eunkyung P, Kyung YH, Hyun KD (2002). Antithrombotic activity of Sunghyangjunggisan. Nat. Prod. Sci. 8(2): 71-75.

Figueiredo SFL (2001). Micro propagation of *Rollinia mucosa* (Jacq.) Baill. *In Vitro* Cell. Dev. Biol. Plant. 37: 4 71-475.

George EF, Hall MA, Klerk GJD (2008). Plant propagation by Tissue culture: Volume 1. The background. Third Edition, Springer Publisher: Dordrecht; London.

Hasegawa Y, Tajima K, Toi N, Sugimura Y (1992). An additional constituent occurring in the oil from a patchouli cultivar. Flav. Fragr. J. 7: 333-335.

Hembrom, Emanuel M, Martin KP, Patchathundikandi, Suneesh K, Madasser Y, Joseph (2006). Rapid *in vitro* production of true to type plants of *Pogostemon heyneanus* through dedifferentiated axillary buds. *In vitro*. Cell. Dev. Biol. Plant. 42(3): 283-286.

Hirata T, Kukreja AK (1990). Volatile monoterpenoids constituents of the plantlets of *Mentha spicata* produced by shoot tip culture. Phytochemistry, 29: 955-959.

Kageyama Y, Honda Y, Sugimura Y (1995). Plant regeneration from patchouli protoplasts encapsulated in alginate beads. Plant Cell Tissue

Organ Cult. 41(1): 65-70.
Kukreja AK, Mathur AK, Zaim M (1990). Mass production of virus free patchouli plants [*Pogostemon cablin* (Blanco) Benth.] by *in vitro* culture. Trop. Agrc. 67: 101-104.
Maheswari ML, Vasantha Kumar T, Neelam Sharma, Chandel KPS (1993). Patchouli- An Indian perspective. Indian Perf. 37: 9-11.
Misra M (1996). Regeneration of Patchouli (*Pogostemon cablin* Benth.) plants from leaf and node callus and evaluation after growth in the field. Plant Cell Rep. 15: 991-994.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Obul RB, Giridhar P, Ravishankar GA (2001). *In vitro* rooting of *Decalepis hamiltonii*. Wight and Arn, an endangered shrub, by auxins and root promoting agents. Curr. sci. 81(11): 26-29.
Padilla MG, Encina CL (2004). Micro propagation of adult Cherimoya (*Annona cherimoya*). *In vitro* Cell. Dev. Biol. Plant. 40(2): 210-214.
Padmanabhan C, Sukumar S, Sreerangaswamy SR (1981). Patchouli plants differentiated *in vitro* from stem tip and callus cultures. Curr. Sci. 50: 195-197.
Pattnaik S, Subramanyam VR, Kole C (1996). Antibacterial and antifungal activity of ten essential oils *in vitro*. Microbios. 86: 237-246.
Pawar PK (2002). A technique for rapid propagation of *Solanum surrattense* Burm. F. Indian. J. Biotechnol. 1: 201-204.
Rajan GB, Shakila A, Rajasekaran LR (1997). Mass propagation of *Pogostemon patchouli* through somatic embryogenesis. South Indian Hort. 45: 45-49.
Reddy PS, Rodrigues R, Rajasekharan R (2001). Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. Plant Cell Tissue Organ Cult. 66: 183-188
Sastri KS, Vasantha Kumar T (1981). Yellow mosaic of patchouli (*Pogostemon patchouli*) in India. Curr. Sci. 50: 767-768.
Sumi H (2003). Fibrinolysis-accelerating activity of the essential oils and Shochu aroma. Aroma Res. 4(3): 264-267.
Yang D (1996). Michel, Mandin andriamboavonjy, Poitry, Chaumont, Mellet C Antifungal and antibacterial properties *in vitro* of three patchouli oils from different origins. Acta Botanica. Gallica. 143(1): 29-35.