It took 3–7 days for the algae to grow in the media. A total of seven species of algae, belonging to Bacillariophyceae (four species) and Chlorophyceae (three species) (Table 2) grew on the soil samples. One way completely randomized ANOVA showed that the representation of different species of algae was significantly higher ($F_{1,82} = 18.7$, $P < 0.0001$) in the samples from the entrance zone (73.68%) compared to twilight zone (26.32%) of all the caves. No green algae or diatoms were found in the culture of samples collected from the dark zone of any of the caves. Among the green algae, *Chlorococcum* sp. and among the diatoms *Navicula* sp. were more dominant at the entrance zone of all the caves.

While the Pannian cave (C2) had all seven species of algae at its entrance zone, only three to five species of algae were found at the entrance zone of other caves. The soil samples taken from the twilight zone of various caves had less number of species: three in cave 2; two in caves 1, 3 and 6; one in cave 5; and none in cave 4.

The presence of seven species of algae in Pannian cave (C2) is presumably because the entrance of this cave faces the zenith and thereby receives more light compared to other caves (Table 1). Some genera like *Chlorella*, *Scenedesmus* and *Chlamydomonas* can grow also chemorganotrophically in dim light, using simple substrates[12]. The present study confirms the earlier reports with regard to the distribution of algae in caves[4], in response to light as the limiting factor.

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In vitro studies on some non-coniferous gymnosperms

Gymnosperms, comprising conifers and non-conifers, are generally evergreen trees and shrubs. A number of reports are available on the application of tissue culture technology for multiplication of coniferous plants[1] unlike the case with non-conifers[2]. The non-conifers, such as cycads and *Ephedra*, differ strikingly from conifers in their habit, structure, and habitat. They are important in terms of evolutionary, scientific, ornamental and medicinal values. The presence of ciliate sperm (primitive feature) in cycads, and double fertilization (advanced feature) in *Ephedra* adds further to their distinct identity. Cycads which were once common in mid-Mesozoic, are now present only as relics of the past and are aptly referred to as 'Dinosaurs of the Plant Kingdom'[3]. Cycads propagate themselves either through seeds or asexually by means of adventitious shoots. Further, all cycads are slow growing. The plants are dioecious and the seed set is very poor. Even the few seeds that mature, rapidly lose their viability. Disturbances in their ecosystem have led to a steady decline in the population size of cycads, thereby causing serious concern.

Cycads are notorious for their recalcitrance to tissue culture[2]; and thus are a challenge to investigators. The generally used explants in cycads are from embryo or endosperm[4]. Paucity of seeds and a single massive shoot apex (pachycaulous) are additional disadvantages for their propagation by tissue culture technique. Some workers have used leaf explants[5–8].

The present report deals with responses obtained from different explants of *Cycas*, *Zamia*, and *Ephedra foliata* cultured on Murashige and Skoog medium (MS) with various plant growth regulators (PGRs) added to the medium individually as well as in combination. Regeneration using nodal segments has been reported in a few species of *Ephedra*[9], and from haploid tissues of *E. foliata*[10]. Micropropagation of *E. foliata* using embryo explants is also described here.

Segments of petiole and rachis (1.0–1.5 cm long portions split lengthwise) of unfolding leaves of cycads and seeds of *E. foliata* were obtained from the Botanical Garden, Department of Botany, University of Delhi. Embryos and portions of endosperms of *C. circinalis* were excised from seeds collected from Nagamangala, Karnataka State. Explants were sterilized with 0.1 to 0.2% $HgCl_2$ solution for 2 min in a laminar flow cabinet prior to inoculation. MS medium[11] with different concentrations of auxins (2,4-D, NAA) and cytokinins (BAP, Kn) singly or in combinations was used. Embryos of *E. foliata* were excised from mature seeds, and medianly-cut half portions were cultured on the MS medium with 2% sucrose and 10% coconut water (CW) + PGRs. For each experiment a minimum of 24 cultures were raised and the experiments were repeated at least twice. Cultures were maintained at 25 ± 2°C under 16 h light photoperiod provided by cool fluorescent light (15 $mE/m^2/s$).

The results obtained in response to different treatments are summarized in Table 1. The leaf explants of *C. revoluta* produced a friable callus. The callus obtained from leaf cultures of *C. rumphii* was compact and nodular, though the amount of callus formed was less than was observed in *C. revoluta* (see Table 1). 2,4-D when used alone in leaf explants of *C. rumphii* caused the formation of unorganized callus, but in combination with BAP or Kn, it stimulated shoot bud differentiation (Figure 1 *a*). Green leaf-like structures developed on the medium containing 2,4-D + BAP. *Z. furfuracea* leaf explants yielded meagre to profuse amount of callus on auxin-containing medium. Addition of cytokinin (BAP or Kn) induced somatic embryogenesis after 12 to 14 weeks (Figure 1 *b*).

The female gametophyte of *C. circinalis* yielded varying amounts of callus depending on the concentrations of the growth regulators added to the MS medium. Nodular structures were obtained in response to NAA + Kn. The female gametophytes of *Z. integrifolia* showed meagre callusing only in response to 2,4-D. In treatments with 2,4-D + Kn, nodular structures were formed in the callus after four months. However, their further development was arrested.

The hypocotyl portion of cultured embryos of *C. circinalis* on MS + 1 µM 2,4-D with 2 µM BAP gave rise to a nodular structure. When this structure was allowed to enlarge and was subsequently transferred to the MS basal medium, it developed into a bulbil (Figure 1 *c*) with numerous leaves, each with a curved tip.

The embryo explants (half embryos) of *E. foliata* were subjected to the same combination of treatments as shown in the column 1 of Table 1. Only the responses to a few treatments are described below. On MS + 2,4-D (2 to 10 µM) + Kn or BAP (2 to 10 µM) the explants gave rise to a good amount of callus which subsequently differentiated roots, shoot buds and somatic embryos (Figure 1 *d*). In treatments containing

Table 1. Summarized data on *in vitro* responses from different explants of cycads

	Treatment	Callusing	Somatic embryogenesis	Shoot bud formation	Other responses
Leaf					
Cycas revoluta	MS + 2,4-D (0.1–10 µM)	++→++++	–	–	–
	MS + NAA (0.1–10 µM)	+→+++	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	+→+++	–	–	–
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	+→++++	–	–	–
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	+→+++	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	+→+++	–	–	–
C. rumphii	MS + 2,4-D (0.1–10 µM)	–→++	–	–	–
	MS + NAA (0.1–10 µM)	–→++	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	+→+++	–	–	Leaf-like structures
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	+→+++	–	++	–
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	–→++	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	–→+++	–	–	–
Zamia furfuracea	MS + 2,4-D (0.5–2 µM)	++→++++	–	–	–
	MS + NAA (0.1–10 µM)	+→+++	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	++→+++	++	–	–
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	++→+++	+	–	–
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	+→+++	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	–→+++	–	–	–
Female gametophyte					
C. circinalis	MS + 2,4-D (0.1–10 µM)	+→+++	–	–	–
	MS + NAA (0.1–10 µM)	+→+++	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	–→+	–	–	–
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	–→++	–	–	–
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	–→+	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	–→++	–	–	Nodular structures
Z. integrifolia	MS + 2,4-D (0.1–10 µM)	–→+	–	–	–
	MS + NAA (0.1–10 µM)	–	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	–	–	–	–
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	–→+	–	–	Nodular structures
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	–	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	–	–	–	–
Embryo					
C. circinalis	MS + 2,4-D (0.1–10 µM)	+→+++	–	–	–
	MS + NAA (0.1–10 µM)	+→++	–	–	–
	MS + 2,4-D (0.5–2 µM) + BAP (1–5 µM)	+→+++	–	–	–
	MS + 2,4-D (0.5–2 µM) + Kn (1–5 µM)	+→+++	–	–	–
	MS + NAA (0.5–2 µM) + BAP (1–5 µM)	+→++	–	–	–
	MS + NAA (0.5–2 µM) + Kn (1–5 µM)	+→++	–	–	–

– Nil, + meagre, ++ moderate, +++ good, ++++ profuse, *see text for details.

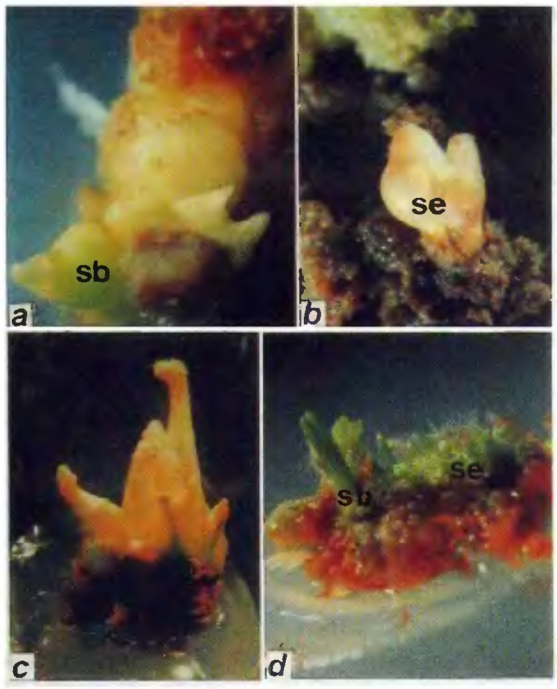

Figure 1. *a*, 10-week-old culture of leaf of *Cycas rumphii* on MS + 1 µM 2,4-D + 2 µM BAP showing shoot buds (× 4.6). *b*, 15-week-old culture of leaf of *Zamia furfuracea* on MS + 1 µM NAA + 2 µM Kn showing a somatic embryo (× 4.7). *c*, 5-week-old sub-culture of embryo of *Cycas circinalis* on MS basal medium showing bulbil having leaves with curved tip (× 2.4). *d*, *Ephedra foliata* embryo explant culture; callus after transfer from MS + CW + 1 µM 2,4-D + 5 µM Kn to basal medium showing elongated shoot buds and somatic embryos after 1 week (× 3.4). (sb, shoot bud; se, somatic embryo.)

NAA + Kn, profuse amount of callusing occurred but only roots were differentiated. Treatments with NAA + BAP induced good callusing followed by the differentiation of roots and shoot buds. Further development of these structures remained arrested in the same medium. However, when portions of the callus were transferred to the MS basal medium, the *in vitro* regenerated adventitious shoots could be rooted on a medium containing 5 µM IAA. The somatic embryos germinated on the basal medium to form 'emblings'. *In vitro* rooted-shoots and emblings were transferred to pots containing sterilized mixture of coarse sand and garden soil (1 : 1). During the process of gradual hardening, nearly 70% plants survived.

Rinaldi and Leva[12] cultured embryos of *C. revoluta* and reported organogenesis from epicotyl explants. The present report describes regeneration ability of embryo of *C. circinalis* which showed formation of mini plantlets or bulbils arising from the nodular structures in the hypocotyl portion. Other cycads in which shoot formation or somatic embryogenesis from cultured embryos has been possible are mainly the species of *Zamia*[13] and *Encephalartos*[14]. So far, the formation of plantlet has not been possible.

Somatic embryogenesis using leaf explants has been reported in *Ceratozamia mexicana*[5], and *C. hildae*[6]. The present work is the first report on the leaf culture of cycads; *C. rumphii* exhibited caulogenesis and *Z. furfuracea* showed somatic embryogenesis. The use of somatic tissues in cycads opens up the possibility of mass-scale propagation from leaf tissues. Protocols have to be developed for rapid micropropagation of these slow-growing cycads. Plants, if produced, could be used for *in vitro* conservation of endangered germplasm, and their re-introduction into natural habitats.

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