5. DISCUSSION

The aspects of investigation in all the three plants studied are similar. The results obtained also follow a similar trend. Therefore it is appropriate to combine the discussion of the results obtained in the present investigation and they are presented together.

5.1 Callogenesis

5.1.1. Explant Selection and callogenesis

In Cycads, explant selection involves only very few options. In the present investigation, vegetative organs, young and mature foliage leaves, scale leaves of suckers and roots were cultured, as they are easily available explants. There are only limited reports on callus induction and somatic embryogenesis in Cycads using explants from vegetative parts (Dhiman et al., 1998 a.; Chavez et al., 1995). Other reports deal with embryogenic or ovular explants (La Rue, 1948, 1954; Norstog and Rhamstine, 1967; Webb and Santiago, 1983; Chavez et al., 1992c; Norstog, 1965; De Luca et al., 1979; Chavez et al., 1992 b, 1995; Rinaldi and Leva, 1995; Rinaldi, 1999).

Though the use of reproductive materials as explants have also been reported, the availability of such material is severely restricted. Therefore the utilization of somatic tissues as explants was preferred.

Though there are reports on induction of organs directly from megagametophytes (Norstog, 1965) direct organogenesis from vegetative organs has not been reported so far. When the objective is to regenerate plantlets, the route of regeneration, directly or indirectly mediating a callus phase is not important. Therefore, profuse callusing is an essential requirement for obtaining sufficient materials for experimentation. In species of Zamia and Cycas, young flushes of
leaves are not available through out the year. In such situations initial production of callus is necessary for the continuation of experiments. More than that, production of embryogenic callus is significant in obtaining somatic embryos.

Callogenec and embryogenic potential is a function of explant, therefore the interaction of the explants with the medium is critical for callogenecis and embryogenesis. Present experiments are directed to obtain profuse callusing using easily available explants within limited period of time. In Cycas circinalis, among the six different explants studied, the megagametophyte from young ovule showed higher callogenec response. The biochemical studies show that the megagametophyte explants are high in starch, soluble sugar and protein content, than any other explants studied. At the same time the resin content in the megagametophyte explant was very meagre or nil. This condition with high metabolite content of the megagametophyte indicates its high callogenec potential. The young rachis and bulb scales stand next to the megagametophyte in callogenec potential. In Cycas revoluta, as in the case of Zamia furfuracea, due to the unavailability of ovules, the callogenec, embryogenic and organogenic potential of the megagametophyte could not be exploited.

Proliferation of embryogenic callus has been reported from leaf cultures of Ceratozamia mexicana (Chavez et al., 1992 a, 1995, 1998); Ceratozamia hildae (Litz et al., 1995) and bulb scale cultures of Cycas revoluta (Chaplot and Jasrai, 2000).

Juvenile tissues have a high degree of morphogenic competence than older tissue. The youngest and less differentiated tissues are found in plant meristems and the culture of these tissues has been successful in a wide range of species. Dhiman et al. (1998) also noticed a three fold increase in callus production when the rachis or
petiole was split longitudinally prior to inoculation. But in the present study, repeated trials in this line showed no increase in callus production.

The size of the explants also has an effect on the nature of response. Explants from lower portion of young rachis of approximately 1 cm length when inoculated vertically on the semisolid culture medium showed considerable higher callus production. As Narayanaswamy (1994) stated very small explants may not proliferate but callus induction is optimum with slightly larger explants.

5.1.2. Effect of different Basal Media

In the present study, callusing was observed in all the seven basal media used. The best response was observed on medium consisting of B5 major salts and MS minor salts.

The callogenetic potential of B5 + MS medium has already been reported for different explants of different cycads (Chavez et al., 1992c; Chavez et al., 1998; Litz et al., 1995). The difference between the major salts of B5 and MS is the difference in the ammoniacal nitrogen content. The result indicates that the lesser ammoniacal nitrogen in the medium favours enhanced callogenetic response in Z. furfuracea. Nitsch and Nitsch (1969) reported that higher concentration of ammoniacal nitrogen could be unsuitable in plant tissue culture.

White’s medium also contains higher concentration of ammoniacal nitrogen. Norstog (1965) reported callogenesis in Zamia integrifolia with an average of 68% on modified White’s medium. But when the same medium was tried for Cerratozamia mexicana, embryos were induced (De Luca et al., 1979).

In the present study SH medium showed only less response when compared to B5, MS and a combination of B5 + MS. Though Rinaldi and Leva (1995)
obtained in vitro responses in *Cycas revoluta* on SH basal medium a comparative study for different basal media was not carried out.

Though the composition of the major salts of SH basal medium (Shenk and Hilderbrant, 1972) is comparable to that of B5, it has a higher concentration of Ca, Mg and P. Comparatively WPM medium is substantially lower in macronutrient concentration but higher in sulphur content.

Webb et al. (1983) and Dhiman et al. (1998 a) reported callogenesis in *Zamia pumila* and *Z. furfuracea* on MS basal medium. Embryogenic calli were reported in different species of *Ceratozamia* from embryogenic explants on basal medium involving B5 major salts and MS minor salts.

### 5.1.3. Effect of Plant Growth Regulators

The present study dealt with a comparative analysis of different PGR combinations on callus induction from the young rachis of *Zamia furfuracea*. The results obtained are indicative of the fact that all auxins tried (2,4-D, NAA, IAA) are capable of inducing callus more or less at similar intensity. However, further proliferation of the induced callus was slightly better with NAA as evidenced by a higher callus index.

In *Zamia furfuracea*, when auxins were tried individually (2,4-D, NAA, IAA) callus induction was observed after 20-23 days, whereas in a combination, NAA + Kinetin, the induction time was reduced to 10 days. NAA in combination with BAP and 2iP also showed lesser time for callus induction than 2,4-D or IAA. The callus induction time is much less than what is reported by Dhiman et al. (1998 a), where callus induction from the rachis explants of *Zamia furfuracea* was observed only after six weeks when NAA alone was incorporated in the medium.
In *Cycas revoluta* and *Cycas circinalis*, individual auxins (2,4-D, NAA and IAA) showed callus induction from the rachis explant after 22 - 25 days, whereas on megagametophyte of *Cycas circinalis*, callus induction was observed within 19 - 21 days.

When auxins were combined with cytokinins the time taken for callogenesis was only ten days in *Zamia furfuracea*, but in *Cycas* the time is not much reduced. Even for megagametophyte, 18 - 20 days were taken for callus induction in the optimum PGR combination.

Growth pattern of callus from the explant was similar in all the three individual auxin treatments. The growth was steady up to 30 days, an increase between 30-45 days and after which the growth rate gradually decreased. Similar trend in growth pattern was observed in all auxin + cytokinin combinations but the callus mass produced in auxin + cytokinin combinations was much higher than the quantity of callus produced in individual auxins.

Callus growth pattern in *Cycas* explants was also different from that of *Zamia*. Up to 45 days the growth was slow and increase in growth rate was noticed after 45 days in all explant of *Cycas* species. The mass of callus proliferated was also much less than that of *Zamia furfuracea* in all the PGR combinations tried.

When the effect of individual auxins (2,4-D, NAA and IAA) on callogenesis was compared, young rachis of *Cycas revoluta* showed highest percentage of callogenic response in 2,4-D but callus index was better with IAA. Whereas in *Cycas circinalis* NAA was better both in megagametophyte and young rachis explants.

Earlier reports showed wide use of 2,4-D for induction of callus. Dhiman *et al.* (1998 a) reported callus initiation from young rachis explants of *Z. furfuracea* in
all concentration of 2,4-D varying from 0.1 – 10 μM within 5 weeks after inoculation. On lower concentration of 2,4-D (0.1 – 0.5 μM), 73.3 – 82.57 % cultures showed callusing. When 2,4-D concentration was increased up to 10 μM, 100 % cultures showed callusing. O'Dowd et al. (1993) reported induction of callus in Ephedra species when IAA was used alone or in combination with cytokinin. The application of IAA is not so far reported in the in vitro studies of Cycads.

The callus induction medium in the previous studies with embryo explants of Zamia species (Norstog, 1965; Norstog and Rhamstine, 1967) contained alanine, glutamine, asparagine and adenine in addition to myo-inositol 2,4-D and Kin. Norstog (1965) reported good callogenic response in two month old cultures of megagametophyte explants in Zamia integrifolia when inoculated on a medium containing glutamine 100 ppm., asparagine 100 ppm. and alanine 100 ppm. in addition to 2,4-D 1 ppm. + Kin 1 ppm. and adenine 10ppm. He also reported that in media lacking auxins and cytokinins very little callusing occurred even after six months. In this study, callus formation occurred in similar media lacking any amino acids or nucleotides.

The PGR combinations in the culture medium are very important in massive callus production. From the experiments involving growth regulators alone and in combinations, the best treatments, as evidenced by statistical analysis showed that combinations of auxins and cytokinins were found more effective in callus induction and proliferation than single auxins. Webb et al. (1983) is also of the opinion that single auxins alone can induce callusing but callus is proliferated better in the presence of cytokinins.
In most of the previous studies on callogenesis in cycads, only one combination of PGR (2,4-D + Kin) was tried (Litz et al., 1995; Norstog, 1965; Chavez et al., 1992 b, 1995, 1998; Jager and Staden, 1996 a, b, c).

It was reported earlier that callusing was observed after approximately 2 - 4 weeks in media containing a wide range of Kin and 2,4-D concentration, regardless of the explant type in Zamia species (Chavez et al., 1992c). They also reported the highest frequency of callus initiation and growth in media containing 4.5 - 9 μM 2.4-D, regardless of the concentration of Kin.

Jager and Staden (1996 a, b, c) reported callus induction from embryo explants of Encephalartos dyerianus, E. natalensis, E. cycadifolius and E. ferox on medium containing 4.5 μM 2,4-D combined with 4.6 μM Kin.

Litz et al. (1995) reported transient callus initiation on leaf explants of Ceratozamia hildae in contact with plant growth media containing 2,4-D alone at a range of 0 - 9.04 μM and combined with Kinetin (0 - 9.3 μM). No callus growth was noticed on control medium and on treatments containing Kin alone. Non-embryogenic callus was initiated in all cultures when PGR combinations involved 4.52 μM 2,4-D, 4.52 μM 2,4-D + 1.16 μM Kin, 2.26 μM 2,4-D + 2.32 μM Kin, 2.26 μM 2,4-D + 4.64 μM Kin.

In other cycads like Ceratozamia and Encephalartos callogenic and embryogenic responses were observed extensively from embryonic explants. Chavez et al. (1992 b) reported callogenesis from cultured zygotic embryos and megagametophytes after 2 - 4 weeks in Ceratozamia hildae and C. mexicana. Highest frequency of callus initiation and growth occurred on media containing 2,4-D with or without Kin. In the present study also a combination of 2,4-D and Kin
induced callus from the young rachis of *Zamia furfuracea*. But the optimum callogenesis was in NAA + Kin combination, not in 2,4-D + Kin combination.

Callus induction was noticed within a period of 10 – 12 days. NAA, in combination with any one of the other cytokinins (BAP and 2iP) also showed comparatively better response related to other combinations tried. The results agree with the findings of Dhiman *et al.* (1998 a) that the degree of callusing was quite high when Kin was used in the medium with any of the auxins.

As reported in tobacco (Syono and Furuya, 1972) Kin has a remarkable ability to maintain endogenous levels of auxins in the explants. When the concentration of NAA was increased in the medium (above 3 mg L⁻¹), the response percentage was correspondingly decreased as reported by Dhiman *et al.* (1998 a). The decline in callus formation at higher NAA levels could have been due to the production of ethylene (ethane) which is sometimes formed at supra optimal auxin concentration (Leopold and Kriedemann, 1975). Since cytokinin levels greater than 0.1 mg L⁻¹ have been shown to stimulate auxin biosynthesis (Syono and Furuya, 1972), the inhibitory effects of cytokinins could have been due to a stimulation of endogenous auxin synthesis resulting in supra optimal levels within the explants.

In *Zamia furfuracea* delayed callus induction and the low percentage of response were observed when NAA + BAP was used in the medium. This is contradictory to the findings of Dhiman *et al.* (1998 a), who observed that NAA + BAP was better than NAA + Kin combination.

Among the nine different PGR combinations tried, NAA + BAP combination showed better result in callogenesis from the explants of *Cycas revoluta* and *C. circinalis*. Highest callus index was observed from the rachis explants of *C. revoluta* when 5 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP was included. Rinaldi and Leva
(1995), reported callogenesis from cultured embryo explants in a combination of 0.9 μM 2,4-D and 0.5 μM 2iP was used with SH basal medium. Chaplot and Jasrai (2000), though tried for shoot bud differentiation from bulb scales of *Cycas revoluta*, obtained callusing when 2.21- 4.43 μM BAP was used in the MS basal medium.

In *Cycas circinalis* also NAA+ BAP combination showed highest callus index, though NAA with Kin or 2iP was also good. Induction and proliferation of callus from rachis explants was higher when 5 mg L⁻¹ NAA + 1 mg L⁻¹ BAP was used in the medium. Whereas in megagametophyte highest callusing was obtained at 3 mg L⁻¹ NAA +1 mg L⁻¹ BAP was used in the medium. When megagametophyte was used as explant, highest callusing was obtained at 3 mg L⁻¹ NAA + 0.5 mg L⁻¹ BAP.

Norstog and Rhamstine (1967) obtained callus from megagametophyte of *Cycas circinalis* on modified MS medium supplemented with 10 mg L⁻¹ 2, 4-D with or without 1 mg L⁻¹ Kin + 100 mg L⁻¹ Alanine + 400 mg L⁻¹ Glutamine. De Luca *et al.* (1979) obtained callogenesis from megagametophyte of *Cycas revoluta* at the same medium combination.

In *Z. pumila*, a closely related species of *Z. furfuracea*, callogenesis was reported from explants derived from embryo when NAA and BAP were added separately or together. In all these instances, NAA was required for callus initiation but BAP was not always required. However, callus proliferated better in the presence of both hormones than with NAA alone (Webb *et al.*, 1983).

### 5. 1.4 Callus morphology

Callus, the dedifferentiated unorganised mass of cells produced from the explants, exhibited different colour and texture. The texture of the callus is much
dependent on the type of PGR combinations in the medium from where the calli have been generated. The colour of the callus may vary depending on whether they carry pigments or secondary metabolites. In the present study, though different PGR combinations have been used to generate callus, the texture of the callus produced was friable in nature and with light yellow colour. The colour change of the callus to brown can be due to the accumulation of resin in the cells. Chavez et al. (1992c) reported similar type of friable callus with yellow brown colour to light brown from the embryo explants of Z. furfuracea. From embryonic explants of Z. pumila, Webb et al. (1983) reported the proliferation of intermediate of friable and compact callus. In species of Ceratozamia friable yellow - white callus from zygotic embryo explants and compact callus from megagametophyte explants were reported (Chavez et al., 1992b, 1998). In Ceratozamia hildae, callus derived from leaf explants was friable and off-white to slight yellow in colour, although it darkened with age becoming light brown (Litz et al., 1995). In Encephalartos cycadifolius and E. natalensis the callus nature was friable yellow, while the callus in E. dyerianus was first orange in colour, later changing to whitish - brown as in E. ferox (Jager and Staden, 1996 a, b, c).

5.1.5 Histology of callus

In the early reports of callogenesis and morphogenesis from ovular and vegetative explants of cycads, the callus mass and regenerants were described only on the basis of morphological features. Anatomical studies on embryogenic cultures were not reported in any of the Zamia species. Among Cycads, a histological study in the in vitro developmental stages was reported for somatic embryogenesis in Ceratozamia mexicana (Chavez et al., 1995).
In *Z. furfuracea*, *Cycas revoluta* and *Cycas circinalis* callus induction in the young rachis explants was observed as slight swelling at the cut ends. Histological investigation revealed that the growth of callus by cell division started from the sub epidermal region, close to the cut ends that were in close contact with the nutrient medium.

The friable light yellow callus derived from the sub epidermal cortical tissue was uniformly composed of nearly spherical, vacuolated cells with prominently stained nuclei. Similar callus tissues were observed in *Ceratozamia mexicana* (Chavez et al., 1995).

In *Cycas revoluta* and *C. circinalis* also the callus cells in the rachis explants were derived from the sub epidermal cortical tissue. The cells of the callus tissue were almost isodiametric and compactly arranged with prominently stained nuclei. The calli developed from the megagametophyte consisted of nearly spherical, loosely arranged vacuolated cells.

As evidenced anatomically, the darkening of the callus was due to the resin deposition in the cells. The browning of callus with age was reported in *Ceratozamia hildae* also (Litz et al., 1995).

### 5.1.6 Metabolite mobilization in callus

The calli derived in all PGR combinations were more or less similar in morphology and physiology. That may be the reason for the similar pattern of metabolite accumulation in callus. In the present investigation, in calllogenesis, there was a decline in the protein content as the callus ages in culture. This may be due to the depletion of the nutrients, which lead to a stress condition. Singla *et al.* (1994) reported that the rate of protein biosynthesis shows a gradual decline during stress condition.
In all the three plants studied, starch and soluble sugar showed similar trend of accumulation in callus cultures. The medium could be the only source of carbohydrate since there is no photosynthesis during calllogenesis. Sucrose is the carbohydrate source in the medium, which is a simple sugar. It could be assumed that the tissue would prefer simple sugar for its respiration. This is also evident by the increasing soluble sugar profile. This clearly shows that there is starch digestion, which is on the increase. It could be inferred that the sucrose obtained from the medium is getting converted to starch, which is stored and later digested by amylase to release soluble sugar as required.

The high callogenic response in megagametophyte of Cycas circinalis may be correlated with the high food reserve in the component cells. The initial decline in the resin content can be because of the newly formed cells, which might not have started secondary metabolite production. The accumulation of the resin in the callus cells at a later stage leads to the death of the callus.

In in vivo conditions, the resin is seen in epithelial cells and resin canals. In actively dividing callus cells, secondary metabolism may be negligible. But when the culture reaches the steady phase of growth, they may turn in favour of secondary metabolite synthesis – resin, and the cells loose their morphogenic potential.

5.2 Callogenesis

Achieving redifferentiation from the callus in the form of shoot buds or somatic embryos is the most critical challenge to be undertaken for the success in the cycad tissue culture. Past reports reveal that complete recovery of plantlets and transfer to the field has not been achieved in cycads.

In Z. furfuracea, Chavez et al. (1992 c) first reported somatic embryogenesis and organogenesis from megagametophyte and zygote embryo explants, but failed
to produce mature plantlets even in the culture conditions. Somatic embryogenesis in *Z. furfuracea* was also reported by Dhiman *et al.* (1998 a). They succeeded only up to the germination of embryoids in petri plates but did not develop into plantlets. In *Z. pumila* detailed *in vitro* studies resulted in the differentiation of 'plant like' structures from embryo explants but sustained growth of plants was not obtained (Webb *et al.*, 1983). Chavez *et al.* (1992 c) also reported the failure of germination of similar embryoids to develop into plantlets in *Z. pumila* and *Z. fischeri*.

Norstog (1965) reported the induction of apogamy in the megagametophyte of *Z. integrifolia* in the culture conditions. However the apogamous organs have not been grown to any stage approaching maturity.

*Encephalartos* is another cycad on which *in vitro* studies have been carried out extensively. Somatic embryogenesis and organogenesis have been achieved (Jager and Staden, 1996a, b, c), but maturation or germination of these embryoids with shoot and radicle was not observed in any of the species. The most successful report was in Ceratozamia where somatic embryos have been converted to plantlets but not transferred to the field level (Chavez *et al.*, 1998; Litz *et al.*, 1995).

So far, the *in vitro* differentiation in cycads has been achieved mainly through somatic embryogenesis.

### 5.2.1 Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells develops into plants, through a series of stages characteristic of zygotic embryo development. Somatic embryogenesis has been reported in many higher plants and represents the most striking confirmation of totipotency.

It has been reported that somatic embryogenesis in cultures proceeds from cells, which are already committed for embryogenic development (Vasil and Vasil,
1986). This requires growth regulators and favourable conditions to allow these predetermined cells to undergo cell division and expression of embryogenesis (Sharp et al., 1980).

Embryogenic potential is largely a function of the explant acquired through its interaction with the growth medium and supplements. Choice of appropriate explant is, therefore, critical for differentiation as the embryonic potential vary from explant to explant.

Though most of the in vitro organogenic studies in gymnosperms show that somatic embryos appeared with efficiency from ovular explants, in the present investigation, somatic embryos are produced from juvenile, friable calli derived from young leaf rachis segments of Zamia furfuracea when steered by different PGR combinations. In Cycas circinalis embryogenic induction was obtained only from megagametophyte explant.

5.2.1.1 Basal media

Modifications to medium components and culture conditions can significantly affect induction of embryogenic tissues and play a major role in enhancing initiation from more mature explants. Important factors are light regime, concentration of basal media and particularly sucrose, nitrogen level, composition of mineral elements, agar, PGR and pH (Tautorus, 1991), Krogstrup et al. (1988), Simola and Santanen (1990) reported that different genotype even within a species have different culture requirement for production of embryogenic tissue.

In the present study of the seven different basal media studied, medium with major salts of B5 and minor salts of MS was found better for the induction of somatic embryogenesis in Z. furfuracea. Full strength B5 + MS basal medium was found better than half strength medium. High ionic strength may be beneficial for in
vitro response in Zamia and Cycas though it was found that half strength MS with low ionic strength was beneficial in many angiosperm plants. Growth and morphogenesis of plant tissue in vitro are largely governed by the composition of culture media.

Evans et al. (1981) noted that 70% of the explants of various crop plants were cultured on MS medium or a modification of MS. A key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate. The present study shows that the high levels of nitrogen, in the reduced form such as ammonium nitrate are not required for somatic embryogenesis in Zamia species, though it is beneficial in other plants.

In Cycas circinalis, embryogenic induction was observed on megagametophyte explant when cultured on medium containing major salts of B5 and minor salts of MS. Rinaldi and Leva (1995) obtained putative embryogenic masses from seedling explants of Cycas revoluta when cultured on SH basal medium, where the composition of the major salts is comparable to that of B5.

De Luca et al. (1979) observed some globular outgrowths (pseudobulbils) from the megagametophyte explants of Cycas revoluta, when cultured on modified MS medium, which showed a disputed ontogeny.

5.2.1.2 Plant Growth Regulators

Choice of basal medium is decisive in the induction of embryogenic callus / embryoids in the cultures. The basic requirements of the cultured plants are similar to those of whole plants, in practice, nutritional components promoting optimum growth of a tissue may vary with respect to particular plants. Media compositions are therefore formulated considering specific requirements of a particular culture system.
In the present investigation, embryogenic response in *Zamia furfuracea*, as indicated by the formation of globular somatic proembryos, was observed only in 40 treatments among a total of 325, which involved different combinations and concentrations of PGR.

Several studies on the importance of auxin for somatic embryogenesis have shown that the process of embryogenesis takes place with the induction of cells with embryogenic competence in the presence of high concentration of auxin, and the development of embryogenic competent cells into embryos takes place in the absence of or in the presence of low concentration of auxin (Sahrawat and Chand, 2001).

The application of individual auxin (2,4-D, NAA, IAA) did not show any effect on embryogenesis in *Zamia furfuracea* or *Cycas* species. The juvenile callus originally derived from young rachis explants, only proliferated as friable creamy callus in all the auxin-containing media. In conifers several authors have reported the use of 2,4-D for the induction of embryogenic growth (Gupta, 1986a; Beckkaoni et al., 1987). A similar effect for 2,4-D in somatic embryogenesis has been reported for *Gnetum ula* (Augustine and D’souza, 1997). However the present study as well as in the reports of Dhiman et al. (1998) auxins alone could not bring about any induction except callusing in *Z. furfuracea* and *Cycas* species.

Cytokinins individually and in pairs (Kin, BAP and 2iP) have showed no embryogenic induction in *Zamia* callus. But upon exposure to cytokinin rich medium the friable calli became compact – a tendency for morphogenesis but further development in that direction was not achieved. Correspondingly low cytokinin containing medium produced only friable nodular calli. According to Chavez et al. (1992 c) embryogenic induction in the calli of *Z. furfuracea* and *Z.*
*Punica* was noticed in the presence of cytokinin alone. Similar observations were made by Schuller et al. (1989) with *Abies alba*. In *Gnetum nitida*, the callus induced with BA was embryogenic and numerous embryo initials were protruding out of embryogenic calli after three months of culture (Augustine and D'Souza, 1997).

In medium containing combinations of one cytokinin and one auxin, the juvenile friable calli of *Zamia furfuracea* became compact with nodular morphology.

Even if 288 PRG combinations were tried, calli derived from rachis of *Cycas* species failed to produce somatic embryos.

Embryogenic callus was obtained from megagametophyte explant when cultured on medium involving one cytokinin and one auxin. A large number of globular structures were developed from the megagametophyte explant. BAP in combination with NAA or 2,4-D showed higher induction percentage.

Histological studies revealed that the initial stages of the globular structure development resembled that of proembryos but were not differentiated into embryos with shoot pole and root pole. Webb and Osborne (1989) observed similar results in *Ceratozamia* megagametophyte and suggested that these structures resemble shoots rather than embryos. An earlier report by De Luca et al. (1979) described the development of such structures as induction of somatic embryogenesis from cultured megagametophytes of *Ceratozamia mexicana*, however in later studies De Luca and Sabato (1980) De Luca et al. (1980) reported such globular proembryonic 'pseudo bulbs' identified as root primordial.

Optimum embryogenic induction was obtained when medium involving any two cytokinins (Kin, BAP, 2iP) were combined with any one auxin (2,4-D, NAA, IAA). The proembryos were developed in combination involving 1-2 mg L⁻¹ Kin
and 0.5-1 mg L⁻¹ of BAP or 2iP with and any one of the auxins. This indicates that induction of embryony in *Zamia* is Kin dependent. Generally Kin is believed to have a major regulatory role in somatic embryogenesis in gymnosperms especially in cycads (Chavez et al., 1992b).

A consolidated report of somatic embryogenesis in coniferales (Attree and Fowke, 1993) has highlighted the requirement of high cytokinin as sole source for induction. But continued proliferation requires an auxin supplement. The present study also supports this fact.

Kin together with 2iP also showed a synergistic effect on induction of somatic embryogenesis in *Zamia* in the presence of an auxin.

In *Cycas circinalis* embryogenic induction appeared BAP dependent. Though induction was observed in medium involving Kin, higher percentage was noticed in medium involving BAP with any one of the auxin. Promisory effect of BAP in particular on somatic embryogenesis is well documented (Wilson et al., 1996; Van der Walk et al., 1995).

Cytokinins are known to foster somatic embryo development (Fujimura and Komamine, 1975) and higher cytokinin levels are believed to counteract residual auxin effect to prevent inhibition of somatic embryogenesis (Wilson et al., 1996).

In *Zamia furfuracea*, when BAP was included in the medium, individually or in combination, the embryogenic induction was poor and the proliferated calli became greenish yellow in colour. Attree and Fowke (1993) reported that green callus of Conifers grew for up to one year before yielding defined immature somatic embryos. This supports with the findings of Koran et al. (1994), who reported an inhibitory effect of BAP on somatic embryogenesis in Cassava.
NAA, 2,4-D and IAA individually showed an enhancing role in embryogenic induction on *Zamia* callus when combined with the cytokinins. Among them NAA was better. But similar effect of NAA was not observed in *Cycas circinalis*.

After embryo induction, the role of auxin changes as that the embryos start to synthesise their own auxin. Several studies have shown that proper polar transport of auxin is a pre – requisite for normal embryogenesis beyond the globular stage (Sahrawat and Chand, 2001).

Dhiman et al. (1998 a) reported that callus obtained on 2,4-D with BAP or Kin combinations and NAA with Kin on prolonged cultures formed somatic embryos. Chavez et al. (1992c) reported somatic embryogenesis from calli of *Zamia* species only on 2,4-D and Kin combination. The present study suggests that NAA and IAA are also effective in inducing somatic embryos from *Zamia* callus when combined with one or more cytokinain.

**5.2.2 Indirect Organogenesis**

Only few reports are there on induction of shoots and roots from the callus in cycads. In *Zamia* species adventitious shoot induction was reported only from callus derived from megagametophyte and zygotic embryo explants (Chavez et al., 1992 c). There are no reports of organogenesis from the calli derived from somatic explants in *Zamia*. In the present investigation, organogenic potential was observed in the calli derived from young rachis explants of *Zamia furfuracea*. Out of the 325 different media compositions tried, only fifteen combinations showed positive response.

However, the calli developed from rachis explants of *Cycas circinalis* and *C. revoluta* failed to induce organogenesis. It can be presumed that the calli derived from vegetative explants may have a low potential in organogenesis. As reported by
Chavez et al. (1992 c), after a comparative study of *Z. pumila, Z. furfuracea* and *Z. fischeri*, the organogenic response varies with species and different explants of the same species.

In the present, study the shoot or root meristem formation was detected between 9–11 weeks of culture. This is similar to the findings of Chavez et al. (1992c) and Webb et al. (1983) where zygotic explants were used.

In all the fifteen combinations, which showed positive response, characteristically Kin and 2iP were the cytokinins. Though the medium containing Kin and 2iP showed the initiation of organogenesis, morphogenesis was improved when any one of the auxins (2,4-D, NAA, IAA) was included in the medium. As embryogenesis was obtained in the medium containing Kin + 2iP + NAA / IAA / 2,4-D in lower concentration, it is likely that higher concentration of same PGR directed a deviation of the pathway to a shoot induction route. The auxin component, IAA in the medium also takes an important role in the induction of roots. Inhibition of shoot pole development by early root formation is reported as a problem encountered in several tissue systems (Grewal and Rani, 1999).

Shoot development was not observed even after six months in cultures, where roots were initiated. PGR combinations involving IAA as the auxin component showed the best response in organogenesis with respect to the average number of shoot and root produced.

Chavez et al. (1992 c) obtained indirect shoot budding from megagametophyte explants of *Zamia* species when 4.6 – 9.2 μM Kin with 2.3 – 4.5 μM 2,4-D was used in the medium. Norstog (1965) reported that highest percentage of root initiation on a medium containing 2,4-D, Kin, Adenine, Glutamine, Asparagine and Alanine from megagametophyte explants of *Zamia*. 
Webb et al. (1983) reported that when BAP + NAA was involved in the medium, friable callus derived from megagametophyte explants of *Ceratozamia* species became nodular and produced root, shoot and embryo like structures after transfer to basal medium.

5.2.3 Direct organogenesis

In *Zamia furfuracea*, direct organogenesis has not been reported so far. In the present study also though 325 treatments involving a wide variety and range of hormones have been tried, positive results was obtained only in one or two instances, which did not show repeatability. This reinstates the difficulty in inducing direct organogenesis in *Zamia*.

However, in *C. revoluta*, shoot induction was observed from sucker scale leaves. Though 20-30% of cultures showed shoot induction, none of the cultures produced more than one shoot. The induced shoots did not show further growth even after transfer to fresh medium. Chaplot and Jasrai (2000) obtained bud induction from bulb scales of *Cycas revoluta* when cultured on ½ MS medium supplemented with 2.21 µM BA. But as in the present study, further growth was not achieved.

5.2.4 Organogenesis from megagametophyte of *Cycas circinalis*.

The megagametophyte collected from different ovules varied in their responses to the medium. In some of the megagametophyte, germination of zygotic embryo was observed as small protuberance from the micropylar region. However in majority of cases shoot initiation or callus growth was noticed from the cut ends, the micropylar cavity region remained unchanged. The longitudinal section of these explants revealed that zygote had not been developed in these ovules.

This reveals the fact that the ovules of the same female cone may not be simultaneously pollinated and eggs of majority of ovules remain unfertilized.
Chaplot and Jasra (2000) reported that the products of fertile seeds in cycads is limited either due to poorly developed pollination mechanism or due to the absence of pollinators in the native strands.

Direct shoot induction from the megagametophyte of *C. circinalis* has been observed in the PGR combinations, in which the zygotic embryos germinated. The percentage of shoot induction was high when the PGR combination included 1 mg L\(^{-1}\) Kin + 0.5 mg L\(^{-1}\) BAP + 0.5 mg L\(^{-1}\) 2,4-D or NAA or IAA. Though multiple shoots were obtained in 85-88% of cultures, the growth did not continue beyond a length of 2 cms in any of the PGR combinations. Root induction was also not achieved.

Norstog (1965) pointed out an auxin – Kin interaction in initiation of roots and leaves from megagametophyte explants of *Zamia* species. It is not clear whether the auxin or Kin or both are responsible for the inhibition of root initiation and elongation. Skoog and Miller (1957) found that Kin counteracted auxin inhibition of root elongation. On the other hand they reported that Kin itself in a higher concentration inhibited root elongation.

Rinaldi and Leva (1995), in their studies on *Cycas revoluta*, confirmed the capacity of megagametophyte tissues in the regeneration of plantlets and pointed out the relation between explant morphogenic competence and its developmental stage. Dehgam and Yuen (1983) reported that the *Cycas* embryo is at a very early stage of development at the time of seed abscission. It follows that germination is hindered by incomplete development of embryo (Dehgam and Schutzman, 1983). This agrees with the reports demonstrating that it is possible to obtain shoot regeneration from mature zygotic embryos (Rinaldi and Leva, 1990).
As reported by Rinaldi and Leva (1995), the germinating embryos when cultured on medium containing 0.5 mg L\(^{-1}\) BAP + 0.5 mg L\(^{-1}\) NAA the shoot is elongated to a length of more than 5 cm with the formation of adventitious roots within a period of 60 days. But multiple shoot formation and establishment of plantlets could not be achieved. Rinaldi and Leva (1995) also stated that the effectiveness of BA in inducing shoot from the mature embryo explants was partially antagonized by NAA and explained that NAA would appear to inhibit bud induction on *Cycas revoluta*. A similar situation is reported in *Pinus elliottii* also (Bermudez and Sommer, 1987).

A low auxin-cytokinin ratio appears to promote root formation from the megagametophyte cultures. In cycads variation in the PGR requirement is noticed between different species of same genus (Chavez et al., 1992b). The geoclimatic difference of the region of the explant may be reflected in the PGR requirements of *in vitro* organogenesis in *Zamia*.

### 5.2.5 Effect of additives

#### 5.2.5.1 Coconut Water

The use of Coconut Water (CW) in *in vitro* cultures of cycads has not been reported so far. In the present study 10 % coconut water in the medium was found to enhance somatic embryogenesis and organogenesis to 30 – 40%. Below and above 10 % in the medium was less responsive. The level of phytohormones in CW is unpredictable; growth promoting substances like diphenyl urea and meso inositol are present. It has been reported that CW acts as a source of reduced nitrogen in the medium (Tulecke et al., 1961). Steward et al. (1964) reported the importance of CW in the medium for cell division and embryo formation in carrot cell culture. Litz et al. (1982) reported that CW is required in inducing somatic embryogenesis in
different varieties of *Mangifera*. According to Mathews and Litz (1992), CW can even replace plant growth substances in inducing somatic embryogenesis in preembryogenic determined cells in *Mangifera*. Guha and Maheswari (1964), the first report of pollen embryos, have also utilized CW in the medium.

### 5.2.5.2 Megagametophyte extract

Since various additives from natural sources are practiced in *in vitro* studies with different plant species, extract prepared from the megagametophyte of *Cycas* was used in the present study. The organogenic and embryogenic response was obtained only when megagametophyte extract was used in the medium.

20% extract showed optimum organogenic induction. This indicates that the synthetic PGR used in the medium alone are not capable of inducing organogenesis and plants own components facilitate organogenic induction in Cycads.

This has not been tried in the previous *in vitro* studies in Cycads. Promising results were not obtained in *Zamia* and *Cycas* species when similar trials were carried out using young rachis extracts of *Zamia furfuracea*, and *Cycas* species.

### 5.2.5.3 Sucrose

Many previous reports suggest that sucrose has both a nutritive and an osmotic effect on embryogenesis in several species of plants (Xie and Hong, 2001). Sucrose has been reported to be the most effective reduced carbon source for the initiation of somatic embryos (Verma and Dougall, 1977). The present study shows that sucrose level between 2 – 4% in the culture medium is supportive effect for the induction of somatic embryos on *Zamia* callus. This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated (Chong and Pua, 1985). The concentration of sucrose
above 4% shows no effect in embryogenic induction. This indicates that a high osmotic condition of the medium is not preferable in embryogenic induction in Zamia.

A low osmotic condition, provided by 1-3% sucrose, in the medium is reported to be beneficial for the embryogenic induction in conifers (Attree and Fowke, 1993). Dewall et al. (1989a) reported that 6% sucrose is the ideal concentration for the initiation of somatic embryos in different varieties of mango. Lu and Ozias (1982) stated that raising the sucrose concentration in the primary medium to 12% favoured the formation of embryogenic callus from the scutellum of immature embryos of Zea mays.

In the present studies, sucrose was provided in the medium for optimising the maturation and recovery of somatic embryos. The effective concentration was 3%. Previous reports have shown that a moderately higher concentration of sucrose is necessary for embryo maturation. Somatic embryo maturation in Cycads was observed when a concentration of 6% sucrose was used in the medium (Chavez et al., 1992 b,c, 1995; Litz 1995; Jager and Staden, 1996 a, b).

The increased osmotic potential provided by moderately higher concentration of sucrose might have contributed to the increased size of embryos. Sucrose acts as the carbon and energy source for the growth of embryos. In the present study, maturation was optimum at 3% of sucrose. The presence of an additional amount of sugar from the coconut water supplement can be the reason for the requirement of a lower quantity of sucrose in the medium for optimum embryo development in Zamia furfuracea. Webb et al., (1983), Dhiman (1998) and Norstog (1965) reported embryogenesis and maturation when 3% sucrose was used in the medium.
5.2.5.4 Glutamine

Reduced nitrogen in the basal medium is necessary for the initiation of somatic embryos (Evans et al., 1983). Reduced nitrogen was available in the form of Glutamine, CH and CW, which may be more readily metabolised than inorganic nitrogen. The amino acid, Glutamine, supplements the existing ammonium to nitrate ratio in the medium, thereby influencing initiation of somatic embryogenesis and morphogenesis (Litz and Gray, 1992).

In the present study, Glutamine up to 200 mg L\(^{-1}\) in the medium containing 10% CW and 100 mg L\(^{-1}\) CH, showed an enhancing effect on embryogenesis. Chavez et al. (1992 c) reported somatic embryogenesis and organogenesis in *Z. furfuracea* and other species on media containing glutamine at concentration of 400 mg L\(^{-1}\). Embryos were induced at the same concentration of Glutamine in *Ceratozamia* spp. (Chavez et al., 1992b, 1995; Litz et al., 1995) and in *Encephalartos* spp. (Jager and Staden, 1996a, b, c). Requirement of comparatively lower concentration of Glutamine in the present investigation might be due to the availability of additional reduced nitrogen in the medium from the CW supplement.

5.2.5.5 Casein Hydrolysate

Inclusion of CH at a concentration of 100 mg L\(^{-1}\) in the medium enhanced the percentage of initiation and number of embryoids in *Zamia*. CH is a non-specific organic nitrogen source and serves as an amino acid supplement (Skoog and Miller, 1957). The current studies show that a concentration above 100 mg L\(^{-1}\) CH has no positive response in somatic embryogenesis and organogenesis in *Zamia* and *Cycas*. This indicates that the organic nitrogen in excess quantity is not beneficial for embryogenesis in *Zamia* and *Cycas*. Embryogenic induction in cycads on media supplemented with 100 mg L\(^{-1}\) CH was reported in species of *Zamia, Ceratozamia*
and *Encephalartos* (Chavez et al., 1992 a, b, c, 1995; Jager and Staden, 1996 a, b, c). Durzan, (1973) has reported that higher amounts of CH and L-Glutamine were found to be toxic in the case of somatic embryogenesis in *Gnetum ula*. But there are reports that CH at a concentration of 500 mg L⁻¹ in the initiation medium shows promising embryogenic response in *Mangifera* (Jana et al., 1994) and other angiosperm plants (Mauro et al., 1986). The contradictory opinion in the level of CH required might be because of the fact that they belong to two distant taxa.

5.2.5.6 Activated charcoal

Activated charcoal has the property to absorb a wide range of compounds present in the explants and the culture medium that inhibit growth of tissues and facilitate easy organogenic response (Jana et al., 1994). In the present study, addition of activated charcoal in the embryogenic media showed no positive response in embryogenesis. Somatic embryo development in *Taxus brevifolia* was reported in medium involving 1% activated charcoal (Chee, 1996).

5.2.5.7 ABA and GA3

Abscisic acid (ABA) and its antagonist, Gibberelllic acid (GA3) have been demonstrated to initiate somatic embryo development beyond the earlier stages and initiate maturation. (Nadel et al., 1990). ABA increases the uniformity of somatic embryoids and reduces the development of abnormal forms (Ammarito 1983). The normalizing effect of ABA on embryo maturation has also been observed with *Daucus carota* (Kamada and Harada, 1981) and *Pennisetum americanum* (Vasil and Vasil, 1981). Kochba et al. (1978) reported that ABA promoted somatic embryo development in *Citrus* ovular callus. All these findings reveal that ABA might serve to promote successful somatic embryo maturation. Hence its addition to culture medium may permit somatic embryo maturation to proceed under conditions where
it normally would not occur. In the present study, exposure of embryogenic cultures to ABA did not show any effect on embryo maturation or recovery.

Abscisic acid (ABA) and moisture stress are important for the maturation of the seeds of many angiosperm species (Zeevart and Creelman, 1988; Kermode, 1990; Hetherington and Quatrano, 1991). In general, ABA accumulates during mid-to-late stages of seed development and prevents the developing embryo from germinating precociously (Kermode 1990). The activation of genes responsive to ABA (rab genes) leads to the accumulation of mRNAs, which control the accumulation of storage products such as proteins, lipids and starch. The abundance of protein during embryogenesis may also thought to be involved in the acquisition of desiccation tolerance (Skriver and Mundy, 1990). ABA levels often decline in the mature dry seeds, enabling the mature embryo to germinate upon imbibition. Physiological and biochemical studies concerning ABA during maturation of conifer zygotic embryos and seeds are currently scanty nonetheless, ABA has been found to be essential for inhibiting cleavage polyembryony and promoting the synchronous maturation of somatic embryos of many conifer species (Attree and Fowke 1993).

Augustine and D’Souza (1997) reported the enhancing effect of ABA in the development of immature embryos to form mature embryos in Gnetum ula. Embryos of cycad seeds are of recalcitrant type. Embryogeny and germination are therefore a continuous sequence of developmental events. Although studies with conifers (orthodox-type embryos) have indicated that somatic embryo maturation and plant recovery from somatic embryos can be facilitated by treating proembryos with ABA (Becwar et al., 1989; Dunstan et al., 1988; Arnold and Hakman 1988), neither ABA nor its antagonist GA3 have so far been shown to have a similar effect with recalcitrant somatic embryos of C.hildae and C.mexicana (Litz et al. 1994,
1995). ABA showed no promotive effect for occurrence and development of somatic embryogenesis in *Acacia mangium* (Xie and Hong 2001). Even though ABA has a known history of promoting embryo recovery in many plants, the reasons for the failure in *Zamia* and *Cycas* is not understood.

**5.2.5.8 Thidiazuron (TDZ)**

TDZ is known to induce shoot formation in a broad range of plant species (Malik and Saxena, 1992) and somatic embryogenesis in many plants (Gill and Saxena, 1993; Murthy and Saxena 1998).

A substituted phenyl urea compound has been demonstrated to stimulate *in vitro* meristems and shoot formation at usually low concentrations (Briggs *et al.*, 1988). These compounds have strong cytokinin like effects on a wide range of species and on species that respond little to cytokinin. However in the present instance, the use of Thidiazuron was not helpful in embryo maturation and recovery in *Zamia* or in the organogenic development in *Zamia* and *Cycas* species studied.

The results indicate that the rate of organogenic growth was very slow even in the presence of various growth promoting agents and there is an underlying problem, which blocks the complete development of plantlets from the embryoids. Similar problems were observed in grapes (Tournand *et al.*, 1983) and Flax (Tejavathi *et al.*, 2000). Since root development occurs in the embryo at an early stage, these precursors might have inhibited the development of shoot pole. Inhibition of shoot pole development by early root formation is a problem encountered in several other plants (Novak *et al.*, 1989). Unsuccessful conversion of somatic embryos into plantlets may be because of the aberration of shoot meristem as reported by Ammarito (1987).
5.2.6 Effect of Light on embryogenesis / organogenesis

In the present investigation, normal light intensity favoured the somatic embryogenesis and organogenesis in species of Zamia and Cycas. Favourable results in somatic embryogenesis of Ceratozamia mexicana were observed in normal light condition (De Luca et al., 1979; Norstog and Rhamstine, 1967). Studies of Torne et al. (1997) showed that normal light condition has a positive effect on somatic embryogenesis in Araujia sericifera. However Chavez et al. (1992b) stated that light is not essential for organogenesis in the in vitro cultures of Ceratozamia mexicana.

5.2.7 Maturation of proembryos

Embryo maturation is often associated with a reduction or omission of auxin from the medium. In the present study embryo maturation was not achieved in Cycas species. In Zamia furfuracea, the proembryos formed in the induction medium, showed differentiation and maturation in a medium containing Kin, 2iP, BAP, NAA, CW and Glutamine. The major change in the maturation medium is the presence of BAP, which had no effect on embryo induction.

Globular proembryos developed to a bipolar structure after 12 -14 weeks. Chavez et al. (1992 c) reported differentiation of somatic embryos in Z. furfuracea only after 4 - 5 months in cultures though in Z. fischeri and Z. pumila fully differentiated somatic embryos were observed after two months of subculture.

Generally PGR free medium is known to induce maturation of proembryos. In the present investigation in Z. furfuracea, even after prolonged cultures of proembryos in PGR free medium for more than six months, maturation of proembryos was not achieved.

Suspensor was not reported in earlier studies of somatic embryogenesis in cycads (De Luca et al., 1979; Norstog and Rhamstine, 1967; Webb et al., 1983)
except in Ceratozamia spp. (Chavez et al., 1992b). Differentiated embryos took 20–28 weeks (5–7 months) for maturation. The observation confirms the reports of Dhiman et al. (1998). However in other cycads like Ceratozamia, it took 15–18 months (Chavez et al., 1992a; Litz et al., 1995).

After 4–5 months on the maturation medium, the somatic embryos became hard, creamy-white and opaque in appearance. Chavez et al. (1992c) report similar observation in Zamia species, where the embryos had a creamy white or pink pigmentation and the pink color persisted after the cotyledons had differentiated. In Ceratozamia also embryos became hard, white to pink and opaque in appearance on maturation (Litz et al., 1995; Chavez et al., 1992b, 1998).

Most of the somatic embryos in the present study were dicotyledonalous, although very occasionally monocotyledonalous somatic embryos developed. As the somatic embryos matured, they become characteristically light pink to white in color. The number of cotyledons in cycads is variable even within a species. According to Chamberlain (1935) there are two cotyledons but the number may vary from one to six. In Ceratozamia (De Luca et al., 1979, Chavez et al., 1992a) the somatic embryo seems to have a single cotyledon. However, in C. hilàae the somatic proembryos undergo due successive cleavage to form many cotyledons (Chavez et al., 1992b). According to Chavez et al. (1992 a,b), the single cotyledon may be resulted either from fused cotyledons or the cotyledons may be surrounded by a 'coleoptile-like' sheath, thus making it appear like a monocotyledonalous. In Z. furfuracea also the number of cotyledons varied from one to as many as eight, single cotyledon being predominant. Polycotyledony in Encephaliarios has been known to arise due to branching during early development of proembryo (Saxton, 1910).
Chavez et al. (1998) reported entirely white and opaque precotyledonary somatic embryos in *Ceratozamia euryphyllidia* that had ceased to proliferate. Two cotyledon primordia usually developed at the distal region of the embryonic mass of each precotyledonary somatic embryo, although growth of one of the cotyledons was suppressed. Dicotyledony was also described in somatic embryos of other *Ceratozamia* species. (Chavez et al., 1992a, Litz et al., 1995).

The embryoids, at a later stage in the maturation medium, started showing erratic growth. Elongation of the shoot and root did not take place and a chimeric structure was developed. Similar anomalous structures were obtained in *Ceratozamia hildae* (Litz et al., 1995). The normal growth of embryoids failed because the shoot pole was inhibited from further growth. The reason for this is not understood.

Sharp et al. (1980) has reported that the loss of bipolarity may be associated with perturbation in steady ionic flow along the development axis of somatic embryos.

### 5.2.8 Histology

#### 5.2.8.1 Embryogenic and Organogenetic cultures.

Somatic embryogenesis is considered to be a model system for studying plant embryo development (Warren, 1993). Two distinct type of somatic embryogenesis, direct and indirect have been recognised (Xie and Hong, 2001). In Indirect somatic embryogenesis, an embryo develops from one or more cells of an embryogenic callus. In the present study, indirect type of somatic embryogenesis was obtained in *Cycas circinalis* and *Z. furfuracea*. 
In the current study, because of the low frequency of somatic embryogenesis from the calli derived from the rachis explants in *Z. furfuracea*, it was not possible to follow the sequence of developmental events from a single cell through maturation.

The embryogenic capacity could be expressed either as meristematic cells that started the cellular division giving embryo like structures or from meristematic centers composed of high mitotic activity cells, prominent nuclei and high protein synthesis. **Histological sections** in the present investigation revealed the second type of differentiation and somatic embryos development from a group of embryogenic initials in the outer most layer of embryogenic callus. Similar observations were reported in *Ceratozamia mexicana* (Chavez *et al.*, 1995).

These results are also in agreement with Arruda *et al.*, (2000), Michaux-Ferriere *et al.*, (1992), who observed that somatic embryogenesis could be correlated with meristematic center formation. Haperin and Wetherel (1964), Haccius (1978), Williams and Maheswaran (1986), Michaux-Ferriere *et al.* (1987), Maataoui *et al.* (1990), also reported the somatic embryo development from meristematic centers on the callus periphery.

Histological examination of the callus cultured for organogenesis indicated that embryonic calli consisted of cells characterized by the accumulation of starch grains, conspicuous nuclei and dense cytoplasm and thick cell wall. These features of embryogenic callus cells were also reported for grape vine (Nakano *et al.*, 2000), *Quercus suber* (Maataoui *et al.*, 1990), *Coffea canephora* (Berthouly and Michaux-Ferriere, 1996) and *Prunus avium* (Garin *et al.*, 1997).

In cycads, in the *in vivo* condition, there can be as many as 10 free nuclear divisions, resulting in a coenctyium of more than 1000 free nuclei, depending on the species (Chamberlain, 1935). A free-nuclear stage during the early phase of somatic
embryo development was not observed. A cell of this magnitude probably could not occur or survive in vitro.

Histological studies of the embryoids produced by the megagametophyte of *Cycas circinalis* reveal that though the cells were divisionally active at the early stages, the cells of the center gradually lost its dividing nature and the cell wall became lignified. Lignified cells are physiologically dead and that can be the probable reason for the discontinuation of growth of embryoids in *Cycas circinalis*.

In *Zamia furfuracea*, the group of embryogenic initials developed after 4 weeks resulted in the formation of proembryonic structures. The somatic proembryonic structures developed further into globular and heart shaped embryo. In the light of histological study, it is suggested that the continued anticlinal divisions of outer embryogenic initials develops into epidermal cells of the somatic embryo while the periclinal and anticlinal initials of embryonic initials develop into somatic embryo body. The inner neighbouring callus cells of the embryonic initials by anticlinal divisions form a layer of cells to isolate the developing somatic embryo from embryonic callus, which can be called the demarcation layer.

On the induction medium, embryo development stopped at the heart shaped stage. Through maturation globular embryos developed into torpedo and precotyledonary stages.

In the longitudinal sections precotyledonary somatic embryos consist of two distinct regions, a suspensor composed of tiers of elongated vacuolated cells surmounted distally by the embryonal mass which is composed of small highly cytoplasmic cells that were actively dividing. Similar observations have been reported in *Ceratozamia mexicana* (Chavez et al., 1995). However on maturation, the embryos showed irregular branching as reported in *C. hildae* (Litz et al., 1995).
5.2.8.2 Histology of non-organogenic cultures

The histological study of the non-organogenic compact callus showed extensive development of xylem cells. Xylem cell development is also a differentiation process and therefore it can be considered that though not towards embryoids formation, the cells were tuned for differentiation in medium containing one auxin and one cytokinin. Therefore it may be presumed that if the xylem differentiation were not resulted, the cells would have developed into embryoids. That is, the differentiation phenomenon started in the cells must have taken a deviation. It is well known that behaviour of plant cells in tissue culture is unpredictable. When a tissue differentiates, there is difference in metabolic pathways. The requirements of metabolites to bring about altered development come through the medium. The hormones in the medium primarily influence the differentiation by activating early response genes or are involved at relatively late stage such as during cell expansion or morphogenesis.
Increasing concentration of sucrose in the medium enhances the number of xylem elements formed in the callus (Krishnamurthy et al., 1999). They also reported that BAP in the medium composition that promoted xylogenesis in the callus cultures of Cucumis.

The darkening of the callus to light brown colour was evidenced anatomically by the presence of cells with resin deposition in 45 – 60 days old callus. Intense deposition of resin in the parenchyma cells makes the cells physiologically inactive. Therefore in the present study the accumulation of resin in the cells of in vitro cultures is an inferred factor for the inhibition of embryogenesis and organogenesis.

The common occurrence of the calcium oxalate crystals in the form of druses in non-embryogenic callus cultures of Zamia furfuracea may be because of the presence of excessive quantity of its source elements in the medium. The abundance of druses was high in compact calli, which have deviated from organogenesis after xylem differentiation. The embryogenic calli were devoid of druses.

It is reported that calcium oxalate formation is a rapid and reversible process, controlling soluble tissue and cell - calcium levels in plants. (Trachtenberg and Mayer 1982; Franceschi, 1989). Pedroso and Pais (1995) reported that the increase in cell wall thickness of those cells becoming embryogenic and the increase in the accumulation of starch and calcium oxalate crystals in the surrounding parenchyma cells is a common feature in embryogenic callus induced from leaves of Camellia japonica. After the initial divisions of proembryos both starch and calcium oxalate crystals disappeared.
5.2.8.3 Histology of differentiated Structures

The transverse sections of *in vitro* roots of *Cycas* and *Zamia* showed no difference with the *in vivo* counterpart. However the number of resin cells was more in the *in vitro* structures. This may be because of the enhanced resin synthesis in the culture conditions, due to the physiological stress.

5.2.9 Histochemical Localization

In *in vitro* culture systems, characterization of biochemical events leading to organogenesis can be studied by the histochemical and histoenzymological techniques. There are only few reports of similar nature in gymnosperms and no reports in cycads. Some histochemical studies (Thorpe and Murashige, 1970; Brossard, 1975; Tran Thanh Van and Dien, 1975) during organogenesis in tobacco callus showed distinct changes in metabolism prior to the histological appearance of shoots. Patel and Berlyn (1983) reported increased levels of cellular constituents as well as increased intensity of staining for several enzymes prior to and during multiple shoot formation in embryonic explants of *Pinus coulteri*.

5.2.9.1 Starch

Starch reserves were less in most cells of the explant, but comparatively sub epidermal layers maintained abundant starch grains, which disappeared only when those cells became meristematic. The histochemical localization revealed that the actively dividing areas are devoid of starch deposition and by the aging of the callus starch is deposited in the cells.

Heavy localization of starch was observed at the regions of tissue differentiation in the callus. As the starch grains disappear from these regions embryos or shoot buds differentiate; it is clear that starch is utilized in organogenesis as reported by Sarasan (1992) in *Piper*.
During the redifferentiation phase, the degradation products of starch were probably utilized for elongation and growth of cells, possibly in the building up of new cell wall material, as well as for energy production. The observation agrees with the earlier reports in shoot forming tobacco callus (Thorpe and Murashige, 1970; Maeda and Thorpe, 1979).

In the non-organogenic calli, the starch grains are evenly distributed showing no signs of utilization. Similar reports showing strong starch accumulation in non-organogenic callus tissues of *Humulus lupulus* has been reported (Maeda and Thorpe, 1979).

During the redifferentiation phase, the stored starch in the cultures is utilized for the cell expansion and division, which culminates in embryogenesis or organogenesis. Similar observations are reported in tobacco (Ross *et al.*, 1973) and in pepper (Sarasan, 1992).

**5.2.9.2 Protein**

Protein localization reflects the metabolic activity in the tissues. Protein localization at the region of active callus development was evident by histochemical staining in the young rachis explants of *Z. furfuracea*. The intensity of the protein was higher at regions where embryogenic or organogenic induction was noticed. Embryoid and organogenic initials are active sites of metabolic processes and therefore these regions naturally possess higher protein. The low intensity of protein in non-organogenic callus supports this fact. Jose *et al.* (1999) reported similar observations in *Bacopa monieri*. In non-organogenic cultures with extensive xylogenesis, proteins are not localized because lignified elements are physiologically dead cells.
In embryonic explants of *Pinus columbia*, Patel and Berlyn (1983) also observed the formation of organogenic domains that could easily be distinguished because of higher levels of nucleic acid and protein.

The proportion of cells with higher levels of protein gradually increased as the meristemoids and subsequently the shoot apices and leaf primordia developed in *Zamia*. Tanimoto and Harada (1982) found that total amount of amino acid incorporated in the protein was significantly more in shoot forming, epidermal and sub epidermal layers than in the remaining inner tissues of *Torenia* stem segments. These observations and those of Villalobos *et al.* (1984) in *Pinus radiata* indicate localized synthesis of new structural and/or enzymatic proteins required for shoot initiation.

5.2.9.3 Lipid

In *Zamia furfuracea* and *Cycas* species, lipid content activity was nil in the callus or in vitro differentiating structures. It is speculated by Sayuri *et al.* (1986) that cell cultures derived from non-fat storing plants do not show accumulation of lipids during organogenesis or differentiation.

5.2.9.4 Acid phosphatase

The histochemical localization of enzymes showed different distributional patterns in explants and callus cultures during various stages of embryogenesis and organogenesis.

Acid phosphatase activity was distributed evenly in the cells of explants but became intense in the dividing cells of the callus. The developing shoot apices and the proembryos also exhibited strong acid phosphatases activity. Therefore, the sites of future shoot formation could easily be distinguished by the strong localization of acid phosphatase. These observations are in accordance with the findings of Saka
and Maeda (1974) who showed a 1.5 fold increase in acid phosphatase activity in shoot forming regions of rice. Fosket and Miksche (1966) also found a close relation between the pattern of histochemical staining of acid phosphatase and the development of cytohistological zonation within the shoot apex of Pinus lambertiana.

Jose et al. (1999) also observed meagre acid phosphatase activity in the callus cells of Bacopa monnieri, and an intense activity in the meristemoids in compact callus and vascular nodules of shoot bud-forming callus.

An intense localization of acid phosphatase in differentiating vascular tissue is also consistent with its role in lignification of xylem. The localization of acid phosphatase activity was very intense in developing vascular elements. Van Fleet (1959) has reported the association of acid phosphatase with differentiation and morphogenesis in vitro conditions.

5.2.9.5 Succinic Dehydrogenase (SDH)

SDH activity is associated with mitochondria. High SDH activity is indicative of pronounced respiration in living cells. Increased respiration is essential in the cells undergoing expansion and differentiation to provide energy for the different metabolic process besides being involved in the synthesis of enzymes concerned with growth and differentiation (Malik and Usha, 1977).

Patel and Thorpe (1984) reported high SDH staining activity during the formation of organic centers and also during further growth and differentiation of shoot and needle primordia in in vitro cultures of Pinus radiata. They reported the SDH activity in the cytoplasm as round or rod shaped particles, which are presumably the sites of mitochondria. Viilalobos et al. (1982) reported the presence of numerous mitochondria in shoot forming explants of Pinus radiata. This is an
indication of the high rate of respiration during the stages of organogenic development.

Though the SDH activity in the explants of *Zamia* and *Cycas* was uniformly distributed, it was more intense in the dividing cells of the callus and in the organogenic regions. The pronounced SDH activity observed at the organogenic regions is indicative of site-specific respiration rate required for organ differentiation. Josc et al. (1999) reported similar intense activity of SDH in the shoot developing callus of *Bacopa monneiri*.

Increased staining intensities of acid phosphatase, succinic dehydrogenase, protein, and starch were found in shoot forming regions of explants. These findings agree with the hypothesis that the initiation of organized development *in vitro* involves a shift in metabolism that precedes and is coincident with the process. The present observations suggest that the newly synthesized proteins could be enzymatic, at least a part of which is concerned with energy release. The breakdown of reserve materials and enhanced enzymatic activities alters the normal development pathway of cell division in callus and brings about embryogenesis and organogenesis. These findings reinforce the view that organ formation *in vitro* has a high-energy requirement as reported by Thorpe (1980).

In general, the analysis of several enzyme activities reveal that during early stages of development with high mitotic activity show good accumulation of physiologically active substances and high enzyme activity. During the phases of maturity and differentiation, enzyme activity was also different. These changes may be attributed to the changes in the accumulation of the metabolites in the cells depending upon natural utilization and depletion.

5.2.10 Metabolic Profile during cell differentiation
In the present investigation, when embryogenic and organogenic cultures of *Zamia* and *Cycas* were analyzed for biochemical changes, a correlation between developmental events and biochemical quantities of protein, starch, soluble sugar and resin was noticed. Time taken for the induction of organogenesis was PGR related. In all cases, up to the induction phase, quantity of all the primary metabolites increased. Rapid depletion of starch reserves, consequent rise and fall in protein and soluble sugar are related to the embryogenic and organogenic development. Gopalan *et al.* (1992) reported similar studies in *Cymbidium* hybrid. At the time of induction and during the maturation phase of the embryoids the decline in the metabolites observed may be correlated with its utilization during this period. This indicates the high physiological activity during cell morphogenesis. Scandalios (1974) emphasized the relation between morphogenesis and biochemical parameters.

**5.2.10.1 Starch**

Starch metabolism associated with embryogenesis and organogenesis has been investigated in depth in tissue culture and these studies indicate a positive correlation between starch accumulation and development (Thorpe, 1974). Starch depletion after the induction phase is because of its utilization for the energy requirements during the growth of embryoids.

In the present study also, there is demarcating difference in starch mobilization between embryogenic and non-embryogenic callus cultures. The callus cells after it gets ‘conditioned’ with media, starts accumulating starch but when the embryogenesis / organogenesis sets in, the accumulated starch gets used up. Though the time taken for induction varied according to the PGR in the medium, starch utilization was observed at every situation of embryo induction. During
organogenesis also metabolic profile shows a similar trend. In contrast to this, the non-organogenic callus did not show any decline in the starch content, as there is no induction of embryoids and the subsequent energy requirements. Since the cells need energy during morphogenic events, carbohydrate metabolism must be activated (Arruda et al., 2000).

Malik and Usha (1977) reported that stored starch with in a cell indicates a source of energy, which could be possibly utilized during various phases of growth and differentiation.

Ross et al, (1973) explains that the disappearance of starch in tobacco callus preceded meristemoid formation in the cells and directly involved in the organogenic process. Sarasan (1992) reported that, in Piper, the deposition of starch on tissue differentiation in organogenic callus is an indication of the requirement of starch in organogenesis. Mendoza et al. (1993) reported that shoot initiation was associated with rapid depletion of starch and protein in Mungbean, indicating that they are utilized from cells close to the developing shoot. Stamp (1987) and Redway (1991) state that the starch metabolism must contribute to the satisfaction of the intense energy requirement of dividing meristematic cells.

Secondary metabolism also requires energy, however, slow starch utilization was observed in non-organogenic callus, where resin accumulated.

5.2.10.2 Soluble Sugar

Soluble sugars are the products of photosynthesis. When plant cells accumulate sugar beyond a level, it is either converted to the polysaccharide starch or inter-converted to other primary products or translocated to other organs for the synthesis of secondary compounds. Once it is in the form of starch, further transformations are slow or limited (Raj et al. 2001).
Sugars are known for their participation in many biochemical events and differentiation processes involving the activation or deactivation of large activities of protein (Arruda et al., 1999).

5.2.10.3 Protein

In all the responsive media combinations, after an initial decline, the protein increased and maximum quantity was noticed between 4-10 days before the cultures showed embryogenic induction. The pattern of distribution of protein in embryogenic cultures indicates that during the development of somatic embryos, there is the utilization and mobilization of protein metabolites. At the morphogenic level, protein synthesis and high cellular activity are related to the embryogenic process (Scandalios, 1974). Guru et al. (1999) also reported that redifferentiation and dedifferentiation involve the metabolism of various proteins at different stages. Reduction in the protein content may affect differentiation processes and the morphogenic responses (Arruda et al., 2000).

After embryogenic induction the protein level gradually decreased. In non-embryogenic cultures, the protein profile showed an irregular pattern of distribution. Arruda et al. (2000) reported that an increase in calcium supply in the medium was correlated to an increase in protein content in in vitro callus morphogenesis of Eucalyptus europhylla. This suggests the participation of calcium in metabolic events such as protein synthesis (Hepler and Wayne, 1985; Grover et al., 1998).

5.2.10.4 Resin

Resin is a common secondary metabolite in many gymnosperms. When the cells are programmed for secondary metabolite production, it loses its differentiating potential. All the cells are not involved in the resin production and therefore it can
be speculated that those cells, which are not involved in resin synthesis, may be competent for embryogenesis or organogenesis.

Continuous increase in the accumulation of resin can be correlated with carbohydrate metabolism. Raj et al. (2001) reported that in in vitro culture systems, sugar is either utilized for tissue growth and differentiation or converted to primary product or translocated for synthesis of secondary compounds.

In the present investigation, resin accumulation is common in the cells of the callus derived from the explant. Reports have shown that compounds either identical with or chemically related with those of whole plants have been frequently detected in plant tissue and cell cultures (Pu Hang and Martin, 1971).

In non-organogenic cultures of Zamia and Cycas, the accumulated sugar might be used for the synthesis of resin, instead of being deviated for organogenesis. Similar correlation between secondary metabolites and carbohydrate metabolism was reported in species of Barleria and Sida (Raj et al., 2001). When the resin is continuously accumulated in cells of cultures, the concentration of sugar and starch declined. Therefore it can be interpreted that sugar, instead of being converted to polysaccharide or other primary product, is translocated for the synthesis of resin.

The whole ‘episode’ reveals that the failure in morphogenesis leading to poor frequency regeneration in cycads is due to the activation of biosynthetic pathway leading to the accumulation of the secondary metabolite – the Resin. The accumulated resin in the embryogenic cultures is in no way utilized for the development and differentiation of the induced embryos. In non-embryogenic cultures, cells fail to induce somatic embryos due to the excessive resin accumulation.
Generally a number of energy demanding stages are required for the biosynthesis of various secondary compounds (Hillis, 1976) similar is the case for the production of new elements of xylem and phloem. In the present investigation, all the plants studied showed the synthesis and accumulation of resin in the cells in the in vitro condition. Since a major share of energy is utilized for the biosynthesis of resin the energy demanding stages leading to morphogenesis is interfered.

The production of secondary metabolites by cell culture has shown to be most active during a restricted phase of growth cycle and such production is often markedly influenced in its intensity by the phytohormone composition of the culture medium (Carcellar et al., 1971; Kaul and Staba 1967; Ogunuga and Northicote, 1970; Tabata et al., 1971).

Street (1976) argues that progress towards induction of particular specialized patterns of cytodifferentiation in cell cultures can only be achieved if the synthesis of volatile oils, resins, latex and other constituents do not take place. Therefore it can be inferred that if the resin was not produced in the cells of callus, morphogenesis leading to plant regeneration would have been definitely improved.

5.2.11 Isozyme analysis

The enzyme pattern of an organism changes during development and differentiation. Changes of isozyme pattern in samples of a particular organ or tissue during development are seen by the appearance and disappearance of individual isozymes. Such changes in isozymes suggest that genes involved in the synthesis of these enzymes are differently activated in development (Chawla et al., 1988). Rao et al. (1992) report that more number of bands and increased intensities indicate increased enzyme activity.
in the present study, marked differences in isozyme patterns was observed in rachis explants, proliferating calli, embryogenic cultures and differentiated in vitro structures of *Zamia* and *Cycas* species.

5.2.11.1 Peroxidase

The peroxidase isozyme pattern in embryogenic and differentiated organs of *Zamia furfuracea* exhibited three distinct bands, which distinguished them from non-embryogenic callus. However, in explant the activity was detected but showing more bands than non-embryogenic callus. In the case of *Cycas circinalis* also pronounced peroxidase activity in embryogenic and organogenic cultures was observed. However, in *Cycas revoluta* there is no distinction in peroxidase activity between rachis explant and in vitro developed calli. *Rao et al.* (1992) reported that the enzyme activity of individual isozyme in a specific tissue depends on maturity and cellular environment.

Histological studies in the in vitro cultures of the three cycads in the present study showed rapid lignification of tissues, in non-organogenic cultures. Higher number of bands and their higher intensity indicate the higher peroxidase activity.

Siegel *et al.* (1960) reported that higher peroxidase levels could be associated with precocious lignification and prevention of cell elongation resulting in dwarf stature. They also reported the role of peroxidase as precursors of lignification. Birecka *et al.* (1999) reported that peroxidase is one of the most reliable indicators of senescence, which involves in plant senescence mainly due to its ability to oxidize IAA and to participate in lignin formation. So a logical conclusion can be that the high peroxidase activity shows inhibition of normal growth.
5.2.11.2 Esterase

Esterases have a definite role to play in plant growth and development, cell wall expansion and somatic embryogenesis and have been implicated in hydrolysis of secondary metabolites (Dubey, 2000).

In the present study, a total of two sharp bands were detected in the embryogenic cultures, which were intense than those of the non-embryogenic cultures and mature explants. Higher esterase activity was also noticed in the embryogenic and differentiated organogenic cultures of Cycas circinalis. Chibbar et al. (1988) reported that esterases have been used as a cytochemical marker of differentiation in stelar lines and somatic embryogenesis in cultured carrot cells.

The detected esterase activity in in vitro differentiated structures can be explained by the active role of these enzymes in developing tissues as reported by Veerabharappa and Upadheya (1979).

5.2.11.3 Acid phosphatase

The appearance of the acid phosphatase bands, though feeble and narrow, in all the samples of Zamia furfuracea, megagametophyte and organogenic cultures of Cycas circinalis suggests the involvement of these enzymes in the developmental stages. The activity in the organogenic regions explains its role in development.

The reduced number and intensity of bands indicate the low rate of enzyme activity compared to those of peroxidase and esterase. This can be correlated with the reports of Zink and Veliky (1979) that acid phosphatase secretes into the medium in culture conditions.

In the present study, acid phosphatase banding patterns are not helpful in distinguishing embryogenic and non-embryogenic callus. This finding is in agreement with the reports of Coppens and Dewitte (1990) who are also of the view
that acid phosphatase was not suitable for discrimination between embryogenic and non-embryogenic callus in *Maize*. However, the appearance of acid phosphatase isozyme bands in the explants are suggestive of high metabolic activity in regions of higher nuclear transcription as reported by Deltous et al. (1981) in *Maize*.

Isozyme pattern vary with tissue organisation during development and differentiation. The isozymes of esterase, peroxidase, investigated in this study were found appropriate to distinguish compact embryogenic calli from friable non-embryogenic one in *Zamia furfuracea* and *Cycas circinalis*. Acid phosphatase was not suitable for discrimination between embryogenic and non-embryogenic callus

To conclude, the present investigation into the *in vitro* response of selected cycads provides insight into the following conclusions.

Profuse callusing can easily be achieved from vegetative explants in all the species studied. However, organogenesis, which is the expected goal in Micropropagation, is not as easy as callogenesis. Despite the stupendous effort, using more than 300 hormonal combinations, frequency of somatic embryogenesis, its recovery, shoot induction and subsequent growth was poor. This shows that result depends not only on basal media and hormonal regime. The cells respond to media and other supplements in an unpredictable way. Induced organs failed to grow into complete plantlets. Mitotic activity was stopped with the untimely lignification of the cells involved in morphogenesis. Cycad cells in culture show intense deposition of secondary metabolite, resin which probably would be the major hindrance of the cell morphogenesis. Therefore, future research in cycads tissue culture to achieve cell differentiation and growth may be focused on blocking the secondary metabolite pathway along with stimulating the cells for morphogenesis.