Establishment of callus cultures of *Nyctanthes arbor-tristis* from juvenile explants and detection of secondary metabolites in the callus

R. Indira Iyer, V. Mathuram* and P. M. Gopinath

Department of Genetics, Dr A.L.M. Post-Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai 600 113, India

*Captain Srinivasa Murti Drug Research Institute for Ayurveda, Arumbakkam, Chennai 600 016, India

Excised cotyledons, hypocotyls, roots, leaves and bases of internodes of plantlets of *Nyctanthes arbor-tristis* callused readily on culture. Plantlets were raised *in vitro* from isolated immature embryos. Calli from cotyledons, hypocotyls and roots exhibited faster growth compared with those from leaves and internodal bases when cultured in Murashige and Skoog's medium with 2,4-D, NAA and coconut milk. Growth potential of the calli was not found to decline on repeated subcultures, even after twelve months. Hexane and chloroform extracts of callus were found to contain β-sitosterol and oleanolic acid. Alcohol extract of the callus showed the presence of iridoid glycosides by thin layer chromatography.

NYCTANTHES arbor-tristis (Coral jasmine) of Oleaceae is a small ornamental tree with fragrant flowers. The plant is used in traditional system of Indian medicine for the treatment of chronic fever and rheumatism. It is also used as an anthelmintic and as a liver and nervine tonic[1]. Earlier investigators have reported the presence of β-amyrin, β-sitosterol[2], friedelin, nyctanthic acid, lupeol and oleanolic acid[3] in the leaves. Iridoid and phenylpropanoid glycosides have been isolated from leaves and seeds. The iridoid arbor-tristoside A has been reported to have pronounced anticancer activity[4–7].

Plant tissue culture techniques are widely employed to obtain a stable supply of bioactive secondary products[8,9], including anti-cancer drugs like taxol[10]. Culture of tissues of ligneous species is difficult[11]. There is no report on the *in vitro* propagation of *N. arbor-tristis*. The present communication details culture of excised tissues of immature embryos of this plant, establishment of culture from juvenile explants taken from axenically grown plantlets and an analysis of secondary metabolites obtained from the callus.

Seeds excised freshly from ripe and unripe fruits were washed in running tap water and surface sterilized in a laminar flow cabinet by immersing in 0.1% $HgCl_2$ for 10 min followed by several rinses with sterile water. The zygotic embryos which were aseptically isolated either from directly surface-sterilized seeds or from aseptically isolated seeds from the unripe fruits were cultured. For callus initiation, the explants from the *in vitro* grown plantlets obtained from embryos were cultured on Murashige and Skoog (MS) basal medium[12] containing sucrose (3%) and 0.8% agar (Ranbaxy) and supplemented with various combinations of growth regulators (Table 1). While the segments of cotyledons, hypocotyls, roots and leaves were placed horizontally on the surface of the medium, the internodal explants were cultured with their bases inserted into the medium. The pH of the media was adjusted to 5.7 prior to autoclaving at 121°C and 103 kPa for 15 min. Cultures were incubated in continuous light from cool white fluorescent lamps at an intensity of 2000 lux at 25 ± 2 °C. Subcultures were carried out at intervals of four weeks.

Phytochemicals were extracted from calli (13 g) derived from cotyledons dried at room temperature and also from fresh calli (8.8 g) derived from cotyledon, hypocotyl and root explants. The dried and powdered cotyledon callus was refluxed with *n*-hexane, chloroform and methanol successively. The fresh callus was refluxed directly with methanol. The extracts were evaporated to dryness.

Table 1. Supplements of growth regulators to the medium of Murashige and Skoog

Medium	2,4-D (mg/l)	BAP (mg/l)	NAA (mg/l)	GA_3 (mg/l)	Coconut milk (%)
A*	5	–	1	–	10
B*	–	2	–	2	–
C	5	–	1	–	15
D	–	3	–	–	–

*Contained activated charcoal (0.25%).

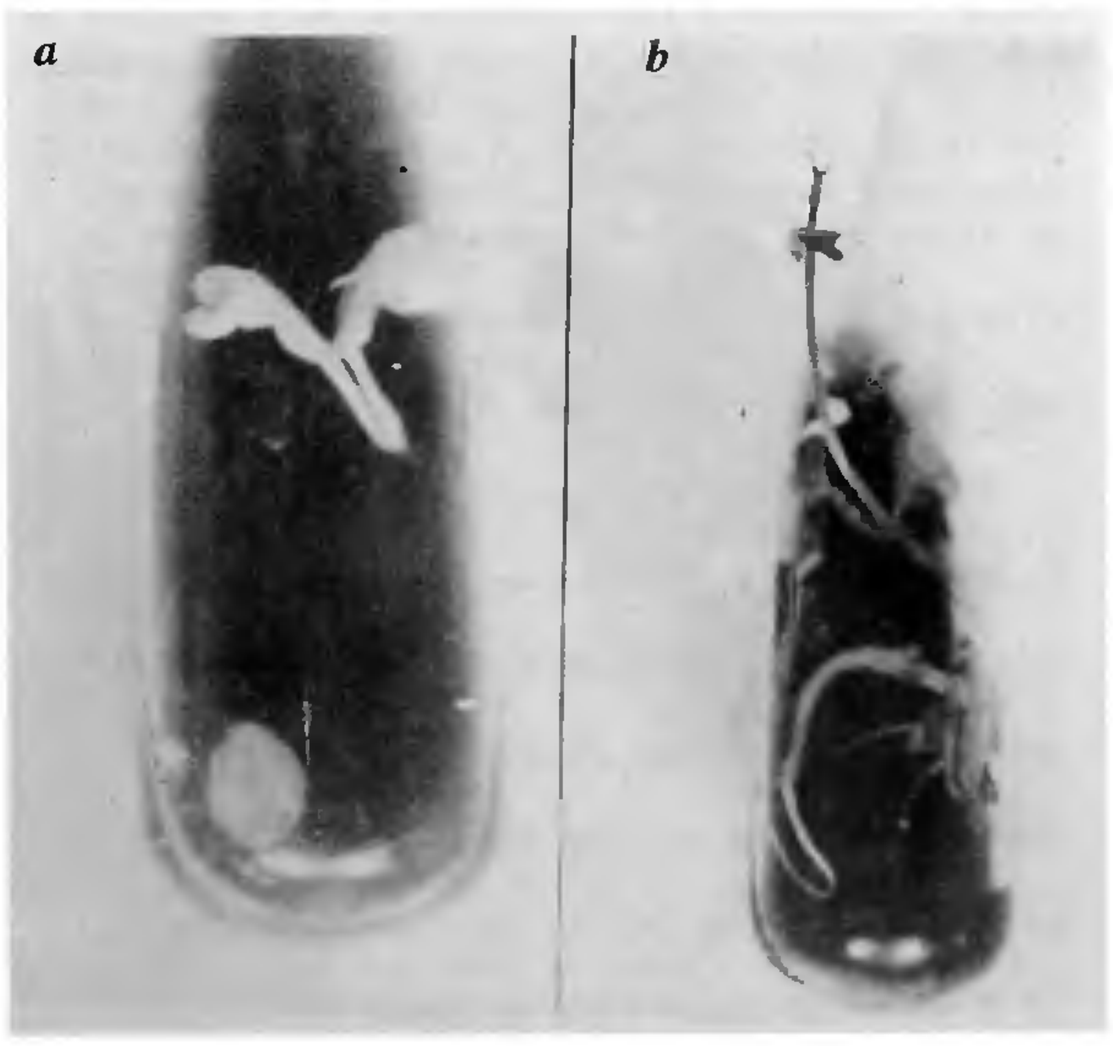

Figure 1. *a*, Germination of excised immature embryo of *N. arbor-tristis* in medium A with activated charcoal; *b*, Rapidly elongated shoot on medium B with GA_3.

Thin layer chromatography (TLC) was carried out on silica gel plates in the solvent systems: (i) benzene-ethyl acetate (4 : 1) and (ii) ethyl acetate-methanol (9 : 1). The chromatograms were sprayed with H_2SO_4 (50%). The R_f values were recorded and were compared with those of authentic compounds. Qualitative tests for sterols (Liebermann–Burchard test)[13], triterpenoids (Noller test)[13], glycosides (Anthrone test)[14] and iridoids[15] were performed. The test for iridoids was performed by employing a concentrated methanolic solution of the substance to which a pinch of vanillin was added followed by a few drops of concentrated HCl. The mixture was heated and the development of pink colour indicated the presence of iridoids.

The immature zygotic embryos from unripe fruits germinated in 3–4 days in medium A (Table 1) with or without activated charcoal. The cotyledons turned green and plantlets were formed in about two weeks. Activated charcoal was reported to be necessary for the *in vitro* germination of embryos of *Carcica papaya*[16]

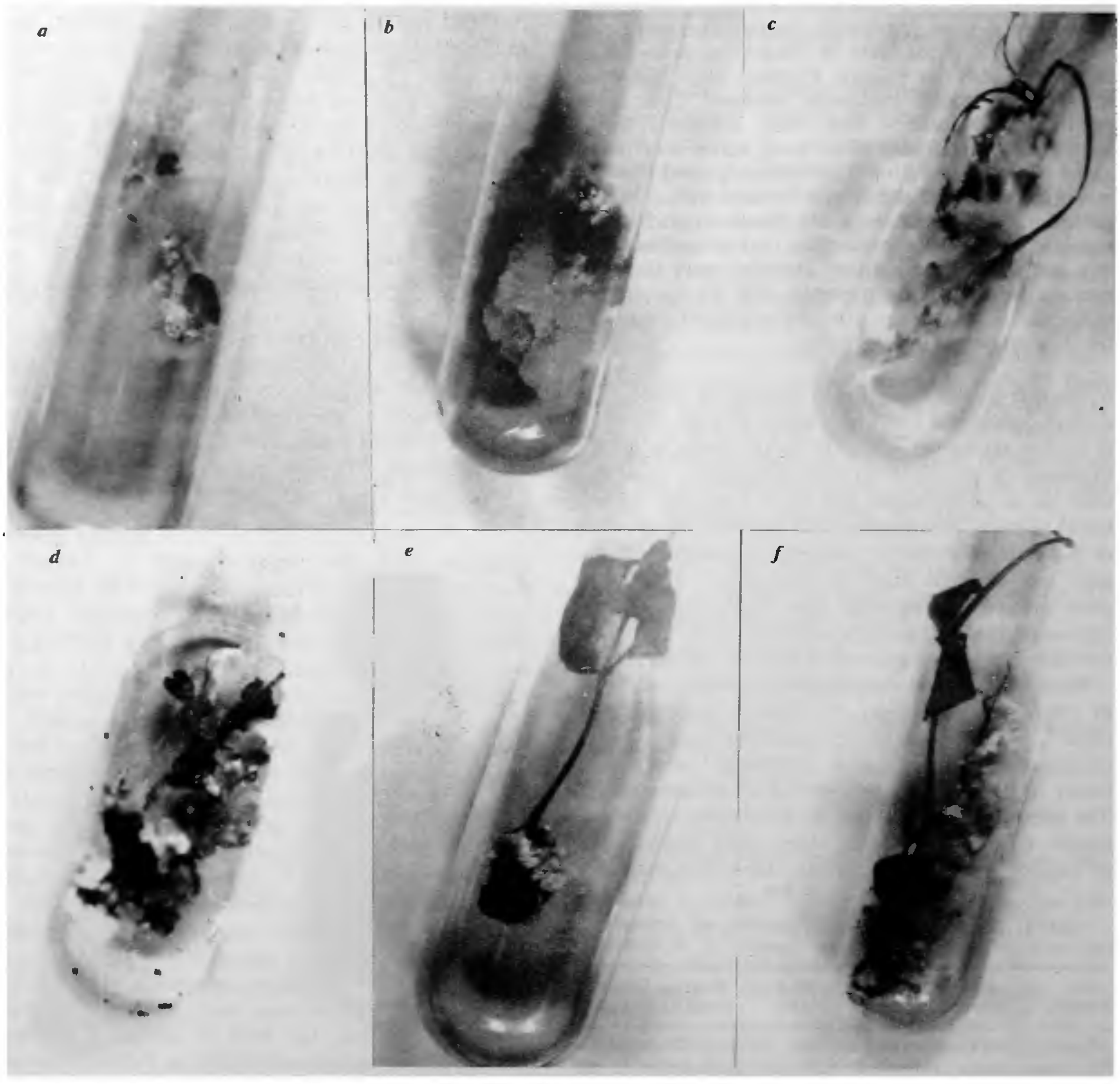

Figure 2. *a*, Formation of callus from immature cotyledons in medium C; *b*, Growth of callus after four weeks in medium C; *c*, Callus formation in hypocotyl explants in medium D; *d*, Callusing of leaf explants; *e*, Formation of hard granular callus at the internodal base; *f*, Callusing on hypocotyl and roots of intact plantlets.

Table 2. Growth of the callus

Hypocotyl	++++++++
Root	+++++++
Cotyledon	++++
Leaf	++
Stem	+

+ Relative measure of the callus size.

and *Phoenix dactylifera*[17] and stimulated root formation as a result of its soil-like properties. Isolated embryos of *Nyctanthes arbor-tristis* were found to germinate even in the absence of charcoal. Transfer of plantlets to medium B (Table 1) with gibberellic acid (GA_3, 2 mg/l) resulted in remarkable increase in the elongation of the shoot by about 4 cm when observed after one week (Figure 1 *a* and *b*). There are reports on germination of isolated embryos *in vitro* in various ligneous species[18–22] and is found to be influenced by the composition of the media[22].

Callus was readily initiated from cotyledons, hypocotyls, leaf and internodal explants obtained from the plantlets directly put into the medium and also from the hypocotyls and roots of intact plantlets (Figure 2 *a–e*). The growth was, however, rapid in calli from hypocotyl, root and cotyledons compared with that from stem and leaf (Figure 2*f*, *g*; Table 2). The callus from leaf explants was amorphous and green in colour. All the calli could be maintained for over twelve months by subculturing at regular intervals without a decline in their growth potential. The growth of the callus in medium C with a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) at 5 mg/l, naphthaleneacetic acid (NAA) at 1 mg/l and coconut milk (15%), was faster than that seen in media A, B and D. The root and hypocotyl calli grown in medium C doubled in a week.

Both the hexane and chloroform extracts of the dried calli derived from cotyledons on examination through TLC showed two spots corresponding to β-sitosterol and the triterpenoid, oleanolic acid, which have been reported also from the intact plant[2,3]. The alcohol extract of the dried callus obtained from cotyledon answered the test for iridoids and sugar[15] and on TLC in ethyl acetate–methanol (9 : 1) showed a spot with R_f value of 0.65. This, however, did not correspond to any of the arbor-tristosides isolated earlier. Yield of secondary metabolites from cultured plant cells has been reported to be low[9] as in the present case which precluded chromatographic separation and isolation of the iridoids. β-Sitosterol and oleanolic acid were also detected in the methanolic extract of the fresh callus. TLC of the methanolic extract in ethyl acetate–methanol (9 : 1) revealed two prominent spots with R_f values 0.24 and 0.29 respectively. The response to the test for iridoids was positive. The R_f values, however, were different from any of the iridoid glycosides isolated from the intact plant.

The results are consistent with the fact that the plant cell cultures produce a spectrum of compounds different from those produced by intact plant[23–25] though low yield under culture conditions may not be favourable for commercial exploitation[23].

The study thus demonstrates the possibility of obtaining rapidly growing calli from juvenile explants of *Nyctanthes arbor-tristis* excised from axenically grown plantlets raised *in vitro* from isolated zygotic embryos. β-Sitosterol and oleanolic acid were detected in the extract from the calli. Application of precursors[26,27] or changes in the culture conditions[28] may result in significant increase in the yield of desired compounds. Large-scale production of the callus will enable isolation and characterization of the unidentified compounds detected.

1. Kirtikar, K. R. and Basu, B. D., *Indian Medicinal Plants* (rewritten by Blatter, B., Caius, J. F. and Mhasker, K. S.), Ms. Bishen Singh Mahendrapal Singh, Dehradun, 1975, vol. 2, pp. 1526–1528.
2. Sen, A. B. and Singh, J. P., *J. Indian Chem. Soc.*, 1964, **41**, 192–194.
3. Anjaneyulu, A. S. R., Murthy, Y. L. N. and Ramchandra Rao, L., *J. Indian Chem. Soc.*, 1981, **58**, 817–819.
4. Purushothaman, K. K., Mathuram, V. and Sarada, A., *Phytochemistry*, 1985, **24**, 773–776.
5. Mathuram, V., Kundu, A. B., Banerjee, S. and Patra, A., *J. Indian Chem. Soc.*, 1991, **68**, 581–584.
6. Stuppner, J., Muller, E. P., Mathuram, V. and Kundu, A. B., *Phytochemistry*, 1993, **32**, 375–378.
7. Mathuram, V., Bhima Rao, R., Halder, S., Banerjee, A. and Kundu, A. B., *J. Indian Chem. Soc.*, 1994, **71**, 215–217.
8. Bajaj, Y. P. S., Furmanowa, M. and Olszowska, O., in *Biotechnology in Agriculture and Forestry 4. Medicinal and Aromatic Plants I* (ed. Bajaj, Y. P. S.), Springer, Berlin, 1988, pp. 60–103.
9. Yeoman, M. M. and Yeoman, C. L., *New Phytol.*, 1996, **134**, 553–569
10. Edgington, S. M., *Biotechnology*, 1991, **9**, 933–938.
11. Etienne, H., Sotta, B., Montoro, P. and Miginiac, E. and Carron, M. P., *Plant Sci.*, 1993, **92**, 111–119.
12. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **16**, 473–497.
13. Overton, K. H., in *Technique of Organic Chemistry* (ed. Bentley, K. W.), Interscience Publishers, New York, 1963, vol. IX, pp. 42–50.
14. Hawk, P. B., in *Hawk's Physiological Chemistry* (ed. Oser, B. L.), Tata-McGraw Hill, New York, 1979, pp. 224–232.
15. Bianco, A., Passacantilli, P. and Righi, G., *J. Nat. Prod.*, 1983, **46**, 314–319.
16. Sharma, N. K. and Bedi, S., *Ann. Bot.*, 1990, **66**, 597–603.
17. Tisserat, B., *J. Exp. Bot.*, 1979, **30**, 1275–1283.
18. Scott, E. S., Rao, A. N. and Loh, C. S., *Ann. Bot.*, 1988, **61**, 233–238.
19. Moura, I. and Carneiro, M. F. N., *Plant Cell, Tissue Org. Cult.*, 1992, **31**, 207–209.
20. Emershad, R. L. and Ramming, D., *Plant Cell, Tissue Org. Cult.*, 1994, **37**, 55–59.
21. Flores, T., Wagner, L. J. and Flores, H. E., *In Vitro Cell Dev. Biol.*, 1993, **29P**, 160–165.
22. Liskova, D., Ordonez, J. R., Lux, A. and Lopez, A. P., *Plant Cell Tissue Org. Cult.*, 1994, **36**, 339–343.
23. Yeoman, M. M., Holden, M. A., Corchete, P., Holden, P. L., Goy, J., Hobbs, M. C., in *Secondary Products from Plant Tissue Culture* (eds Charlwood, B. V. and Rhodes, M. J. C.), Oxford University Press, Oxford, 1990, pp. 139–161.

24. Yeoman, M. M., Miedzybrodzka, M. B., Lindsey, K., McLauchlan, W. R., in *Plant Cell Cultures. Results and Perspectives* (eds Sala, F., Parisi, B., Cella, R. and Ciferri, O.), Elsevier, Amsterdam, 1980, pp. 327–343.
25. Lindsey, K., Yeoman, M. W., *Cell Culture and Somatic Cell Genetics of Plants. A Comprehensive Treatise* (ed. Vasil, I.), Academic Press, New York, 1985, pp. 61–101.
26. Lindsey, K. and Yeoman, M. M., in *Plant Biotechnology – Society for Experimental Biotechnology Seminar Series 18* (eds Smith, H. and Mantell, S. M.), Cambridge University Press, Cambridge, 1983, pp. 39–66.
27. Chowdhury, A. R. and Chaturvedi, H. C., *Curr. Sci.*, 1979, **49**, 237–238.
28. Payne, G., in *Plant Cell and Tissue Culture in Liquid Systems* (eds Payne, G., Bringi, V., Prince, C. and Shuler, M.), Hanser Publishers, Munich, 1991, pp. 225–280.

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Photoperiodic regulation of reproduction in migratory bunting, *Emberiza melanocephala*: An evidence for external coincidence model

Manoj Kumar Mishra and P. D. Tewary

Department of Zoology, Banaras Hindu University, Varanasi 221 005, India

Photosensitive female blackheaded buntings (*Emberiza melanocephala*) were exposed to various night-interruption cycles for 42 days. These light–dark cycles consisted of a basic photophase of 6 h and 1 h photointerruption of the 18 h dark phase in 24 h cycle at different points. A control group was also placed under short photoperiod (8L : 16D). Ovarian growth and increased plasma estradiol level were evident in night interruption cycle in which photo-interruption of dark photophase was made 13 h and 15 h after the onset of basic photophase, i.e. 6L : 7D : 1L : 10D and 6L : 9D : 1L : 8D cycles. The present results are consistent with external coincidence model (Bünning hypothesis), suggesting the involvement of an endogenous circadian rhythm in photoperiodic time measurement in bunting.

SEVERAL avian species of temperate, subtropical and tropical zones have been reported to display their reproductive, physiological, morphological and behavioural activities in response to environmental cues in which the role of photoperiod is of great importance[1–5]. Endogenous circadian rhythm and its involvement in control of reproduction in avian as well as in mammalian species with regard to photoperiod have been well defined[2,6–9]. Many powerful methods have been used to test the circadian rhythm of sensitivity to light, of which night-interruption experimental protocol is worth mentioning. It involves the use of repetitive light–dark cycles consisting of a main photophase of non-stimulatory duration combined with dark phase in 24 h cycle which is interrupted by 1 h short pulse of light. Using this protocol, various experiments have been carried out on avian species, particularly on males paying less attention towards females[10–13]. Keeping in view the scanty information about female avian species, we sought to explore the role of photoinducible phase and involvement of the circadian rhythm in photoperiodic time measurement in female blackheaded bunting.

The blackheaded bunting is a migratory finch which visits the Indian subcontinent during winter season (September/October) and returns to its breeding ground when summer approaches (March : April)[14]. Buntings were captured locally near Varanasi (25°18′N, 83°01′E) in January 1995 when they were available in plenty. They were brought to an outdoor aviary, acclimatized for 15 days and then placed in a short photoperiodic (8L : 16D) environment for 60 days to make them photosensitive. Birds were laparotomized periodically to ensure that they maintained regressed ovaries (4–5 mg). These photosensitive birds were divided into six groups (five experimental and one control) and kept in wirenet cages (50 × 30 × 25 cm). The control group was placed under short photoperiod (8L : 16D) while the experimental groups were held under various night interruption cycles such as 6L : 3D : 1L : 14D (9 h), 6L : 5D : 1L : 12D (11 h), 6L : 7D : 1L : 10D (13 h), 6L : 9D : 1L : 8D (15 h) and 6L : 11D : 1L : 6D (17 h) for 42 days (Table 1). The cages containing birds (4 birds/cage) were placed under different photoperiodic chambers (as scheduled above), illuminated with 20 W fluorescent tubes having light intensity of 400 lux at perch level. The food and water were supplied *ad libitum* only during light phase of cycles which commenced in each cage at 0600 h. The birds were laparotomized before the beginning and at the end of experiments and ovarian growth was assessed by comparing the size of ovaries *in situ*, with standard sets of ovaries of known weights. The error inherent in this method is about 20% (ref. 15). In the beginning and at the end, blood was collected in heparinized capillary tubes, centrifuged at 4°C, plasma separated and kept at −20°C till assayed. The circulating plasma estradiol concentration was measured by radioimmunoassay using estradiol Direct Radioimmunoassay kit (Biotexc Laboratories Inc., 6023, Southloop East Huston, Tx 77033). The kit was highly specific for estradiol. The cross reactivity for 17β-estradiol was 100%. Data were analysed using one-way analysis of variance (ANOVA) supplemented with Neuman–Keul's multiple range *t*-test.