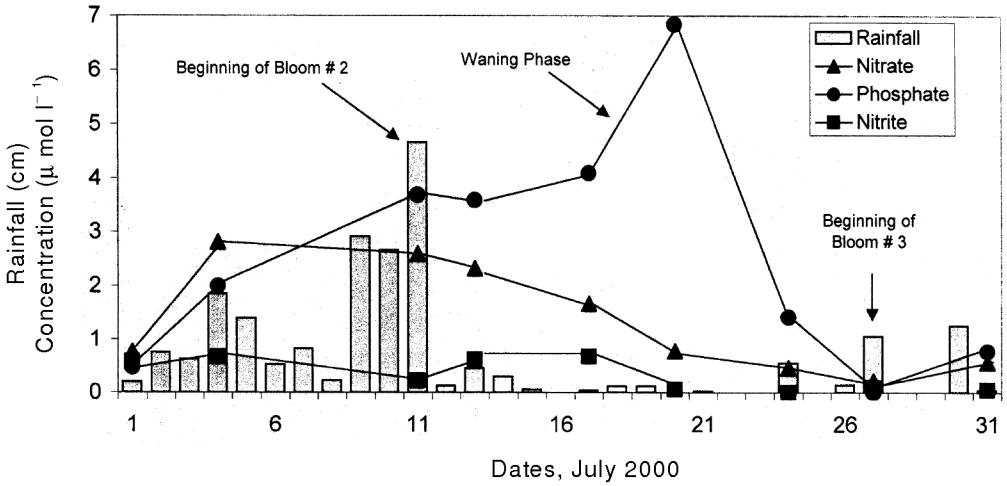

Figure 4. Rainfall and nutrient data for July 2000.

repetitive, permitting an unambiguous progression in the monitoring programme.

To our knowledge, this is the first technical account of plankton blooms from the Andamans. The *Noctiluca* bloom observed in this work was somewhat unique, because it appeared thrice in a time-period of two months. The precise cause for the blooms remains unclear; nevertheless the data in this work suggest that the origin of *Noctiluca* blooms was not always concurrent with nutrient inputs into Port Blair Bay.

1. Prasad, R. R., *Proc. Indian Acad. Sci.*, 1953, **31**, 41.
2. Subrahmanyan, R., *Proc. Indian Acad. Sci.*, 1954, **B39**, 118.
3. Katti, R. J., Gupta, T. R. C. and Shetty, H. P. C., *Curr. Sci.*, 1988, **57**, 380–381.
4. Sargunam, C. A., Rao, V. N. R. and Nair, K. V. K., *Indian J. Mar. Sci.*, 1989, **18**, 289–290.
5. Naqvi, S. W. A., George, M. D., Narvekar, P. V., Jayakumar, M. S., Shailaja, M. S., Sardesai, S. Sarma, V. V., Shenoy, D. M., Naik, H., Maheshwaran, P. A., Krishnakumari, K. Rajesh, G., Sudhir, A. K. and Binu, M. S., *Curr. Sci.*, 1998, **75**, 543.
6. Grasshoff, K., Ehrdart, K. and Kremling, M., *Methods of Seawater Analysis*, Verlag Chemie, Vienheim, 1983.
7. Strickland, J. D. H. and Parsons, T. R., *A Practical Handbook of Seawater Analysis*, Fisheries Research Board, Canada, Ottawa, 1972.
8. Elbrächter, M. and Qi, Y-Z., in *Physiological Ecology of Harmful Algal Blooms* (ed. Anderson, M.), Springer Verlag, Berlin, 1998, pp. 315–335.
9. Desai, B. N., Ram, M. J., Abidi, S. A. H. and Nair, R. *Mahasagar*, 1984, **17**, 79.
10. Kirchner, M., Sahling, G., Uhlig, G., Gunkel, W. and Klings, K. W., *Sarsia*, 1996, **81**, 45–55.
11. Montani, S., Pithakpol, S. and Tada, K., *J. Mar. Biotechnol.*, 1998, **6**, 224–228.

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In vitro micropropagation of *Lippia alba*

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The study has given a method for rapid multiplication of *Lippia alba* cv. Kavach, the perennial shrub that protects soil erosion when grown on slopes and whose leaves yield a linalool-rich essential oil. Multiple shoots were induced *in vitro* from shoot tips of *L. alba* on Murashige and Skoog (MS) medium containing 2 μg/ml 6-benzyl adenine. The stem nodal segments derived from *in vitro*-grown shoots also gave multiple shoots on the medium of the same composition. The shoots readily rooted upon transfer to basal MS medium. The rooted *in vitro* raised plants established well on soil following acclimatization. The essential oil profiles and morphology of the micropropagated plants were identical to the normal vegetatively propagated plants.

THE genus *Lippia* belongs to family Verbenaceae and consists of nearly 200 species of herbs, shrubs and small trees widely distributed in tropical to semitemperate areas of the American, African and Asian continent. *Lippia alba* and some other species have been reported to be used in traditional medicine[1] and pest control in food grains. Plant extracts from *L. alba* have also been reported to possess cytostatic properties[2]. Medicinal and cytostatic properties of this herb may primarily be

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Figure 1. *In vitro* multiplication of *Lippia alba* var. Kavach. ***a***, Explant, 4 days after culture on MS medium containing 2.0 mg/l BA; ***b***, Multiple shoots, 2 weeks after culture initiation on MS basal medium containing 2.0 mg/l BA; ***c***, Multiple shoots, 4 weeks after culture initiation on MS basal medium containing 2.0 mg/l BA; ***d***, Isolated shoots, 20 days after transfer on MS basal medium for root initiation; ***e***, Rooted shoots of *L. alba*, 20 days after root initiation on MS basal medium; ***f***, Plantlets of *L. alba,* 3 weeks after transfer to soil in glasshouse.

attributed to various components of its essential oil. The essential oil of *L. alba* has been recognized as a potential source of several commercially important terpenoid compounds. Certain *L. alba* clones have been reported to yield essential oil rich in linalool and terpenoids such as citral and cineole[3]. *L. alba* cv. Kavach has been recommended for growing on slopes of ravines, railway tracks and road embankments for protection against soil erosion and as a resource of linalool-rich essential oil. An efficient micropropagation system is required in

Table 1. Effect of type and concentration of plant growth regulators on *in vitro* shoot proliferation from nodal explants of *Lippia alba* after four weeks of culture

Phytohormone concentration (mg/l)			Per cent explant response (mean ± SE)	Number of shoots per explant (mean ± SE)	Shoot length (cm) (mean ± SE)
BA	Kinetin	NAA			
0.5	–	–	86.7 ± 8.8	1.5 ± 0.2	6.3 ± 0.4
1.0	–	–	93.3 ± 1.7	2.2 ± 0.2	7.3 ± 0.3
2.0	–	–	93.3 ± 1.7	6.1 ± 0.5	7.1 ± 0.3
5.0	–	–	96.7 ± 1.7	6.4 ± 0.7	3.3 ± 0.3
–	–	0.5	91.6 ± 4.4	1.1 ± 0.1	3.0 ± 0.2
2.0	–	0.1	78.3 ± 1.7	1.4 ± 0.1	2.7 ± 0.1
2.0	–	0.5	80.0 ± 2.9	1.3 ± 0.1	2.7 ± 0.2
2.0	–	1.0	85.0 ± 2.9	1.4 ± 0.1	3.1 ± 0.2
–	2.0	–	76.7 ± 4.0	1.2 ± 0.1	2.4 ± 0.1
2.0	1.0	–	76.7 ± 4.0	1.7 ± 0.2	2.4 ± 0.1
2.0	2.0	–	68.3 ± 4.1	1.6 ± 0.2	2.3 ± 0.1

Table 2. Effect of NAA on rooting of *in vitro*-derived shoots of *Lippia alba* after 20 days of culture

Effect of medium composition on root induction	
Medium	Rooting (%)
MSO	100
+0.1 NAA	85
+0.5 NAA	10
1/2 MS	90
+0.1 NAA	75
+0.5 NAA	20

Table 3. Main constituents of control versus tissue culture-raised plants in *Lippia alba*

	Concentration (% ± SE)	
Constituent	Control plant	Tissue culture raised plant
1,8-Cineole	3.4 ± 0.3	3.5 ± 0.4
γ-Terpinine	0.8 ± 0.4	0.8 ± 0.1
Linalool oxide	0.4 ± 0.1	0.5 ± 0.1
Linalool	72.2 ± 3.5	69.0 ± 2.2
α-Terpineol	1.4 ± 0.2	1.4 ± 0.1
Geranyl acetate	6.1 ± 0.2	6.1 ± 0.2
Geraniol	2.3 ± 0.3	2.5 ± 0.2

L. alba cv. Kavach to augment the existing process of clonal propagation for rapid multiplication of its propagules. The present work was primarily aimed at development of an efficient micropropagation system for *L. alba*, as such a procedure does not appear to have been standardized.

Shoot tips of *L. alba* cv. Kavach were obtained from CIMAP farm, Lucknow. They were thoroughly washed with tap water and surface disinfected by treating them for 5 min with 0.1% w/v mercuric chloride solution. After five rinses with sterile distilled water, the stem pieces were placed in 150 ml flasks containing MS basal medium supplemented with 3% w/v sucrose and 0.7% w/v agar (Qualigen, India) to obtain aseptic cultures. The pH of all media was adjusted to 5.8 before autoclaving for 20 min at 121°C and 1.06 kg/cm^2 pressure. Cultures were incubated at 25 ± 2°C under light 60 μmol/m^2/s PAR provided by cool white and fluorescent tubes. The shoots obtained served as resource for obtaining further explants.

Leaf discs (1 cm^2) and stem internode and node-bearing segments of about 1 cm size excised from aseptically maintained shoots obtained as above were used as explants for further *in vitro* studies. MS[4] basal medium solidified with 0.7% w/v agar (Qualigens, India) was supplemented with one of the following phytohormone(s) recipes: (i) 0.5 mg/l BA, (ii) 1.0 mg/l BA, (iii) 2.0 mg/l BA, (iv) 5.0 mg/l BA, (v) 0.5 mg/l NAA, (vi) 2.0 mg/l BA and 0.1 mg/l NAA, (vii) 2.0 mg/l BA and 0.5 mg/l NAA, (viii) 2.0 mg/l BA and 1.0 mg/l NAA, (ix) 2.0 mg/l kinetin, (x) 2.0 mg/l BA and 1.0 mg/l kinetin, (xi) 2.0 mg/l BA and kinetin. The pH of the medium was adjusted to 5.8 and dispensed into the 150 ml conical flasks (Borosil, India) before autoclaving at 1.06 kg/cm^2 for 20 min.

The inoculated cultures were transferred to the culture room set at 25 ± 2°C, 60 μmol/m^2/s PAR (provided by cool white and fluorescent tubes) and 16 h photoperiod. The cultures were observed weekly for shoot initiation.

The shoots initiated on the nodal explants were excised after 4–6 weeks of culture initiation and transferred to MS basal medium containing 3% w/v sucrose and 0.7% w/v agar for elongation and root induction for three weeks. The rooted plantlets were transferred to sterile hydroponic Hoagland's solution and incubated for 2 weeks in the laboratory under 16 h photoperiod (60 μmol/m^2/s PAR) at 25 ± 2°C. These were later transferred to water-saturated gravel and maintained in light (60 μmol/m^2/s PAR) at 25 ± 2°C for two more

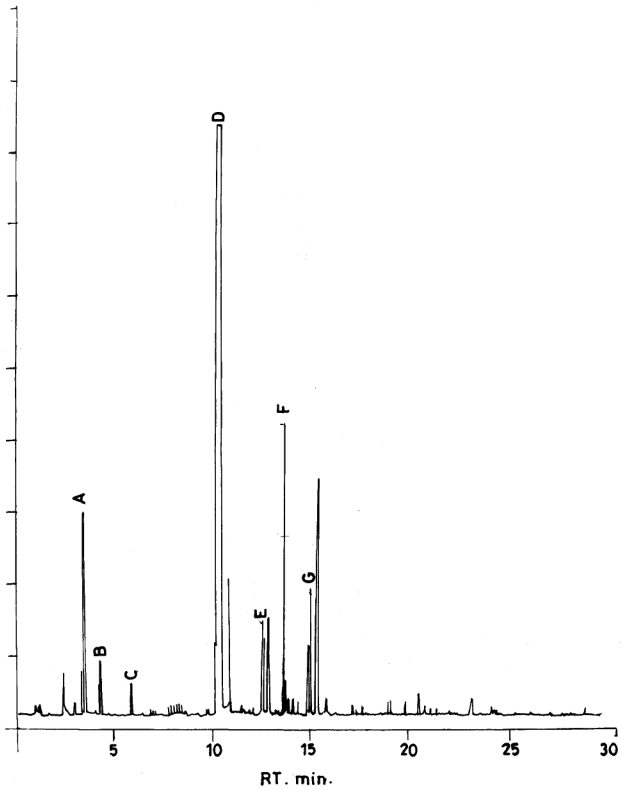

Figure 2. Gas liquid chromatogram of essential oil hydro-distilled from tissue culture-raised plants of *Lippia alba* cv. Kavach. The main constituents identified were: A, 1,8-cineole (RT: 3.30); B, γ-terpinine (RT: 4.19); C, linalool oxide (RT: 5.55); D, linalool (RT: 10.32); E, α-terpeneol (RT: 12.41); F, geranyl acetate (RT: 13.41); G, geraniol (RT: 15.03), where RT was in min

weeks for root proliferation, before transplanting into potted soil in a glasshouse.

Essential oil from five samples from each of *in vitro*-raised plants and glasshouse-grown control plants were extracted by hydro-distillation of shoots. The essential oil was resolved on Varian Cx3400 GLC with flame ionization detector, using Supelco Wax-10 column (30 m × 0.3 mm) packed with fused silica. Initial temperature of the column was 80°C, which was increased at the rate of 5°C/min to a final temperature of 225°C. Flow rate of hydrogen gas was 1 ml/min and the temperatures of injector and detector were 200°C and 220°C, respectively. The main constituents were identified on the basis of their retention time (RT).

Out of the three types of explants, only the stem nodal segments gave shoot proliferation when cultured on MS basal medium supplemented with 0.5 mg/l or higher concentration of BA. On the medium used, both leaf discs and stem internodes failed to regenerate shoots. The dark-brown calli were observed at the cut edges of leaf discs and stem internode explants, after 2–3 weeks of culture initiation on the medium supplemented with NAA with or without BA (Table 1). BA alone, at the concentration 2.0 mg/l or above, induced shoot proliferation from nodal explants. The concentration of BA in the medium affected the number of shoots produced and their growth. Number of shoots proliferated per explant increased with the concentration of BA in the medium (Table 1, Figure 1). Increased number of shoots (1.5 to 6.4) with the enhanced levels of BA (from 0.5 to 5.0 mg/l), confirm the results obtained for *L. junelliana*[5]; in the present material best results were obtained with 2 mg/l of BA supplementation to the medium. However in contrast, addition of an auxin (NAA) along with BA did not improve the shoot proliferation, but favoured callusing at the lower end of the nodal stem explants. Growth of the shoots proliferated in the presence of higher concentrations of BA (at 5.0 mg/l or above) was stunted abruptly.

Kinetin alone or in combination with BA was not effective for *in vitro* shoot proliferation. Instead of multiple shoots, a fast-growing callus was seen at the base of the explants. Kinetin has been reported as an ineffective cytokinin for shoot proliferation in some other cases also[6,7], while the combination of kinetin and BA has been reported to show a synergistic effect for *in vitro* shoot proliferation in some other plant species[8–10].

After four weeks of culture, the regenerated shoots were taken out and transferred to the rooting medium. Root induction could be seen within 3–5 days and prolific roots were initiated within a period of two weeks on MS basal medium with or without 0.1 mg/l NAA. Root initiation was highly frequent as evidenced by 100% root induction observed on the shoots transferred on MS basal medium. Rooting of shoots on half-strength MS medium was poorer than that on MS basal medium (Table 2, Figure 1). Higher concentration of NAA resulted in callusing at the basal end of the shoots.

Survival rate of plantlets transferred to soil after acclimatization was 95 ± 4%, as a total of 57 plantlets survived out of 60 transferred to the soil over a period of two months. The plants developed from *in vitro* cultures were morphologically identical to normal vegetatively propagated plants. GLC-chromatogams of the essential oil revealed that *in vitro* culturing of explants caused no variation in the concentration of major constituents of the essential oil (Figure 2 and Table 3).

The growth of propagules obtained by the micropropagation procedure described above grew was similar to those obtained from cuttings, when transplanted on slopes in the CIMAP farm.

1. Retamer, J. A., in *Essential Oil From Aromatic Plants* (ed. Verghese, J.), Synthite, India, 1988, pp. 124–279.
2. Lopez Abraham, A. M., Rojas Hernandez, N. M. and Jimenez Misas, C. A., *Rev. Cubana Med. Trop.*, 1979, **31**, 105–111.
3. Kumar, S. *et al.*, *J. Med. Aromat. Plant Sci.*, 1999, **21**, 1094–1096.
4. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–479.

5. Julani, H. R. (Jr), Koroch, A. R., Julani, H. R. and Trippi, V. S., *Plant Cell Tissue Org. Cult.*, 1999, **59**, 175–179.
6. Koroch, A. R., Juliani, H. R. (Jr), Juliani H. R. and Trippi, V. S., *Plant Cell Tissue Org. Cult.*, 1997, **48**, 213–217.
7. Santos, M. C., Esquibel, M. A. and dos Santos, *Plant Cell Tissue Org. Cult.*, 1990, **21**, 75–78.
8. Taylor, P. W. J. and Dukic S., *Plant Cell Tissue Org. Cult.*, 1993, **34**, 217–222.
9. Agrawal, D. C., Morwal, G. C. and Mascranhas, A. F., *Lindleyana*, 1992, **7**, 95–99.
10. Agrawal, D. C. *et al.*, *Plant Cell Rep.*, 1997, **16**, 647–652.

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Studies of poly(3-hydroxybutyric acid) inclusions in whole cells of *Azotobacter chroococcum* MAL-201

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Accumulation of poly(3-hydroxybutyric acid) inclusions in whole cells of *Azotobacter chroococcum* MAL-201 has been studied. Cells harvested at the late exponential phase of growth gave bright orange fluorescence when stained with Nile blue A. Electron microscopy of ultra-thin sections of such cells revealed 4–5 granules cell^{-1}, each measuring 0.1–0.44 μm in diameter. The infrared absorption spectra of lyophilized cells confirmed the chemical nature of the polymer, while in *in vivo* conditions the inclusions appeared to be amorphous as observed under wide angle X-ray scattering.

POLY(3-hydroxybutyric acid), [P(3HB)], is the most well-known representative of polyhydroxyalkanoic acids [PHAs] which represent a complex class of bacterial storage polyesters. As reserve material it is synthesized by a wide variety of bacteria grown under conditions of nutrient limitations, but with excess of carbon[1–3]. P(3HB) and copolymers of 3-hydroxybutyric acid and 3-hydroxyvaleric acid [P(3HB-co-3HV)] derived from bacterial sources have been identified as substitutes for petrochemical-based plastics mainly due to their thermoplastic properties, biocompatibility and complete degradation in nature[4,5].

The polymer being insoluble in water is produced *in vivo* in the form of inclusion bodies or granules. The number and size of granules vary considerably depending on the nature of the organism, growth condition, substrate used and the degree of polymer accumulation[1]. Earlier studies have suggested that the native P(3HB) granules are crystalline[6,7]. However, the concept of crystalline polymer existing within native granules has been revised during 1980s. Studies of granules using ^{13}C-NMR spectroscopy, X-ray diffraction and electron microscopy revealed that the polymer in *Methylobacterium* and *Alcaligenes eutrophus* is actually amorphous, at 30°C is mobile and elastomeric[8–11]. The question why the granules remained amorphous has been debated extensively in literature and several hypotheses have been advanced, mostly involving auxillary factors or 'plasticizers'[11–13].

Azotobacter chroococcum MAL-201, a mucoid, free-living, nitrogen-fixing strain isolated from a soil sample of Malda, West Bengal is reported to accumulate P(3HB), accounting more than 68% of its cell dry weight when grown in nitrogen-free medium under laboratory conditions[14,15]. In this communication, an attempt has been made to detect and determine the characteristic feature of the P(3HB) inclusions in whole cells of *A. chroococcum* MAL-201 grown in nitrogen-free medium under batch culture.

Azotobacter chroococcum MAL-201 was grown in modified Norris nitrogen-free medium[16] containing 2% (w/v) glucose. The medium (50 ml per 250 ml Erlenmayer flask) was inoculated with 2 ml of freshly prepared inoculum and incubated at 30°C on a rotary shaker (120 rpm) for 27 h. Cells were harvested by centrifugation (10,000 *g*) for 10 min and washed thoroughly with distilled water. Intracellular accumulation of the polymer was detected following staining with Nile blue A[17]. Cell suspension was smeared on a clean grease-free slide and stained with aqueous solution of Nile blue A at 55°C for 10 min in a coplin jar. The excess stain was removed with tap water and washed with 8% (v/v) aqueous acetic acid and again rinsed with water. After blot-drying, the smear was re-moistened with tap water, covered with cover glass and examined under Leitz-Labour-Lux D microscope with an I_2 filter which provides an excitation wavelength of approximately 460 nm. For transmission electron microscopy, ultra-thin sections of cells fixed in uranyl acetate (0.5%, w/v) were stained with lead citrate and examined in Hitachi H600 electron microscope with an acceleration voltage of 75 kV and photographed using Fuji film.

The infrared spectrum of lyophilized whole cell (in KBr pellate) was recorded with a Perkin-Elmer Model

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