1. INTRODUCTION

In 1825 the Scottish botanist Robert Brown distinguished gymnosperms from the other major group of seed plants, the angiosperms, whose seeds are surrounded by an ovary wall. The first known use of the word 'Gymnosperm' was, in fact, by one of Aristotle’s pupils - Theophrastus - who used it to describe plants whose seeds are unprotected. The seeds of many gymnosperms (literally, "naked seed") are borne in cones and are not visible. These cones, however, are not the same as fruits. During pollination, the immature male gametes, or pollen grains, sift among the cone scales and land directly on the ovules (which contain the immature female gametes) rather than on elements of a flower (the stigma and carpel) as in angiosperms. Furthermore, at maturity, the cone expands to reveal the naked seeds.

Some of the oldest living things on earth are gymnosperms. Although since the Cretaceous period gymnosperms have been gradually displaced by the more recently evolved angiosperms, they are still successful in many parts of the world and occupy large areas of the Earth's surface.

Gymnosperms were considered at one time to be a class of seed plants, called Gymnospermae, but taxonomists now tend to recognize four distinct divisions of extant gymnospermous plants (Coniferophyta, Cycadophyta, Ginkgophyta, Gnetophyta) and to use the term gymnosperms only when referring to the naked-seed habit. Some of the divisions of gymnosperms are not closely related to others, having been distinct groups for hundreds of millions of years.

The modern plant systems regard the living Cycads (Cycadinae) as a separate class in the subphylum Gymnospermae of the Phanerogamous plants.

Subphylum 1. Gymnospermae
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Class 1. Pteridospermae
Class 2. Cycadinae
Class 3. Bennettitinae
Class 4. Cordaitinae
Class 5. Ginkgoinae
Class 6. Coniferae

Of these, the classes Cycadinae, Ginkgoinae and Coniferae are represented among the living plants; the Pteridospermae, Corditinae and Bennettitinae, which had first arisen in the Paleozoic, had disappeared already in the Mesozoic, and their place was gradually taken by the Angiosperms.

1.1 Cycads

The Cycads are ancient plants, which developed during the Triassic and were most abundant during the Jurassic, when they were found on all continents; Cycads have survived up till our time, but they are threatened due to destruction of their habitats and by the fact that they are collectors items (Greguss, 1968).

It is generally conceded that from the Pteridospermae arose members of the division Cycadophyta. The first cycads appeared in the Permian period (286 to 245 million years ago). Some of these presumed cycads differ from extant members in that megasporophylls were undivided, unlike those of Cycas, considered to be primitive among cycads, in which the distal portion of the megasporophyll may be pinnately divided. Other Permian megasporophylls from China are more like those of Cycas. Cycad remains, especially leaves, are abundant in Mesozoic rocks. For this reason paleobotanists often refer to the Mesozoic era as the "age of cycads." The earliest well-known cycads appear to have had slender stems, sometimes branched, and with leaves not borne close together, unlike the situation in extant
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cycads in which leaves are densely crowded at the apex of the plant. There is evidence that these earliest cycads were deciduous. Megasporophylls of Mesozoic cycads are essentially like those of extant cycads. The megasporophyll of the Triassic Palaeocycas is like that of Cycas. Jurassic megasporophylls are like those of most other cycads. Extant cycads are now limited in geographic distribution to the warmer parts of the earth.

Cycads are among the most primitive spermatophytes (Gymnospermae, Cycadales) and have been described as living fossils. Cycads have changed very little in the last 200 million years. Norstog (1967) has noted that Cycads are repositories of information concerning the biology and evolution of higher plants.

Cycads existing at the present time are the surviving remnants of an ancient time of plants that was common in the early Mesozoic period. Today Cycads are restricted to East Africa, Madagascar, sub-tropical and tropical America, India, Southern China, Southern islands of Japan, some islands of the south Pacific ocean and Australia. Most Cycads are in danger of extinction and several species have almost disappeared, the only survivors being the 9 genera and 132 species, which now constitute the entire order. They are not only of remarkable botanical and horticultural interest, but they also represent a natural source of antimicrobial and pesticidal biochemicals (Rinaldi, 1999).

Cycads occupy a unique botanical position. They are morphologically the intermediate stage in the plant evolution from ferns to the flowering plants, and the only gymnosperms living in symbiosis with Cyanobacteria. Most of the members among the existing 132 species are under severe environmental stress. Consequently more than half of the species have been classed as ‘vulnerable’, ‘rare’ or ‘endangered’ (Osborne, 1988; Webb and Osborne, 1989).
Their slow growth rate, the paucity of viable seeds and limited potential for vegetative reproduction severely limit both the natural regeneration and the controlled propagation of the Cycads.

The class Cycadinae is divided into three families:-

Family I. Cycadaceae  Family II. Stangeriaceae  Family III. Zamiaceae  

Most Cycads inhabit the tropical zone. In the Mesozoic, they were widespread also in the northern hemisphere, the temperate zone of the Europe of today. At present they generally live in the equatorial region, the zone between the Tropics of the Capricorn and of the Cancer. They nowhere form continuous closed stands, but occur sporadically, at most in smaller groups, in the savannah regions and partly in the virgin forests of the mountains.

At present, about 132 species of living Cycads are known. Although all these are rather similar in external habit, in the appearance and structure of the flowers, in being dioecious, and also in their anatomical structure, it is easy to classify them into smaller or larger natural groups.

Though Cycas circinalis L. is the only native species of cycads in Kerala, Cycas revoluta Thunb. and Zamia furfuracea L. are among the costly ornamental plants in great demand.

1.1.1 Zamia furfuracea L.

This is one of the ‘living fossil’ plants, of its kind surviving on earth since the time of the dinosaurs. Zamia, popularly called Cardboard palm, belongs to the Cycad family. Cardboard palm has 3 to 4 foot leaves that emerge from a central point forming a rosette. They resemble Tree Ferns, but produce cones as a pine tree does. Male and female reproductive structures (cones) form on separate plants. Even very young plants produce these interestingly shaped cones. When ripe, the
female cone breaks to reveal an array of tightly packed, bright red 1-inch long seeds.

The Seminole Indians, as a main source of starch, once used this plant. The root-like stem was chopped into pieces, crushed into powder, washed and drained. The residue was left to dry into yellowish-colored flour. The raw stem is poisonous and should not be eaten. These tender perennials are from tropical and sub-tropical countries.

1.1.2 Cycas revoluta Thunb.

_Cycas revoluta_, one of the most primitive living seed plants, is very unusual and therefore ornamental. The plant is with a rugged trunk, topped with whorled feathery leaves and known by the common name "Japanese Sago". Often called "living fossils", Cycads have changed very little in the last 200 million years.

The growth habit of _Cycas revoluta_ displays an upright trunk with a diameter from 1" to 12" depending on age, topped with stiff feather-like leaves growing in a circular pattern. This is the most popular and widely cultivated of the cycads. It makes an excellent landscape plant, as well being very well suited to pot culture, and even bonsai.

It is a very hardy plant (like most cycads), tolerating dry periods, and light frosts. It prefers a sunny, well-drained spot, with deep soil, but will still thrive in less than ideal conditions. Though it can be propagated from seeds, the seed setting is scarce and therefore the common method of propagation is through offshoots or 'pups'. As the plant is slow growing and the development of offshoots is rather limited, the conventional method of propagation never cope up with the demand.

1.1.3 Cycas circinalis L.
Cycas circinalis also is a palm like tree, commonly known as 'queen sago'. It is a larger and more graceful version of its more commonly encountered cousin, Cycas revoluta. The queen sago's solitary trunk can grow to 20 feet in height, which is twice that of the Japanese sago. In older specimens some branching may occur to produce plants with multiple crowns. The dark green pinnate leaves grow to 8 feet in length with narrow 12 inch leaflets that give a gracefully drooping aspect.

The large cone that grows out of the crown of the male tree is tan-colored. Modified leaves or megasporophylls grow out in a rosette from the crown of the female tree bearing ovules in their margins.

As in Cycas revoluta, this also can be propagated through seed. However, availability of fertilized seeds is less due to the unscientific maintenance of male and female plants.

1.2 Cycad Tissue Culture

The IUCN has classified more than half of 132 species of the order Cycadales (Stevenson and Osborne, 1993) as endangered, vulnerable or rare (Gilbert, 1984). This is mainly because of the destruction of native habitat and over collection. Cycads are usually grown from seeds. The erratic seed germination, rapid loss of seed viability, slow growth and low morphogenic potential have aggravated the problem of conservation (Rinaldi and Leva, 1995). Most Cycad seeds are large, have a high water content and possess a short viability period. There is a long delay between pollination and fertilization and by protracted embryo development in some species of Cycads. Rate of multiplication is limited because fertile ovulate sporophylls are very few in number. Pollination mechanisms are poorly developed or pollinators are absent from native strands. Cycads are dioecious and there is no synchrony between the maturation of ovulates
and staminate reproductive structures and even in dense strands, the production of fertile seeds is limited.

Growing cycads from seed is commonly practiced as techniques for doing so have been developed. It is a more time consuming approach for propagation, but one, which yields good results. A more elaborate technique but one that appear to have a good chance of success in the future is tissue culture (Gilbert, 1984).

Micropropagation using tissue culture techniques provides a promising method for mass multiplication of any such valuable species (Dhiman et al., 1998 a).

Cycads are notorious for their recalcitrance to tissue culture (Webb and Osborne, 1989) and therefore a challenge to investigators. The generally used explants in Cycads are from embryo or megagametophyte (La Rue, 1954). Some workers have used leaf explants also (Dhiman et al., 1998 a). Paucity of seeds and a single massive shoot apex (pachycaulous) are additional disadvantages for their propagation by tissue culture techniques.

Though induction of somatic embryogenesis has been achieved from zygotic embryos and leaf callus of adult plants of Zamia species (Chavez et al., 1992 c) and Ceratozamia (Chavez et al., 1992a; Litz et al., 1995 b), complete plant recovery has not been achieved. Micropropagation was also attempted from haploid (megagametophytes) and diploid embryo explants of Zamia species (La Rue, 1954; Norstog, 1965) and Ceratozamia (De Luca et al., 1979; Chavez et al., 1992a ). The first haploid plants experimentally produced in vitro were derived from the megagametophyte of Z. pumila (Chavez et al., 1992 c).
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In cycads other than *Zamia pumila* and *Cycas revoluta*, adventitious shoot regeneration was reported only from cultured zygotic embryos of *Ceratozamia hildae*, *C. mexicana* and *C. euryphyllidia* (Chavez et al., 1992c, 1998).

Dhiman et al. (1998 b) reported mini plantlets or bulbils arising from the nodular structures in the hypocotyls portions of embryogenic explants in *Cycas circinalis*. Except in an isolated report of complete plant recovery via somatic embryogenesis in *Ceratozamia euryphyllidia* has been reported (Chavez et al., 1998), successful recovery of plantlets in the field condition has not been reported so far in any of the cycads.

1.3 Objectives of the present study

Though the past reports of cycad tissue culture is not showing promising results, considering the importance of conservation and economic importance of *Zamia furfuracea*, *Cycas revoluta* and *Cycas circinalis*, in the present study renewed attempts have been made to establish *in vitro* protocols using haploid and diploid tissues.

The main objective was to experiment with the different explants with a wide variety, range and combinations of plant growth regulators, which have not been tried before so as to get an efficient protocol for *in vitro* propagation of cycads.

The objective was also focused at elucidating the causes of poor morphogenic response of cycad genotype in tissue culture.

Histological techniques are widely used in many areas of research. Structural analysis is an important step in the study of organization and changes in the plant body and it is an extremely useful approach in the study of plant morphogenesis. Different histological methods have contributed significantly to the understanding of *in vitro* culture systems (Yeung, 1999). Histological techniques have been
employed in the present study to investigate and document the origin and developmental stages of somatic embryogenesis and organogenesis.

Histochemistry and histoenzymology are applied to correlate physiological aspects with anatomical and developmental patterns. Basic knowledge of histoenzymology has collated morphologic, ultrastructural, biochemical and physiological data in relation to development of tissues and organs in several systems. Within a plant, different tissues exhibit anatomical diversity and histochemistry, which suggest physiobiochemical basis of particular pattern of differentiation and morphogenesis (Malik and Singh, 1980).

In this investigation, biochemical and histoenzymological procedures have been applied to localize and thereby identify the characteristics of cells involved in differentiation and to study different enzyme changes during development and differentiation of tissues and organs of selected cycad plants exposed in the in vitro conditions.

The metabolic pathways, operating in a cell, are well connected with cell differentiation process. It is expected that a periodical assessment of primary metabolites like proteins, starch, soluble sugars and secondary metabolites like resin would underlie the morphogenetic events in tissue culture. Therefore, periodical evaluations of metabolites were also carried out in the cultured samples.

Since electrophoretic techniques have become a tool of general usage in plant genetic studies, the number of works dealing with the ontogenic changes of isozymes has continuously increased (Ono and Nakano, 1978).

The primary use of isozyme in plant tissue culture to date has been in relation to physiological studies. Isozyme patterns frequently vary in intact plants as a function of various physiological and developmental states (Scandalios, 1974) and
one expects similar responses in cultured cells. Therefore in the present study analysis of isozymes was conducted for biochemical identification and characterization of different developmental and morphogenetic events, which occur during regeneration in Cycad cultures.