

## REGENERATION OF SOMATIC EMBRYOS AND PLANTLETS FROM STEM CALLUS PROTOPLASTS OF SANDALWOOD TREE (*SANTALUM ALBUM* L)

V. A. BAPAT, RAVINDER GILL and P. S. RAO

Bio-Organic Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

### ABSTRACT

Protoplasts were successfully isolated from various explants such as leaf mesophyll, stem and hypocotyl callus of *Santalum album*. Protoplasts isolated from stem callus underwent divisions, formed colonies and regenerated somatic embryos which developed into plantlets.

### INTRODUCTION

SEVERAL reviews have amply demonstrated the application of protoplast technology for making parasexual crosses between species where hybridization through conventional breeding proved difficult<sup>1-3</sup>. In woody plants, sexual hybridization poses certain problems which are not encountered in other plants. Somatic hybridization through protoplast fusion may overcome some of these. In addition, protoclone variability would possibly help selection of suitable genotypes required in tree improvement programmes. However, although success in protoplast isolation, culture and regeneration has been achieved in a large number of herbaceous species<sup>1,2</sup>, investigations on protoplast culture of tree species are relatively few.

We have investigated extensively the morphogenetic potentialities of organ cultures of Sandalwood (*Santalum album* L), a tree species of commercial value in Asia because of its fragrant wood and oil, and have devised a protocol for clonal propagation through tissue culture<sup>4-8</sup>. The present studies were undertaken to explore the regenerative potentialities of hypocotyl, stem callus and mesophyll protoplasts of sandalwood and report the success obtained in this direction. Regeneration of the whole plants from protoplasts derived from cell suspensions of sandalwood is reported elsewhere<sup>9</sup>.

### MATERIALS AND METHODS

#### (a) Protoplasts isolation from hypocotyl callus

Hypocotyl segments (5 mm long) were excised from *in vitro* grown 4-week-old seedlings and were cultured on MS, Murashige and Skoog<sup>10</sup> supplemented with 2,4 dichlorophenoxyacetic acid, 2,4-D or BA, 6-benzylaminopurine (1 mg/l each). Two types of calli were

established (i) non-embryogenic and (ii) embryogenic. One gram callus tissue of both types was transferred to 15 ml conical centrifuge tubes, gently macerated with a glass rod in 10 ml nutrient medium and centrifuged to obtain the pellet. Different enzyme mixtures containing combinations of Cellulase (R-10, Onozuka), Hemicellulase (Sigma), Pectinase (Sigma) and Macerozyme (Kinki Yakult) were employed in various concentrations along with 0.55 M sorbitol and 0.9%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  at 30°C for 7 hr on a metabolic shaker (40 rpm). The pH of the enzyme mixture was adjusted to 5.8. Following incubation, the suspension was passed through a nylon mesh (100  $\mu$ ) and centrifuged at 40 rpm for 3 minutes. The enzyme was decanted and the pellet was washed twice with the nutrient medium. Protoplasts were cultured in liquid or agar-jelled medium at a concentration of 4 to  $5 \times 10^5$ /ml. The cultures were grown under continuous fluorescent light (1000 lux) at  $23 \pm 2^\circ\text{C}$  and with a relative humidity of 55–60%. The medium used to culture the protoplasts has been described earlier<sup>4</sup>. After a week, the cultures were transferred to 10 ml of fresh liquid medium devoid of sorbitol.

#### (b) Protoplast isolation from stem callus

Stem callus was initiated by culturing young juvenile shoots obtained from 30-year old mature trees obtained from the Forest Research Laboratory's Campus, Bangalore, on MS medium supplemented with 2,4-D (1 mg/l) along or in combination with kinetin, Kn (0.2 mg/l). From rapidly growing callus tissues, 1 g of tissue was removed and incubated in an enzyme mixture (10 ml) of cellulase 1% (R-10 Onozuka), macerozyme 0.5% (Kinki Yakult) with 0.5 M sorbitol or mannitol. The pH of the enzyme solution was adjusted to 5.8. After 16 hr of incubation, debris were removed first by sieve and later by 20.5% sucrose. The subsequent washing was done with

sorbitol or mannitol and the protoplast suspensions were pipetted in thin layer into 6 cm plastic petri dishes in various liquid nutrient media (2 ml). After formation of microscopic colonies, osmoticum was reduced to 0.3 M level by transferring the cultures to the fresh medium. Subsequently 2 ml of nutrient medium containing 0.4 % soft agar was incorporated in petri plates containing 2 ml of protoplast suspension.

### (c) Protoplasts isolation from leaf mesophyll

Young leaves (first 3–4 from apex) from mature trees were used for isolating the protoplasts. The leaves were surface-sterilized with 0.1 %  $\text{HgCl}_2$  and washed repeatedly with autoclaved water. The washed leaves were macerated into small pieces in 0.3 M mannitol and incubated in the enzyme solution. Different enzyme combinations of cellulase, macerozyme and hemicellulase were tried. For some treatments leaves were plasmolyzed in 0.3 M mannitol for 1 to 2 hr.

## RESULTS

### (a) Hypocotyl callus

Among the two types of calli, non-embryogenic callus yielded large number of protoplasts compared with the embryogenic tissue. Maximum release of protoplasts could be obtained by the enzyme mixture comprising cellulase (2 %) + pectinase (1 %) + hemicellulase (1 %) along with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.9 %) and sorbitol (0.55 M) (table 1).

Isolated protoplasts plated on MS + 2,4-D (1 mg/l) were solidified with agar or without the liquid medium. The first division occurred after 5 days in culture; thereafter repeated divisions followed rapidly and at the end of 8 weeks, multicellular colonies resulted. When the liquid cultures bearing the protoplasts were agitated on a rotary shaker further divisions followed and at the end of two weeks clearly distinguishable colonies were formed.

### (b) Stem callus

Maximum yield of protoplasts ( $8.73 \times 10^{-6}$ ) was obtained when cellulase (1 %) was combined with macerozyme (0.5 %) with 0.5 M sorbitol or mannitol (table 1).

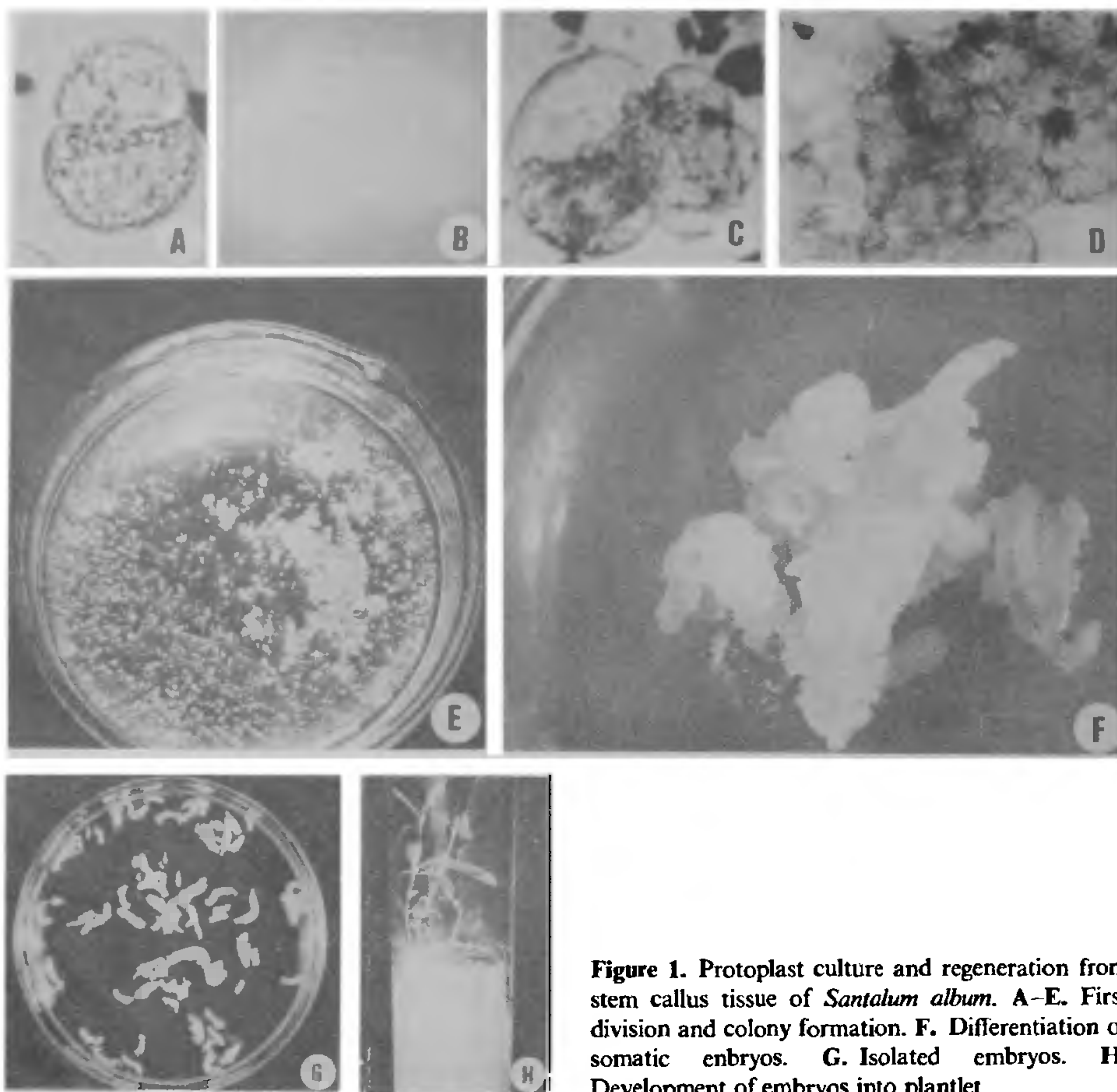
Release of protoplasts was dependent upon the age of callus. Actively growing callus tissue after four weeks of subculture gave maximum number of protoplasts with less debris. Although stem callus dif-

**Table 1** Maximum release of protoplasts from different explant sources of *Santalum album*

Source	Enzyme Combination %	Incubation period (hr)	Yield of Protoplasts
Hypocotyl callus	C(2) P(1)	7	moderate
	C(2) P(1) H(1)	7	high
Stem callus	C(1) M(0.5)	16	intense
	C(2) M(0.5)	16	high
Leaf	C(2) M(1)	8	love
	C(2) M(1) H(1)	8	high

C = cellulase; P = pectinase; H = Hemicellulase; M = Macerozyme.

ferentiated numerous embryos upon transfer to MS + indole acetic acid, IAA (1 mg/l) + BA (1 mg/l), the non-differentiated callus grown on MS + 2,4-D (1 mg/l) was used as a source tissue for protoplast isolation since as in hypocotyl callus, yield of protoplasts was better from non-differentiated callus compared with the embryogenic callus. The cultured protoplasts regenerated cell wall within 36 to 48 hr which was detected by the asymmetrical shape assumed by viable protoplasts as well as by calcofluor staining. Increase in size and formation of prominent nuclei and cytoplasmic strands were observed in the protoplasts which regenerated cell wall and underwent divisions. First division occurred between 4th and 5th day (figure 1A). About 60–70 % protoplasts divided and approximately 50 % of such divided protoplasts showed sustained divisions leading to colonies (figure 1, B, C, D). Among the several nutrient media tested such as MS, V 47<sup>11</sup>, B5<sup>12</sup>, VKM<sup>13</sup> and modified V 47 (table 2) medium containing V 47 major salts, MS minor salts and iron, LS vitamins<sup>14</sup>, sucrose (2 %), glucose (2 %), powdered activated charcoal (0.1 %) and hormones: 2,4-D, naphthalene acetic acid, NAA and BA (1 mg/l) each. The last one was found to be the most suitable medium for enhanced and sustained divisions of isolated protoplasts. Addition of charcoal (0.1 %) proved effective. The colonies grew rapidly and within 8 to 10 weeks developed into microcalli (figure 1E). Subsequent transfer of calli to MS + IAA (1 mg/l), MS + IAA (1 mg/l) + BA (1 mg/l), 1/2 MS + IAA



**Figure 1.** Protoplast culture and regeneration from stem callus tissue of *Santalum album*. A-E. First division and colony formation. F. Differentiation of somatic embryos. G. Isolated embryos. H. Development of embryos into plantlet

(1 mg/l) and 1/2 MS+Coconut milk, CM (10% v/v)+casein hydrolysate, CH (500 mg/l) induced the formation of somatic embryos (figure 1 F, G). The intensity of embryo induction was maximum on an half strength MS+IAA (1 mg/l). The embryos developed into plantlets (figure 1H).

#### (c) Leaf

An enzyme combination of cellulase (2%) + macerozyme (1%) + hemicellulase (1%) containing 0.8 M mannitol gave very satisfactory yield of protoplasts

(table 1). Different incubation periods were attempted and after 8 hr, best yield of protoplasts was achieved. Interestingly release of protoplasts increased with increase in level of osmoticum upto 1 M. However, leaf mesophyll derived protoplasts have so far not shown any divisions. Further trials with various media are in progress.

#### DISCUSSION

The results reported here have shown that protoplasts of sandalwood under the experimental con-

**Table 2** Composition of the culture medium for protoplasts culture of *Santalum album* isolated from stem callus

Major elements (mg/l)		Iron and minor elements (mg/l)	
NH <sub>4</sub> NO <sub>3</sub>	1444	FeSO <sub>4</sub> 7H <sub>2</sub> O	27.85
KNO <sub>3</sub>	1480	Na <sub>2</sub> EDTA	37.25
MgSO <sub>4</sub> 7H <sub>2</sub> O	984	KI	0.83
CaCl <sub>2</sub> 2H <sub>2</sub> O	735	H <sub>3</sub> BO <sub>3</sub>	6.2
KH <sub>2</sub> PO <sub>4</sub>	68	MnSO <sub>4</sub> 4H <sub>2</sub> O	22.3
		ZnSO <sub>4</sub> 7H <sub>2</sub> O	8.6
<i>Organic addenda</i>		Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.25
Thiamine HCl	1	CuSO <sub>4</sub> 5H <sub>2</sub> O	0.025
Nicotinamide	1	CoCl <sub>2</sub> 6H <sub>2</sub> O	0.25
Riboflavin	0.1		
Ca pantothenate	1	<i>Carbon source</i>	
Ca pantothenate	1	Sucrose	20.0
Biotin	1	Glucose	20.0
Choline Chloride	1		
Folic acid	1	<i>Hormones</i>	
Pyridoxine HCl	1	NAA	1
		2,4-D	1
		BA	1
Inositol	100	<i>Other</i>	
		Charcoal	1000

\* Mannitol/sorbitol was added as an osmoticum and osmometer was used to adjust the proper osmotic value of the medium (700 to 750 mOsm), pH was 5.5 prior to the autoclaving.

ditions described above can be manipulated to regenerate into whole plants. This is significant, as protoplast culture work has been attempted only in a restricted number of tree species.

Effects of activated charcoal on the growth and the differentiation have been reported in many tissue cultures. For example, increase in the induction of embryogenesis and root formation has been demonstrated in pollen cultures<sup>15</sup>. The fact that the activated charcoal can adsorb inhibitory substances accounts for the enhanced and sustained divisions of cultured protoplasts from stem callus of *Santalum*. Sugawara *et al*<sup>16</sup>, reported similar results in protoplast culture of *Marchantia*. Release and accumulation of unwanted substances in the medium by regenerating protoplasts has been detected in tobacco<sup>17</sup>.

Profound influence of source tissue on the yield and viability of isolated protoplasts was observed in the present study and those protoplasts derived from stem callus and cell suspensions (established from stem callus) only regenerated into somatic embryos and plantlets<sup>9</sup>. Smith and McCown<sup>18</sup> have reported a correlation between source of tissue and protoplasts yield in woody species.

This demonstration of totipotency and embryogenesis of tree protoplasts reveals that cell and protoplast cultures of trees are similar to other herbaceous species in which protoplasts have been cultured successfully.

This should stimulate vigorous and sustained efforts with other tree species so that the techniques of parasexual hybridisation and genetic modification can be adopted for tree breeding and improvement.

30 May 1985

1. Vasil, I. K. and Vasil, V., *Int. Rev. Cytol. Suppl.*, (ed) I. K. Vasil, New York, 1980, **11b**, p. 1.
2. Rao, P. S., *Embryology of vascular plants*, (ed.) B. M. Johri, Berlin, 1982, p. 231.
3. Cocking, E. C., Davey, M. R., Pental, D. and Power, J. B., *Nature (London)*, 1981, **293**, 265.
4. Rao, P. S. and Bapat, V. A., *Can. J. Bot.*, 1978, **56**, 1153.
5. Bapat, V. A. and Rao, P. S., *Ann. Bot.*, 1979, **44**, 629.
6. Rao, P. S. and Bapat, V. A., In: *Proce. Natl. Symp. BARC*, (eds) P. S. Rao, M. R. Heble and M. S. Chadha, Bombay, 1980, p. 206.
7. Bapat, V. A. and Rao, P. S., *Proc. Indian Acad. Sci. (Pl. Sci.)* 1984, **93**, 19.
8. Rao, P. S., Bapat, V. A. and Mhatre, M., *Proc. Indian Natl. Sci. Acad.*, 1984, **B50**, 196.
9. Rao, P. S. and Ozias-Akins, P., *Protoplasma*, 1985, **124**, 80.
10. Murashige, T. and Skoog F., *Physiol. Plant*, 1962, **15**, 473.

11. Binding, H., *Z. Pflanzenphysiol.*, 1974, **74**, 327.
12. Gamborg, O. L., Miller, K. A. and Ohyama, K., *Exp. Cell Res.*, 1968, **50**, 151.
13. Binding, H. and Nehls, R., *Z. Pflanzenphysiol.*, 1977, **85**, 279.
14. Lin, M. and Staba, E. J., *Lloydia*, 1961, **24**, 139.
15. Tyagi, A. K., Rashid, A. and Maheshwari, S. C., *Physiol. Plant.*, 1980, **49**, 296.
16. Sugawara, Y., Mori, K., Matsushima, H. and Takeuchi, M., *Z. Pflanzenphysiol.*, 1983, **10**, 275.
17. Meyer, Y. and Cooke, R., *Planta*, 1979, **147**, 181.
18. Smith, M. A. L. and McCown, B. H., *Plant. Sci. Lett.*, 1982, **28**, 149.

---

## ANNOUNCEMENT

---

### AWARD OF RESEARCH DEGREES

**University of Cochin, Cochin** – Ph.D. (Physics) Smt. C. K. Valsala Kumari, (Title: '*Growth and characterisation of tin dichalcogenide crystals*') Ph.D (Marine Sciences) Smt. Mercy Thomas (Title: '*Studies on portunid crabs (Crustacea: Decapoda: Brachyura)*')

**Gulbarga University, Gulbarga** – Ph.D (Mathematics) Shri K. H. Gopal Krishna, (Title: '*Viscoelastic flow through dusty media*') Ph.D (Chemistry) Shri N. J. Kalaskar (Title: '*Synthesis and pharmacological evaluation of some heterocyclic compounds*')

**The M.S. University of Baroda, Vadodara** – Ph.D (Chemistry) Shri Umendra Dayal (Title: '*Studies on Organic metal complexes*'), Ph.D (Geology) Shri Girish Chandra Agarwal (Title: '*Further studies on tertiary and quaternary sequences of south Gujarat with reference to their structural style and geomorphic expressions*')

**Utkal University, Bhubaneswar** – Ph.D. (Physics) Shri Prasanta Kumar Jena (Title: '*Investigations in quantum electrodynamics and chromodynamics*'). Ph.D (Chemistry) Shri Shashadhar Samal (Title: '*Grafting vinyl monomers onto wool and silk fibers*') Ph.D (Botany) Shri Radhe Shyam Kashyap (Title: '*Comparative physiology of some pathogenic members of the gymnoascaceae*')

**Sri Venkateswara University, Tirupati** – Ph.D (Physics) Sri M. V. Rama Sarma (Title: '*Pyroelectric, electrooptic and dielectric studies on certain pure and doped ferroelectric crystals*'; Smt K. Swarnalatha (Title: '*Studies in thin film technology—Physical investigations on thin films of oxide mixtures*') Ph.D. (Chemistry) Sri G. Bhaskar Raju (Title: '*Studies on the flotation of fine particles—Electroflotation of chalcopyrite*') Ph.D (Physical Anthropology) Sri K. Rajasekhara Reddy (Title: '*A genetic study of the madigas: A scheduled caste population of Andhra Pradesh, India*') Ph.D (Psychology) Smt D. Jamuna (Title: '*A study of some factors related to adjustment of middle aged and older women*') Ph.D (Botany) Shri C. Venkatramaiah (Title: '*Some physiological studies during fruit growth and development of *Lagenaria ciceraria* (Standl) and *trichosanthes anguina**') Ph.D (Zoology) Smt N. Geethanjali (Title: '*Role of prolactin on testicular metabolism of albino rats during puberal transition*') Shri M. Balavenkatasubbaiah (Title: '*Studies on the effects of some selected heavy metals on hydromineral balance and energy metabolism in freshwater apple snail, *pila globosa* (Swainson)*') Shri S. Venkata Reddy (Title: '*Studies on the effects of carbamate insecticide carbaryl on aspects of biology of silkworm, *Bombax mori* L*')