DISCUSSION

The tissue culture studies carried out on Pterocarpus marsupium and Shorea robusta with the sole objective of unraveling their specific amenability to this useful technique has provided interesting data that has been described earlier and is being discussed below.

i) CALLUSING:

Callus has been defined as the unorganized mass of tissue. It's formation occurs as a result of dedifferentiation of explant cells and their proliferation. Yeoman (1970) had divided the callus development into three stages during which explant cells initiate dedifferentiation, begin to divide and redifferentiate again. The proliferative capacity of the explant may be governed by several factors including endogenous concentrations of growth regulator(s) at the time of excision, capacity to synthesize growth regulators and essential metabolites (George and
Sherrington (1984) and sensitivity to exogenous growth regulators (Trewavas 1982). Generally the callus from juvenile plants grow faster than the callus from mature plants (Flick et al. 1983).

The globular and heart-shaped embryos of *P. marsupium* failed to form callus on the media containing NAA, 2,4-D or kinetin in combination with either of these auxins. The failure of callus formation from the cells of these two types of explants may be attributed to their small size, stress due to excision and to the complex nutritional requirements. The nutritional requirements of young embryos have been reported to be complex (Raghavan and Torrey 1963, Hu and Wang 1986). It appears that globular and heart-shaped embryos being small in size and having complex nutritional requirements could not establish in culture and ultimately turn albino due to loss of green pigments. The torpedo-shaped embryos of *P. marsupium* because of their larger size and comparatively less complex nutritional requirements established on the medium and showed callusing response to NAA (Table 1) and 2,4-D (Table 3). NAA induced white (C1 type) and 2,4-D induced brown (C2 type) callus. Kinetin in combination with NAA as well as with 2,4-D
slightly increased callusing intensity of torpedo-shaped embryos (Tables 2 & 4).

Hypocotyl explants of *P. marsupium* seedlings showed better callusing response to both, IAA and NAA, than cotyledon explants (Tables 5 & 11).

Cotyledon explants showed increased callusing intensity when placed on the medium containing combination of BAP (5.0 mg/l) and IAA or NAA, but the hypocotyl explants did not show any significant change in callusing intensity when BAP was included in the media containing IAA or NAA (Tables 7 & 13).

Combination of BAP with NAA showed significant change in callus morphology. It induced C1 and C2 type callus from hypocotyl and cotyledon explants. The combination of BAP with IAA induced only C1 type callus from both types of explants (Tables 8 & 14).

Callus formation from various explants of *S. robusta* appears to be dependent on the type of explants and kind and concentration of exogenous auxin(s). Explant age doesn't seem to have any definite influence on callusing. Early embryos and embryonic axis of mature embryos were found to exhibit good callusing response to modified MS medium supplemented with 2,4-D, followed by NAA and IAA.
(Tables 22, 28 & 34). Responses of cotyledons and embryonic axis of late embryos were different from that of early embryos and embryonic axis of mature embryos. Late embry explants (cotyledons and embryonic axis) showed best callusing on modified MS medium supplemented with 2,4-D followed by IAA and NAA. In these explants, callus induction was lacking on modified MS medium containing NAA (Tables 22, 28 & 34).

2,4-D appears to be the best auxin for the development of callus from the four types of explants of *S. robusta*. Callus forming efficiency of 2,4-D has been reported higher than IAA and NAA by Singh and Bansal (1993) in *Madhuka indica*. It may be attributed to higher mobility of 2,4-D and lower rate of oxidation and conjugation than that of IAA and NAA (Jacobsen 1983).

Combinations of 2,4-D and NAA enhanced callus formation from cotyledons of late embryos. 2,4-D at 5.0 mg/l concentration showed 50% callus induction frequency and ++ callusing intensity which got increased to 100% and +++ at 4.0 mg/l 2,4-D + 0.5 mg/l NAA (Table 36). The embryonic axis of late and mature embryos showed better callusing at lower levels of 2,4-D by inclusion of NAA in
The most significant effect of the combination of 2,4-D and NAA was found on callus morphology. A variable response in callusing of different explants of *S. robusta* was evidenced in differentiable callus types produced by them. The early embryos produced 5 callus types whereas the cotyledons of late embryos, the embryonic axis of late embryos and of the mature embryos produced only 3 callus types each (Table 36). The early embryos and embryonic axis of mature embryos produced heterogeneous callus whereas cotyledons and embryonic axis of late embryos produced homogeneous callus at similar combinations of 2,4-D and NAA. Colour, texture, and appearance related variations in callus morphology have been reported by several workers (Narayanaswamy 1977, George and Sherrington 1984, Sudha Devi and Nataraja 1987 and Singh and Ransal 1993). Development of homogeneous or heterogeneous callus has been attributed to the proportion of competent cells in explants and their proliferative capacity, site of callus formation, age of explant and the size of explant (Zankowski and Rost 1990).

Inclusion of cytokinin in low concentration in auxin-containing callus inducing medium has been suggested by several workers (Narayanaswamy 1977). Cytokinins are known to stimulate cell proliferation (Jacobsen 1983).
Perhaps due to this reason better callusing was induced in some explants of *P. marsupium* and *S. robusta* by inclusion of RAP in the medium.

ii) **DIFFERENTIATION**:

The callus raised from embryos of *P. marsupium* did not show differentiation on medium containing 2,4-D, TAA, 2,4-D + BAP or TAA + BAP (data not given). Root differentiation occurred in callus derived from cotyledon and hypocotyl explants of *P. marsupium* on MS medium containing different concentrations of IAA or NAA. Cotyledonary callus showed more rooting than hypocotyl callus, irrespective of the kind of auxin tested. Rhizogenesis has been reported to be the most frequently occurring differentiation in callus than any other form of regeneration, irrespective of source of callus (Narayanswamy 1977). Rooting from callus precedes caulogenesis in many plant species; but in this case only rhizogenesis was observed, caulogenesis never occurred in any of the hypocotyl or cotyledon callus. BAP in combination with IAA or NAA suppressed rooting considerably. High conc. (5.0 mg l⁻¹) of RAP in combination with low concentrations (0.5 to 1.0 mg l⁻¹) of IAA or NAA produced callus without rooting. Skoog and Miller (1957) demonstrated that
the relative ration of cytokinin to auxin determine the nature of organogenesis in tobacco pith tissue; high conc. of cytokinin caused shoot bud differentiation, whereas high levels of auxin favoured rooting. In the present study, levels of auxin were decreased in medium II or conc. of cytokinin were increased in medium II but they failed to elicit caulogenic response. Rao et al. (1990) reported shoot bud induction on MS + 2.0 mg l\(^{-1}\) IAA + 1.0 mg l\(^{-1}\) BAP from root callus of P. marsupium raised on MS + 2.0 mg l\(^{-1}\) NAA + 1.0 mg l\(^{-1}\) BAP. It appears that the calli of hypocotyl and cotyledon explants (without axillary bud region) require further manipulation in nutrient medium and physical conditions for stimulating caulogenesis.

Early embryos, cotyledon of late embryos, embryonic axis of late embryos, and embryonic axis of mature embryos of S. robusta showed direct and indirect rhizogenesis on modified MS medium containing TAA, NAA or combinations of BAP with IAA or NAA. In general, the rhizogenesis response in four types of embryos explants of S. robusta, was better than embryo and seedling explants of P. marsupium (Table 2, 6 & 24).

Three types of structures of undefined morphology were differentiated from the callus of embryo ex-
plants of S. robusta on modified MS medium containing various combinations of 2,4-D, NAA and BAP. The S2 and S3 structures were flat and albinic. The S3 structures developed a brown band at their periphery. The browning appeared after sometime even in very small S3 structure (0.5 cm) but not in S2 structures. Combinations of plant growth regulators are important in initiating and maintaining growth and in fostering somatic embryo development (Ammirato 1983). Equally important is the sequence of media (Steward et al. 1967). In the present case combinations of 2,4-D + NAA + BAP were used in medium I and their levels were changed in the medium II (complete withdrawal of auxins from medium II did not elicit any response, therefore data has not been given) but morphogenic response was only observed in medium II containing same concentration of the plant growth regulators (i.e. PGR conc. present in medium I) (Table 39). Somatic embryo induction and development on the same medium has been reported by Inamdar et al. (1990) in Crataeva nurvala. The morphogenic response was found restricted to the medium containing 2.0 - 4.0 mg l\(^{-1}\) 2,4-D + 0.5 - 4.0 mg l\(^{-1}\) NAA + 0.5 to 4.0 mg l\(^{-1}\) BAP. Further manipulation in the medium, particularly change in nitrates,
phosphate and sucrose concentration, addition of amino acids or change formulation of nutrient medium did not help in altering the morphology of these structures (Tables 40, 41, 42 & 43).

These structures may be abnormal somatic embryos. Probably the abnormality initiated during globular stage and instead of forming heart-shaped structure, they got transformed into flat and albinic structure. However, further investigation is required to find out the exact stage at which abnormality starts and appropriate modifications in the composition of medium and sequence of media should be devised to achieve normal somatic embryos for in vitro propagation of this important tree species of India. Abnormalities during development and germination of somatic embryos have been reported by Goebel-Tourand et al. (1993) and Jha (1988).

iii) SHOOT BUD ESTABLISHMENT, SHOOT MULTIPLICATION AND ROOTING:

Best shoot bud induction from cotyledonary node, juvenile node (from 2-month-old plants grown in poly-packs in wire-mesh house) and mature node (10-year-old tree) occurred on MS + 0.05 mg l⁻¹ BAP + 0.5 mg l⁻¹ kinetin, MS + 0.05 mg l⁻¹ BAP + 0.5 mg l⁻¹ kinetin and MS + 2.5 mg l⁻¹ BAP,
respectively. Mature nodes showed good response on MS + 0.05 mg l\(^{-1}\) RAP + 0.5 mg l\(^{-1}\) kinetin also. On this medium, maximum 10 cm shoot length was observed. Inclusion of cytokinin in stage-1 medium has been suggested by Bergman et al. (1984) and Hu and Wang (1983). Occasional shoot bud induction has been reported from cotyledon and hypocotyl segments of *P. marsupium* by Rao et al. (1990). It is presumed that in some of the cotyledon and hypocotyl explants axillary bud regions were present which contain competent cells for shoot bud induction. Shoot bud induction has been reported from cotyledonary node of *P. santalinus* (Patri et al. 1988). In many cases, excision of longest shoot was found to induce adventitious shoots from axillary region, which may be due to the loss of apical dominance (Shanker and Mohan Ram 1990).

Shoots derived from cotyledonary-node, juvenile node and mature node of *P. marsupium* showed best shoot multiplication on MS medium free from PGR. Reduction in the level of cytokinin has reported to be favorable for *in vitro* shoot multiplication and shoot elongation of several forest trees (Youn & Ohba, 1990).

Best rooting in cotyledonary node derived
shoots occurred on MS + 2.5 mg/l IBA and in juvenile node
derived shoots on 1/2 MS + 2.5 mg/l IBA. Rooting in cotyle-
donary node derived shoots of P. marsupium has been
reported by Kapade and Mishra (1993) and P. santalinus by
Patri et al. (1988). It appears that the nutritional re-
quivalents of shoots derived from cotyledonary node and
juvenile nodes vary considerably. Rooting could not be
induced in microshoots derived from mature nodes perhaps due
to seasonal variation in rooting. Reddy and Srivasuki
(1997) reported seasonal variation in rooting of microshoots
derived from mature explants of P. santalinus.

Further investigation is required to accom-
plish rooting in shoots derived from mature nodes of P.
marsupium.

Best multiple shoot bud induction from late
embryos of S. robusta occurred on MS medium supplemented
with 1.0 mg/l NAA + 2.5 mg/l BAP. Ivanicka and Pretova
(1980) reported shoot bud proliferation from immature embry-
os of early ripening cherries on nutrient medium containing
BAP, GA, IBA and phloroglucinol and in vitro rooting of the
isolated shoots with NAA containing medium. Hisajima (1982)
stimulated multiple shoots from almond embryos by using BAP
containing medium and also achieved multiple shoot formation
from cut shoots. Rooting in late embryo derived was achieved on 1/2 MS medium containing 5.0 mgl⁻¹ NAA. Sharma and Chaturvedi (1997) reported shoot bud induction from hypocotyl segments and nodal stem segments of seedlings of *S. robusta* on medium containing major salts of MS + trace elements of Nitsch medium + 0.5 mgl⁻¹ BAP + 0.1 mgl⁻¹ NAA and 15 mgl⁻¹ adenine sulphate + antioxidants. They achieved rooting on medium containing 0.5 mgl⁻¹ NAA. Late embryos appear to be good explants for initiating shoot bud multiplication for clonal multiplication of one of the most valuable timber and oil-yielding tree species of India.

(iv) VARIATION IN PROTEIN PROFILE AND PEROXIDASE ISOENZYMES

Qualitative differences were observed between the polypeptide bands of undifferentiated and differentiated calli from cotyledon explants of *P. marsupium*. The polypeptide bands of undifferentiated and differentiated calli from late embryonic axis of *S. robusta* showed quantitative differences. The peroxidase isoenzymes exhibited qualitative differences between differentiated and undifferentiated calli of both the species. Variation in isoenzyme pattern and protein profile may be attributed to the differences in
the metabolic patterns of different type of calli of two species. Isozyme pattern appears to be better marker than protein profile for different type of calli of S. robusta. Occurrence of specific protein and isoenzymes have been reported in organogenic and embryogenic calli in rice (Chen & Luthe 1987), sorghum (Wozniak & Partridge 1988 and Coppens & Dewitte 1990), Gardenia jasminoides & Dendranthera ma morifolium (Shen 1990) and maize (Rao et al. 1983 and 1990).