Department to recruit staff, a cordial relationship between the Forest Department and the managements of tea gardens can prove to be effective to carry out wildlife conservation activities.

1. Myers, N., Mittermeier, R. A., Mittermeier, C. G., La Fonseca, G. A. B. and Kent, J., Biodiversity hotspots for conservation priorities. *Nature*, 2000, **403**, 853–858.
2. Menon, S. and Bawa, K. S., Application of geographic information systems, remote-sensing, and a landscape ecology approach to biodiversity conservation in the Western Ghats. *Curr. Sci.*, 1997, **73**, 134–144.
3. Kumar, A., Umapathy, G. and Prabhakar, A., A study on the management and conservation of small mammals in fragmented rainforests in the Western Ghats, South India: A preliminary report. *Primate Conserv.*, 1995, **16**, 53–58.
4. Congreve, C. R. T., *The Anaimalais*, Madras, 1938.
5. Umapathy, G. and Kumar, A., The occurrence of arboreal mammals in the rainforests in the Anaimalai hills, South India. *Biol. Conserv.*, 1999, **48**, 1–9.
6. Karanth, K. U., Status of wildlife and habitat conservation in Karnataka. *J. Bombay Nat. Hist. Soc.* (*Suppl.*), 1986, **83**, 166–179.
7. Krishnan, M., An ecological survey of the larger mammals of peninsular India. *J. Bombay Nat. Hist. Soc.*, 1972, **69**, 26–54.
8. Prater, S. H., *Book of Indian Animals*, Bombay Natural History Society, Mumbai, 1998.
9. Johnsingh, A. J. T., Distribution and status of dhole *Cuon alpinus pallas*, 1811 in South Asia. *Mammalia*, 1985, **49**, 203–208.
10. Prasad, S. N., Nair, P. V., Sharathchandra, H. C. and Gadgil, M., On factors governing the distribution of wild mammals in Karnataka. *J. Bombay Nat. Hist. Soc.*, 1979, **75**, 718–743.
11. Rice, C. G., Observation on predators and prey at Eravikulam National Park, Kerala. *J. Bombay Nat. Hist. Soc.*, 1986, **83**, 283–305.
12. Johnsingh, A. J. T., Large mammalian prey–predators in Bandipur. *J. Bombay Nat. Hist. Soc.*, 1983, **80**, 1–57.
13. Karanth, K. U. and Sanquist, M. E., Prey selection by tiger, leopard and dhole in tropical forests. *J. Anim. Ecol.*, 1995, **64**, 439–450.
14. Kumar, M. A., Singh, M., Srivastava, S. K., Udhayan, A., Kumara, H. N. and Sharma, A. K., Distribution patterns, relative abundance and management of mammals in Indira Gandhi Wildlife Sanctuary, Tamil Nadu, India. *J. Bombay Nat. Hist. Soc.*, 2002, **99**, 184–210.
15. Singh, M., Singh, M., Kumar, M. A., Kumara, H. N. and D'Souza, L., Distribution and research potential of non-human primates in the Aliyar–Valparai sector of the Indira Gandhi Wildlife Sanctuary, Tamil Nadu, India. *Trop. Biodivers.*, 1997, **4**, 197–208.
16. Singh, M., Singh, M., Kumara, H. N., Kumar, M. A. and D'Souza, L., Inter and intra-specific association of non-human primates in Anaimalai Hills, South India. *Mammalia*, 1997, **61**, 17–28.
17. Scheel, D., Profitability, encounter rates and prey choice of African lions. *Behav. Ecol.*, 1993, **4**, 90–97.
18. Ramachandran, K. K., Nair, P. V. and Easa, P. S., Ecology of larger mammals of Periyar Wildlife Sanctuary. *J. Bombay Nat. Hist. Soc.*, 1986, **83**, 505–524.
19. Fox, M. and Johnsingh, A. J. T., Hunting and feeding in wild dogs. *J. Bombay Nat. Hist. Soc.*, 1975, **72**, 321–326.

ACKNOWLEDGEMENTS. This research was carried out during a period when we received grants from Ministry of Environment and Forests, Government of India; Zoological Society of San Diego, and Tamil Nadu Forest Department for various other research projects. We are grateful to the management of Waterfall Estates for providing facilities to establish a field station since 1995. Special thanks are due to Mr Deepak Ganapathi and Mr Vinay Kumar. We thank Dr Divya Mudappa and Dr Shankar Raman for help in identification of plants and for useful discussions.

Received 7 April 2004; revised accepted 30 June 2004

Thidiazuron-induced high-frequency shoot proliferation in *Cineraria maritima* Linn.

Suchitra Banerjee*, Jyoti Tripathi, Praveen Chandra Verma, Prem Dutt Dwivedi, Suman Preet Singh Khanuja and G. D. Bagchi

Central Institute of Medicinal and Aromatic Plants, PO CIMAP, Lucknow 226 015, India

***Cineraria maritima* Linn. is an important medicinal plant of known therapeutic value for the treatment of cataract and corneal opacity. An *in vitro* regeneration protocol has been standardized for large-scale supply of planting material of this otherwise scarce medicinal plant species. The medium for propagation contained MS salts, B_5 vitamins, 30 g l^{-1} sucrose and 8.0 g l^{-1} agar (designated as MSB medium). Addition of thidiazuron [TDZ: N-phenyl-N′-(1,2,3-thidiazol-5-yl) urea] in the culture medium proved superior to the combined treatments of 6-benzyladenine and α-naphthaleneacetic acid. The highest adventitious shoot bud (36 ± 2.34) induction, per nodal explant used, occurred at 4.54 µM TDZ after 6 weeks of incubation. The number of shoots formed per explant increased significantly upon sub-culture of the responding explants on plant growth regulator-free MSB medium, after 8 weeks of culture initiation. *In vitro* produced shoots exhibited good rooting response on half strength MSB medium containing 4.92 µM indole 3-butyric acid. After 3 weeks of hardening of plantlets as hydrophonic cultures, almost 95% of 300 rooted plants could be successfully transferred and acclimatized *ex vitro* under glass-house conditions, followed by their establishment in the field.**

CINERARIA maritima Linn. (Asteraceae), an important annual exotic medicinal herb, is commonly known as 'Dusty Miller' or 'Silver dust' due to its characteristic wooly, silvery-grey foliage and a low mound-like habit. This plant is cultivated in Europe and cooler areas of USA[1]. In India, recently, limited cultivation of *C. maritima* has been taken-up in the Nilgiri Hills of southern India. The plant is used in the preparation of homeopathic drops, applied in various eye ailments[1,2], particularly for treating cataract and corneal opacity[3]. On account of its therapeutic value, the plant is in great demand in the pharmaceutical industry. Due to limited availability of raw materials of *C. maritima* in India, the national requirement is met solely by importing the prepared drug formulations from other countries. In order to meet the internal requirements of our country and at the same time to keep pace with the growing demand of this herb in the global commercial markets, there is an urgent need to develop an efficient multiplication protocol suitable for commercial use. Plant tissue culture techniques have

*For correspondence. (e-mail: suchi_banerjee@yahoo.com)

been put to effective use for obtaining the required quantity of uniform, high quality planting material in a short time in some cases[4,5]. The present communication describes efforts undertaken to standardize an efficient and reproducible *in vitro* micropropagation protocol for *C. maritima.*

Cineraria maritima Linn. plants were collected from Udagamandalam, Tamil Nadu, maintained in the glasshouse in our institute and were used as the source of explants. Nodal segments, collected from the young side branches, were cut into 1–1.5 cm pieces and washed with Teepol (10%, v/v; 2–3 min) followed by thorough washing under running tap water for 2 h. The explants were then surface disinfected with mercuric chloride (0.1%, w/v; 3 min) with intense shaking, followed by several washings with sterile distilled water. Such explants were subsequently cultured on modified basal medium containing MS salts[6], B_5 vitamins[7], 30 g l^{-1} sucrose and 8.0 g l^{-1} agar-agar (hereafter called as MSB medium). For the regeneration trials, varying concentrations of 6-benzyladenine (BAP) (1.11, 2.22, 4.44 and 8.89 μM) along with a fixed concentration of α-naphthaleneacetic acid (NAA; 0.27 μM) or thidiazuron (TDZ) alone (1.14, 2.27, 3.40 and 4.54 μM) were added to the MSB medium. The pH of the medium was adjusted to 5.8 before autoclaving at (121°C) 15 psi for 20 min. Cultures were incubated at 25 ± 2°C under continuous light of cool white fluorescent tubes (approx. 30 –40 μmol m^{-2} s^{-1}).

The nodal explants along with the induced adventitious shoots were subsequently transferred to plant growth regulator (PGR)-free MSB medium for shoot elongation. After 2–3 weeks, the elongated shoots were excised from the explants and transferred onto half strength MSB medium supplemented with either 2.46 or 4.92 μM indole 3-butyric acid (IBA) for rhizogenesis.

The rooted shoots were taken out of the culture vessels after 3–4 weeks, delicately washed under running tap water to remove the adhering culture medium from the roots, placed at the top of culture tubes filled with plain water (the roots were allowed to be dipped into the water). The tubes were kept in the culture room for two weeks for hardening; afterwards these plantlets were further hardened under glass-house conditions for one week, and then transferred to pots containing a mixture of soil : sand : farmyard compost (3 : 1 : 1; w/w).

The shoot regeneration response of nodal explants of *C. maritima* was tested on MSB medium supplemented with various concentrations of either BAP (with NAA) or TDZ. Both the cytokinins promoted shoot proliferation and 40–60% response could be observed with either of them after 4–6 weeks of culture.

As reported in *Glycine max* (L.) Merr.[8], *Hypericum perforatum* cv 'Anthos'[9], *Bacopa monniera*[10], *Rosa damascena*[11] and *Artemisia judaica* L[12], the presence of TDZ in the culture medium proved vastly superior to the treatment with BAP in case of *C. maritima* also, and the rate of proliferation was significantly higher in TDZ-containing media (Table 1).

Proliferation appeared to be by direct organogenesis as callus formation was not observed at any of the tested TDZ concentrations. The list of plant species exhibiting morphogenesis in the sole presence of TDZ has continued to increase over the years, facilitating the improvement of tissue culture technology[13].

The maximum number of adventitious shoot bud induction in *C. maritima* was caused by 4.54 μM TDZ, and an average of 36 ± 2.34 shoot buds were produced per explant after 6 weeks of incubation (Table 1). After a period of 6–8 weeks, a thick mat of shoot buds was found to cover 80–90% surface of the explant in the presence of 3.40 or 4.54 μM TDZ and each explant was transformed into a dense mass of profusely regenerating shoot buds (Figure 1 *a*), which made it impossible to count the number of shoot buds/explant after 8 weeks.

In accordance with earlier observations by several workers[13–16], sub-culturing of the resultant shoot buds on the same medium caused stunted shoot growth, which was

Table 1. Effect of BAP, NAA and TDZ on shoot bud induction and microshoot formation in nodal explants of *Cineraria maritima* L

Concentration of growth regulators	Number of microshoots/explant	
	After 4 weeks	After 6 weeks
Control (PGR-free medium)	No response	No response
BAP 0.25 mg l^{-1} + NAA 0.05 mg l^{-1}	3.25 ± 0.5	4.25 ± 0.8
BAP 0.5 mg l^{-1} + NAA 0.05 mg l^{-1}	5.0 ± 1.0	6.6 ± 1.14
BAP 1.0 mg l^{-1} + NAA 0.05 mg l^{-1}	5.0 ± 0.8	7.4 ± 0.89
BAP 2.0 mg l^{-1} + NAA 0.05 mg l^{-1}	5.2 ± 1.09	7.6 ± 0.55
TDZ 0.25 mg l^{-1}	11.60 ± 1.14	22.0 ± 1.30
TDZ 0.5 mg l^{-1}	12.4 ± 1.82	22.4 ± 1.82
TDZ 0.75 mg l^{-1}	15.8 ± 1.64	30.6 ± 1.34
TDZ 1.0 mg l^{-1}	22.8 ± 1.79	36.0 ± 2.34

Values are means ± SE (n = 30 explants for each treatment); PGR, Plant growth regulator. All experiments were done using MSB medium with or without PGRs.

Figure 1 ***a–h.*** *In vitro* plant regeneration from nodal segments of *Cineraria maritima* Linn. and its acclimatization under glass-house and field conditions. ***a***, Multiple shoot induction from nodal explants on MSB + 4.54 μM TDZ after six weeks of culture; ***b***, ***c***, Proliferation and elongation of *in vitro* regenerated shoots on PGR-free MSB medium after two and three weeks of transfer respectively; ***d***, Induction of roots from shoots on half-strength MSB + 4.92 μM IBA after three weeks; ***e***, Complete plantlet; ***f***, Initial hardening of *in vitro* raised plantlets maintained on water under culture-room conditions; ***g***, ***h***, Glass-house and field establishment of *in vitro* propagated plants of *C. maritima* Linn. after two and three months of *ex vitro* establishment respectively.

followed by hyperhydricity of shoots upon prolonged exposure to the same concentration of TDZ. In contrast, sub-culturing of profusely regenerating shoot mass from TDZ-containing medium to PGR-free MSB medium after 8 weeks of culture initiation stimulated elongation and further growth of each individual regenerant (Figure 1 *b* and *c*), as in other medicinal herbs like *Lavandula stoechas*[17] and *Bacopa monniera*[10]. Moreover, repetitive cycles of shoot regeneration could be observed in *C. maritima* through culturing these profusely regenerating shoot masses on PGR-free MSB medium after initial exposure to the optimal TDZ concentration, as reported earlier[18].

C. maritima shoots of about 2.5 to 3.5 cm height were subsequently separated and transferred to rooting medium for rhizogenesis. The optimal rooting medium contained only half the concentration of basal salts and vitamins along with 4.92 μM IBA on which cent per cent shoots developed roots within 3–4 weeks (Figure 1 *d*). Complete plants were obtained 2 months after the regenerated shoots were transferred to PGR-free MSB medium followed by culture on rooting medium (Figure 1 *e*).

The potential of *in vitro* propagated *C. maritima* plantlets to be used for glass-house or field cultivation was investigated with the plantlets transferred to pots containing a mixture of soil : sand : farmyard compost (3 : 1 : 1) after 2 weeks of initial hardening as hydrophonic cultures under culture-room conditions (Figure 1 *f*). Almost 95% of these regenerants survived under standard glass-house conditions and grew into fully developed plants (Figure 1 *g*), which were morphologically similar to *in vivo* seed-propagated plants. Through the rapid and efficient micropropagation protocol developed in this study, 300 healthy plants could be successfully established under *ex vitro* conditions (Figure 1 *h*). These may be useful for the production of medicinally active constituents for the eventual development of optimized pharmaceutical products having easy accessibility.

1. Moore, M., *Herbal Materia Medica*, South West School of Botanical Medicine, PO Box 4565, Bisben, AZ 85603, 1995, 5th edn.
2. Dwivedi, P. D., Pharmacognostic evaluation and development of cultivation technology for some commercially important traditional medicinal plants. Ph D thesis, Lucknow, University, 2003.
3. Vikramaditya and Joshi, P., *A Guide to Important Medicinal Plants used in Homeopathy*, Homeopathic Pharmacopoeial Laboratory, Ghaziabad, 1971, vol. I, p. 13.
4. Fowler, M. W., Commercial applications and economic aspects of mass cell cultures. In *Plant Biotechnology* (eds Mantell, S. H. and Smith, H.), Cambridge University Press, Cambridge, 1983, pp. 75–108.
5. Aitken-Christie, J., Automation. In *Micropropagation: Technology and Application* (eds Debergh, P. and Zimmerman, R. H.), Kluwer, Academic Publishers, Dordrecht, 1991, pp. 363–388.
6. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15**, 473–497.
7. Gamborg, O. L., Miller, R. A. and Ojima, K., Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 1968, **50**, 151–158.
8. Kaneda, Y., Tabei, Y., Nishimura, S., Harada, K., Akihama, T. and Kitamura K., Combination of thidiazuron and basal media with low salt concentrations increases the frequency of shoot organogenesis in soybeans [*Glycine max* (L.) Merr.]. *Plant Cell Rep.*, 1997, **17**, 8–12.
9. Murch, S. J., Choffe, K. L., Victor, J. M. R., Slimmon, T. Y., Krishna, Raj S. and Saxena, P. K., Thiazuron-induced plant regeneration from hypocotyls cultures of St. John's wort (*Hypericum perforatum* L. cv. Anthos). *Plant Cell Rep.*, 2000, **19**, 576–581.
10. Tiwari, V., Tiwari, K. N. and Singh, B. D., Comparative studies of cytokinins on *in-vitro* propagation of *Bacopa monniera*. *Plant Cell Tissue Org. Cult.*, 2001, **66**, 9–16.
11. Kumar, A., Sood, A., Palni, U. T., Gupta, A. K. and Palni, L. M. S., Micropropagation of *Rosa damascene* Mill. from mature bushes using thidiazuron. *J. Hortic. Sci. Biotechnol.*, 2001,**76**, 30–34.
12. Liu, C. Z., Murch, S. J., Demerdash, M. E. L. and Saxena, P. K., Regeneration of the Egyptian medicinal plant *Artemisia judaica* L. *Plant Cell Rep.*, 2003, **21**, 525–530.

13. Murthy, B. N. S., Murch, S. J. and Saxena, P. K., Thidiazuron: a potential regulator of *in-vitro* plant morphogenesis. *In Vitro Cell. Dev. Biol.–Plant*, 1998, **34**, 267–275.
14. Lu, C. Y., The use of thidiazuron in tissue culture. *In Vitro Cell Dev. Biol.–Plant*, 1993, **29**, 92–96.
15. Hutchinson, J. M. and Saxena, P. K., Acetylsalicylic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* X *hortorum* Bailey) tissue cultures. *Plant Cell Rep.*, 1996, **15**, 512–515.
16. Vijaya Laxmi, G. and Giri, C. C., Plant regeneration via organogenesis from shoot base-derived callus of *Arachis stenosperma* and *A. villosa*. *Curr. Sci.*, 2003, **85**,1624–1629.
17. Jose Nobre, J., *In vitro* cloning and micropropagation of *Lavandula stoechas* from field-grown plants. *Plant Cell Tissue Org. Cult.*, 1996, **46**, 151–155.
18. Kanyand, M., Dessai, A. P. and Prakash, C. S., Thiadiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea* L.) plants *in vitro*. *Plant Cell Rep.*, 1994, **14**, 1–5.

ACKNOWLEDGEMENT. We thank Mr A. P. Dhiman, Photography Division, CIMAP, Lucknow for support.

Received 5 March 2004; revised accepted 12 July 2004

Accumulation of astaxanthin in flagellated cells of *Haematococcus pluvialis* – cultural and regulatory aspects

B. R. Brinda, R. Sarada*, B. Sandesh Kamath and G. A. Ravishankar

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 020, India

The effect of nutrient stress on astaxanthin formation in flagellated cells of *Haematococcus pluvialis* was evaluated under autotrophic conditions. High biomass yield of 3.5 g/l was achieved on phosphate free medium and astaxanthin constituted 1.5% (w/w) on dry weight basis, with a production of 55.6 mg/l. A combination of nitrogen and phosphate limitation was effective in enhancement of astaxanthin content to 2.0 ± 0.2% (on dry weight basis), resulting in its production of 35–38 mg/l. Actinomycin-D inhibited both cell growth as well as carotenogenesis, while chloramphenicol and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea inhibited significantly the secondary carotenoid formation in flagellated cells under low light intensity, with no effect under high light intensity. Cycloheximide inhibited carotenogenesis in both flagellated and encysted cells under low as well as high light intensities. The accumulation of astaxanthin in lipid globules of flagellated cells was detected with staining of algal cells. The results indicated that both cytoplasmic and organellar translational inhibitors play a role in the secondary carotenoid formation in flagellated cells, which depend on light to a greater extent than salinity stress.

*For correspondence. (e-mail: pcbt@cscftri.ren.nic.in)

HAEMATOCOCCUS pluvialis, a green alga, gained commercial importance owing to its ability to accumulate a potent antioxidant, astaxanthin, having nutraceutical and pharmacological applications as well as pigmentation source in farmed salmon, trout and poultry industries[1]. Most of the reports showed astaxanthin accumulation in encysted cells when grown in heterotrophic conditions under high light or due to salinity stress[2,3]. However, astaxanthin accumulation in flagellated cells would be advantageous as the flagellated cells are fragile over the thick encysted cells which are resistant for solvent extraction, enzyme treatment and mechanical breakage. The present study focused to understand the effect of nutrient stress on astaxanthin accumulation in flagellated cells and its regulation in both flagellated and encysted cells using photosynthesis, transcriptional and translational inhibitors.

H. pluvialis (SAG 19-a) was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany. Stock cultures were maintained in autotrophic bold basal medium (BBM)[4]. *Haematococcus* cultures grown in autotrophic medium were used for this study. The cultures were grown in a two-tier flask containing nutrient limiting medium (limiting to 1/4 and 1/10 of original concentration of nitrogen, magnesium and phosphorus individually and in combination) with 10 ml inoculum, as detailed by Sarada *et al.*[3] and Usha *et al.*[5]. The two-tier vessel consisting of two 250 ml narrow-neck Erlenmeyer flasks was used for enriching CO_2 in the culture environment. The lower compartment of the flask contained 100 ml of 3M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask[5]. The upper chamber contained 40 ml of nutrient limiting medium. The cultures were incubated at 25 ± 1°C temperature under multi-directional light source of intensity 2 ± 0.2 klux[6].

Cells were stained with coomassie brilliant blue R 250 (0.25 %) at vegetative, intermediate and encysted stages. Growth in terms of cell count was determined using a haemocytometer (12.6×10^4/ml) at the time of inoculation and also before imposing salt stress (NaCl; 0.04%). At the end of the experimental period, cultures were harvested by centrifugation. Dry weight of biomass was estimated after drying at 60°C in hot-air oven till constant weight was obtained.

Total carotenoid and chlorophyll contents were calculated according to the method of Lichtenthaler[7] by reading the absorbance at 470, 645 and 661.5 nm respectively. Astaxanthin content was estimated by Davies[8] method by reading absorbance at 480 nm. Carotenoid extracts were subjected to HPLC analysis using reversed phase C18