IV. DISCUSSION

Somatic embryogenesis has been reported to be influenced by 50 substances belonging to 10 classes of organic and inorganic compounds, naturally occurring plant juices, plant extracts and some physical factors. Extensive studies on somatic embryogenesis have indicated that hormones and source of nitrogen are among the factors which influence the process most (Rangaswamy, 1986). However, what makes a cell competent for embryogenesis is still empirical.

Somatic embryo has been defined as a non-zygotic embryo arising from a single cell, with no vascular connections with maternal tissues (Street and Withers, 1974; Haccius, 1978). In many cases, on the other hand, apparently bipolar embryoids are formed from aggregates of cells (Raghavan, 1976; Tisserat et al, 1979). Thus an embryo is ab initio a bipolarised entity (Rangaswamy, 1986), bounded by cuticle, since physical or physiological isolation of the cell/cell group is considered prerequisite for them to embark upon the embryogenic pathway (Handro et al 1972; Street, 1979; Vasil and Vasil, 1982; Kononowicz, et al 1984).

The involvement of hormones especially auxins and cytokinins in the process of somatic embryogenesis is well
documented. (Ammirato, 1983; Rangaswami, 1986). Of the auxins tried, 2,4-D has been proved extremely useful, having been employed in nearly 60% of successful embryogenic cultures (Evans et al, 1981). Most protocols call for the exposure of a tissue explant to synthetic auxin, especially 2,4-D, at the initial stage of somatic embryogenesis as it is critical for the cells which are programmed or programmable to express their totipotency respond by dividing (Krikorian et al, 1986). The system we examined, Sapindus trifoliatus (soapnut) required 2,4-D as a prerequisite for the induction of embryogenesis from the callus derived from leaf explants.

Numerous tissue culture experiments have substantiated the pivotal role of exogenously applied auxins mainly 2,4-D in the triggering of embryogenic pathway of somatic cells both in dicots (Ammirato, 1983; Borge et al, 1990) as well as in monocots (Vasil, 1980, 1981, a, b). Auxin can promote calcium efflux (de Guzman and Fuente, 1984) which in turn leads to cell wall loosening. A certain degree of tissue fragmentation is mandatory for the induction of embryogenesis (Haplarin and Jensen, 1967). This is evident in the case of soapnut system in which embryo induction takes place only when the callus becomes friable (Fig. 2 and 3). Auxin is the most important factor for the regulation of induction and development of embryogenesis and it has different effects in different phases of embryogenesis.
(Komamine et al, 1990). It is indeed necessary for competent cells to express totipotency. However, continued presence of 2,4-D at the same concentration i.e. 2 ppm in the media for soapnut callus inhibited the embryogenic calli from undergoing further differentiation. So the friable embryogenic calli of soapnut had to be transferred to a medium with reduced level of 2,4-D (0.5 ppm). On this medium (Stage I, Table 2) the embryoids at globular stage became visible. Numerous studies on different systems have also revealed the requirement of auxin for embryo induction followed by auxin free environment for further differentiation as in carrot (Sung and Okimoto, 1981), white spruce (Attree, et al, 1987), *Picea glauca* (Tremblay, 1990) etc. A lower level of auxin was found to be necessary for the differentiation of embryogenic calli in this system. Since it is a tree species the mechanism might be more complex than the other systems. However, auxin was found to be inhibitory for further development i.e. from globular to heart-shaped embryoids in this system. Certain findings at molecular levels suggest the role of auxins as signal carrier which can trigger the synthesis of certain embryo specific proteins, but its continued presence also prevents the continued synthesis of these proteins necessary for embryogenesis (Raghavan, 1983). Earlier while studying

* see Chapter III Results
nucleic acid metabolism during the induction of somatic embryogenesis in carrot cell suspensions, it was suggested that the control is post-transcriptional. The m-RNA inducing somatic embryogenesis is synthesised in the presence of auxin (2,4-D) and is translated, when the auxin is removed from the medium (Sengupta and Raghavan, 1978). Recently Komamine et al (1990) also demonstrated the essentiality of auxin for the expression of protein to maintain totipotency in carrot cell suspension cultures.

Eventhough there is no compelling evidence for a universal requirement of cytokinins for somatic embryogenesis, in a number of systems, it is found to be essential for somatic embryogenesis. In certain systems somatic embryo development could be accomplished without cytokinin as in carrot (Ammirato, 1984). But, some reports demonstrate the requirement of cytokinins for the maturation of somatic embryos (Fujimura and Komamine, 1982; Ammirato,1984). While certain protocols call for the requirement of only one cytokinin especially BAP as in Picea glauca (Attree et al, 1987), Albizia richardiana (Tomar and Gupta, 1988) etc. in the induction medium for somatic embryogenesis along with auxin, certain systems
demand both (Kn and BAP) for the induction of embryoids like *Hevea brasiliensis* (Carren and Enjalric, 1982). Contrary to this, the presence of cytokinins is found to be inhibitory for auxin induced somatic embryogenesis in *Petunia hybrida* (Rao, *et al.*, 1973), carrot (Kamada and Harada, 1979), *Panax ginseng* (Chang and Hsing, 1980) and *Solanum melogena* (Gleddie *et al.*, 1983).

In the present investigation BAP was found essential because the development of globular embryo into heart-shaped embryo was achieved only when BAP was incorporated at 2 ppm along with 0.5 ppm Kn (stage 2, Table 2* Fig.5 and 6) in auxin free medium. Moreover, Kn at the con of 0.5 ppm was maintained in all the three stage (Stage 0, Stage 1 and stage 2, Table 2)* media. This shows cytokinin evokes promotory effect on embryogenesis in soapnut system. Action of cytokinin may be involved in promotion of cell division (Komamine *et al.*, 1990).

For the maturation, embryoids obtained from tender leaf callus were treated with a tryptophan analogue 5-MT (5 methyl tryptophan) after incubation of embryoids on MS basal

* see chapter III Results
medium for one week as reported by Desai et al (1986) (Table 1). This did not however favour the maturation of embryoids appreciably, hence the frequency of germination remained low (\(\sim 4\%\)).

Experiments imposing stress were carried out to foster maturation with a view to increase the frequency of germination. These involved osmotic and physiological stress: a) addition of sucrose, b) addition of mannitol and c) incorporation of ABA. These three were added separately into stage 3 media as shown in revised protocol (Table 2).

The overall osmotic con of the medium is known to exert profound influence on morphogenesis (Ammirato, 1986). Elevated osmotic levels achieved by the addition of sucrose have favoured the somatic embryo development in a number of plant species including that of pappaya (Litz and Conover, 1983), cacao (Pence et al 1981), millet (Vasil and Vasil, 1981b), etc. When it is added within the range of 4-8\% in the maturation media of the present system, around 20 \% embryoids underwent germination after transfer to the germination media (stage 4, table 2). This favourable

* see chapter III Results
effect can be attributed to the prevention of precocious germination of embryoids (Ammirato, 1986). Eventhough some enhancement was achieved in the rate of germination of soapnut embryoids, it was not high enough.

On the basis of several reports on the modification of embryo maturation by adding various sugar alcohols such as sorbitol (Ammirato and Steward, 1971) or mannitol (Altman et al 1990), the effect of mannitol was checked upon embryo maturation in the soapnut system. Four con were tried, but none of the con was found to be favourable for embryo maturation. This indicated that sucrose was not only acting as an osmoticum but also provided a source of energy.

Several investigators have shown the promotory effect of ABA on somatic embryo development eg. in millet (Vasil and Vasil, 1982), bean (Allavena, 1984) and in stationary cultures of maize (Earle and Grace, 1985) as well as on embryo maturation as reported in carraway (Ammirato 1974, 1977), carrot (Ammirato, 1983) and in interior spruce (Roberts et al, 1990). ABA is demonstrated to inhibit recallusing of developing embryos, aborrent embryo formation and also precocious germination. These observations prompted us to study its effect on soapnut
embryoids. When it was added at the con of 1 mol m$^{-6}$ to stage 3 media (Table 2) the rate of germination increased to 50%.

In our present investigation we used three different criteria to document the maturation of soapnut embryoids: 1) morphological - normal somatic embryoids possess well defined axis with two cotyledons; 2) biochemical - normal somatic embryos registered higher fresh weight and protein content than the aberrant ones; 3) physiological - normal somatic embryos germinated into seedlings.

Eventhough gain in fresh weight and protein content was lower in ABA treated embryoids than in embryoids grown in media with higher sucrose levels (4% and 8%), around 50% of ABA treated embryoids underwent germination. Compared to the control (embryoid grown in 2% sucrose in stage 3 media as in Table 2), there were three fold and 34 fold increase in the fresh weight and protein content in ABA treated embryoids respectively. The ability of ABA to promote the accumulation of embryo specific proteins in zygotic embryos is well established. Crouch and Sussex (1981) demonstrated that ABA promoted embryo growth and protein accumulation in

* see chapter III Results
in vitro cultured rape seed embryos. Later Ackerson (1984) provided the evidence that ABA is indeed necessary for normal embryogenesis. From their studies it was concluded that ABA selectively inhibited the synthesis and translation of certain m-RNAs only. It is conceivable therefore that ABA does not restrict storage protein synthesis (Ackerson 1984), but suppresses at the same time the synthesis of germination enzymes (Ihle and Dure, 1972) thereby inhibiting precocious germination. The enhancement in the frequency of germination of ABA treated embryoids can be attributed to the increased protein content compared to the control embryoids, because it is already shown that ABA can influence the synthesis of embryo specific m-RNAs (Dure, 1985; Galan et al. 1986; Quatrano, 1986). These data were also supported by other observations that ABA not only promoted the accumulation of storage proteins but also inhibited precocious germination of somatic embryos in interior spruce (Roberts et al. 1990) and microspore derived embryos of Brassica napus (Holbrook et al. 1990), etc.

The promotory effect of sucrose on the maturation of somatic embryos of soapnut might be due to the involvement of ABA in response to osmotic stress. Even though the effect
was less pronounced (as only 20% of somatic embryos underwent germination) the frequency of germination was high compared to the control. Under osmotic stress it was reported that the induction of ABA synthesis is promoted in spinach (Creelman and Zeewart, 1985) and in suspension cultures of grape pericarp (Loveys, et al. 1975). It can be suggested that the effect of increased concentration of sucrose in the medium on protein accumulation and embryo maturation may be the result of ABA synthesis under osmotic stress and this in turn favoured the synthesis of embryo specific proteins (mediated by ABA) followed by germination of embryoids. However, direct addition of ABA in to the media is much more effective in regulating embryo maturation. The same view was echoed by Ammirato (1986) in the case of carrot.

Of the three criteria used in order to document the maturation of somatic embryos of soapnut, only the physiological criterion of germination of embryoids correlates well with maturation of embryoids. Using this criterion we have shown that a high degree of maturation can be induced by providing ABA to the heart shaped embryos. A high degree of germination of such mature embryo make the soapnut system a reliable avenue of micropropagation.
The comparative studies of polypeptides in mature embryoids obtained in various treatments (sucrose and ABA) in stage 3 media revealed broad similarity with those of germinated zygotic embryo (Fig.11). Unlike germinated zygotic embryos of soapnut, ungerminated ones possessed three prominent bands of storage peptides (1, 2, 3, in Fig.11). The low intensity of the band corresponding to the storage peptides in somatic embryos (compared to that of zygotic embryos) indicated lesser production of storage protein in somatic embryos. The low level of synthesis of storage proteins in somatic embryo is of usual occurrence. Data reported for Brassica napus by Crouch (1982) indicated that somatic embryos maximally expressed only one tenth of the level of seed storage protein. This is further substantiated by the finding that the synthesis of 11S protein (a storage peptide) in the somatic embryos of alfalfa, was only about one tenth of level observed in seed (Stuart et al., 1988).

Application of stress treatment (sucrose as well as ABA) at maturation stage could not reveal any marked qualitative changes in protein profile in somatic embryos of
soapnut. The technique we pursued here was based on averaging the embryo population rather than direct measurement of proteins from individual somatic embryos. Perhaps in individual embryos higher levels of embryo specific protein may occur (mediated by ABA). This would call for the development of sensitive techniques such as ELISA (Enzyme-linked immunosorbent assay).

Numerous studies have been carried out with regard to salinity tolerance in plants by making use of in vitro techniques to unravel the mechanism/s as it offers a unique opportunity to study cellular level mechanisms and functions of salt tolerance (Rains, et al 1980; Dix et al,1986; Hasegawa et al 1986). Moreover, cell culture techniques can be used as an alternative approach in improving the response to saline stress since the whole plant breeding system has met with limited success. (Raghava Ram and Nabor 1984; Rains, et al 1986) In our system, we attempted to develop salt tolerant embryoids with a view to produce salt tolerant plants. Moreover, we examined the ion status, growth profile, proline content, sterols and phospholipids to understand the basis of salt tolerance in this system.
As a preliminary step the proembryo subcultured in stage 1 medium (Table 2)* was exposed to salinity in stage 2 medium by incorporating different con of NaCl. After 10 days of incubation, they were transferred to maturation medium (stage 3) containing NaCl at different con. In stage 3 medium they were allowed to grow fully and these embryoids were used for the study of salt tolerance.

An interesting observation made by this study was the promotion of somatic embryo growth by lower con of (25 and 50 mol m$^{-3}$) of NaCl (Table 7).* At 25 mol m$^{-3}$ NaCl treated embryoids became remarkably large and turned green (Fig.12). Although salt tolerant embryoids have been isolated in *Vitis* (Lebrun et al 1983) and in wheat (Galliba and Yamada, 1988), growth of these embryoids was not found to be enhanced by NaCl at any of the con tested.

Data pertaining to ion composition show that somatic embryos of this plant possess the intrinsic capacity to exclude Na$^+$ while, at the same time, it can tolerate high con of Cl$^-$ (Table 8).* Moreover, contrary to many reports (Ben-Hayyim et al 1985; Heuer and Plaut, 1989) somatic embryos of soapnut did not show any reduction of K$^+$ content

* see chapter III Results
in response to salinization (Table 8). Tolerance mechanism used by plants to adapt salinity can be separated into those that allow the growing cells of plants to avoid high ion con and those that permit the cell to cope with high ion con upon exposure to salt (Flowers et al 1977; Greenway and Munns, 1980; Hasegawa et al 1986). Interestingly in soapnut system both the mechanisms (Na⁺ exclusion and at same time tolerance to high level of Cl⁻ ion) seem to be operative. Ion accumulation in response to salinity is to provide osmotic adjustment and turgor to maintain growth. This in turn will lead to ion toxicity and induced ion deficiency (Bernstein and Hayward, 1958; Greenway and Munns, 1980; Yeo, 1983). Interestingly this system exhibits discriminative exclusion of Na⁺, hence it can be conceived that somatic embryos of S. trifoliatus survive the salt stress condition partly by excluding the Na⁺. Comparison between NaCl tolerant and sensitive lines in soybean has revealed that salt tolerant lines excluded Na⁺ more efficiently than the unselected one (Rains, et al, 1986).

The exchange of K⁺ by Na⁺ has been observed in saline environment by plants and this may lead to K⁺ deficiency (Wyn Jones, 1983). So conservation of K⁺ is essential for

* see chapter III Results
metabolic roles and it is partly achieved through compartmentalization (Yeo, 1983). Salt grown embryoids of soapnut maintained $K^+$ at levels equal to or higher than that of non-stressed control. There is an appreciable increase in the content of $K^+$ in somatic embryos grown in $25 \text{ mol m}^{-3}$ and $50 \text{ mol m}^{-3}$ NaCl con. Of the many salts present in culture medium, potassium has been shown to be one of the key elements controlling growth and development of somatic embryos (Ammirato, 1983). Further a number of studies have proved unequivocally that higher levels of potassium dramatically promote growth, accelerate the process of greening and augment the production of fresh weight in intact plants as well as in excised plant tissues (Arnold and Fletcher, 1986). From this information and with the data obtained in present studies, it can be suggested that increased growth observed at low con i.e. $25 \text{ mol m}^{-3}$ and $50 \text{ mol m}^{-3}$ NaCl con could be at least partly due to the ability of somatic embryos to maintain relatively high con of $K^+$ ion under saline conditions.

The data also indicated that somatic embryos could tolerate high levels of $\text{Cl}^-$. Similar studies with whole plant system as in *Eucalyptus cammaludensis* and *E.*
microtheca showed greater accumulation of Cl$^-$ than that of K$^+$ and Na$^+$ (Prat and Fathi-Ettai, 1990). The high con of Cl$^-$ ion in salt grown embryos of soapnut showed a partial shift towards a halophytic mode of salt tolerance.

The level of endogenous proline in somatic embryos was found to increase upon salinization (Table 9). Proline has been advocated as a cytosolute in halophytes (Stewart and Lee, 1974; Treichel, 1975). Furthermore the accumulation of this compound is of near universal occurence in response to water stress (Hsiao, 1973; Wyn Jones 1983). The increase in proline content may be because of the increased rate of synthesis or decreased rate of degradation (Levitt, 1980). The effect of salinity on proline level has been investigated in some salt adapted cell lines. Wataf et al (1983) found that proline content increased in selected Nicotiana tabacum and Nigossili cells, while unadapted cell line accumulated less proline. Ricardi et al (1983) showed greater salt tolerance in the mutants of Spirulina plantens and Daucus carota which overproduced proline. Further, in a recent study by the four hydroxyproline resistant lines of Solanum tuberosum grew better at elevated levels of NaCl con (Van Swaaij, et al 1986). The increased accumulation of

* see chapter III Results
free proline in somatic embryos of \textit{S. trifoliatus} exposed to salinity might have also contributed to the salt tolerance observed in this investigation. The beneficial effects of accumulation of proline as a compatible osmotic solute, as a protein stabilizing or solubilizing factor under limiting cell water conditions and as a source of carbon and nitrogen have been proposed by Handa \textit{et al} (1986); Boggess and Stewart, (1986) and Taylor \textit{et al} (1982) respectively. However, the precise physiological significance of proline in stressed plants has yet to be fully elucidated.

The changes in lipid content and lipid composition in response to salinity are considered to have some adaptive value (Wyn Jones, 1983; Kuiper, 1985; Brown and DuPont 1989). Biomembranes are essential for osmo-regulation and have been proven to be salt sensitive (Marschner, 1974). Regulation of membrane permeability by changes in lipid composition possibly plays an important role in adaptation of plants to salinity (Stuvier \textit{et al} 1981). In most of the studies on lipid changes where whole plants were employed and virtually no information was available on lipid changes in \textit{in vitro} developed callus or cell lines tolerant to NaCl. The results in the present study summarized in Table 10:

* see chapter III Results
illustrate that NaCl significantly increased the levels of free sterols, steryl glycosides, steryl esters and phospholipids. This is in agreement with the findings of Kuiper (1968) in grape varieties and also in bean, barley and sugarbeet (Stuvier et al 1981) in which the contents of sterol esters and free sterols of the roots increased with salt resistance. Free sterols are characteristic of plasmamembrane and tonoplast and are effective in the regulation of lipid membrane stability and in the reduction of passive ion permeability (Kuiper, 1985). Moreover, sterols are believed to be involved in the discriminative exclusion of Na\(^+\) against K\(^+\) as they decrease membrane permeability for K\(^+\) relatively to Na\(^+\) (Scarpa and Gier, 1971). This is evident from the fact that salt grown somatic embryos of soapnut maintained K\(^+\) at levels equal to that of control (Table 8)\(^*\). It can be also said that high levels of free sterols may contribute to salt tolerance. This is supported by the fact that NaCl resistant varieties of grape, plantago and sugarbeet maintained higher levels of free sterols compared with sensitive lines (Kuiper, 1985). Recent studies with *Duneliella salina* (one celled marine alga, extra-ordinarily tolerant to high salinity) pointed towards the role of relatively stable plasmamembrane with

\(^*\) see chapter III Results
definite lipid composition because its cytoplasmic enzymes are sensitive to high NaCl con(Browitzka and Brown, 1974); indicating that plasmamembrane of Dunaliella must not only remain functional under high external con of NaCl but also maintain a permeability barrier against the high NaCl con outside the cell (Peeler et al, 1989).

The data presented (Table 10)* also showed an increase in phospholipids in soapnut embryoids with increased salt levels. Increase in phospholipid content in response to salinity in roots of barley and sugarbeat was demonstrated by Fergusen (1966) and Stuvier et al (1981) respectively.

From the changes in the contents of free sterols, steryl esters, steryl glycosides and phospholipids in this study, it appeared that adaptive changes might also be occurring at the membrane level in somatic embryos of S.trifoliatus grown in presence of salinity.

In the present investigation, first we used unselected cell lines i.e. the embryogenic calli (non-salt treated) were exposed to saline medium for a total period of 25 days as described in Materials and Methods ( II.7.d.(a)).** Many

* see chapter III Results
** See also results III.2
workers used unselected cell lines in order to develop salt tolerant plant by exposing them to regeneration medium containing salt. (Mathur et al., 1980; McHughen and Swartz, 1984). While the presence of salt may be inhibitory to regeneration especially by somatic embryogenesis (Rangan and Vasil, 1983; Chandler and Vasil, 1984), it appears that its presence in the regeneration may increase the likelihood that plants which do regenerate from embryoids will be salt resistant (Nabors et al., 1980). Eventhough somatic embryos of soapnut were differentiated and developed in salt containing medium, its continued presence in the regeneration medium inhibited their germination. This led us to develop salt adapted callus by passing soapnut callus through saline medium for several subcultures and this salt adapted callus was then placed on induction medium to obtain salt tolerant embryoids with a view to obtain salt tolerant plants.

Of the two con tried, it was found that NaCl at the con of 100 mol m$^{-3}$ was lethal, because by fourth subculture the callus maintained at this con of NaCl turned brown and died. Studies with unselected cell lines also indicated the sensitivity of soapnut cultures at this con of NaCl. Though embryoids were differentiated on this medium reduction in fresh weight was observed.
The callus maintained in 50 mol m\(^{-3}\) remained healthy (Fig. 13) and somatic embryos obtained from this callus in the presence of salt, showed around 10% germination irrespective of ABA treatment (Fig. 17, Table 15). These embryoids also exhibited the same growth pattern and maintained more or less same level of Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) ions (Tables 11 and 12). A little increase in the accumulation of free sterols and steryl glycosides was noted (Table 14) and they certainly have some significant role in the adaptation of plants to salinity.

Thus, it may be concluded that somatic embryos of *S. trifoliatus* can tolerate NaCl salinity to a considerable extent without growth and development being much adversely affected. The results indicate that somatic embryos of this plant adapt to saline conditions possibly by excluding Na\(^{+}\) and tolerating high levels of cellular Cl\(^{-}\) ions. The results also suggest that somatic embryo of *S. trifoliatus* are able to maintain the functional integrity of the plasma-membrane and thereby helping in regulating passive ion permeability under stressed conditions.

* see chapter III Results