DEVELOPMENTAL PHYSIOLOGY AND BIOCHEMISTRY OF SOMATIC EMBRYOGENESIS IN *PINUS KESIYA* ROYLE EX. GORD.

BY
SANGHAMITRA PURKAYASTHA

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BOTANY

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SHILLONG 793 022, INDIA
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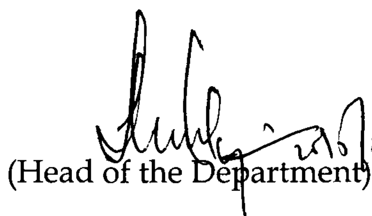
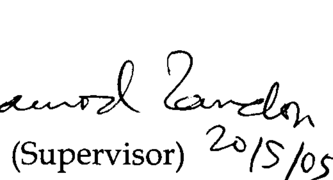
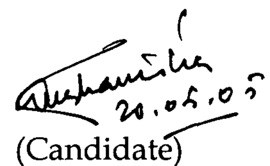
NORTH-EASTERN HILL UNIVERSITY
SHILLONG

MAY, 2005

CERTIFICATE

I, Sanghamitra Purkayastha, hereby declare that the subject matter of the thesis entitled "Developmental physiology and biochemistry of somatic embryogenesis in *Pinus kesiya* Royle Ex. Gord." embodies the record of original work done by me, that the contents of the thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the thesis has not been produced or submitted by me for any research degree for any other University/Institution.

The thesis is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Botany.

(Head of the Department) (Supervisor) 20/5/05 (Candidate)

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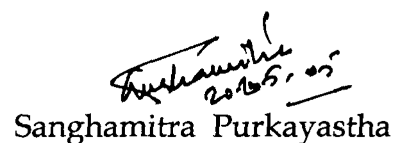
Shillong

20th May, 2005



Sanghamitra Purkayastha

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Chapter I

Introduction - A review of literature

Somatic embryogenesis in conifers has in the recent years been used as a potential tool for the mass propagation of the forest trees. At the same time, it provides an ideal experimental process for investigation of differentiation in the organism and understanding the expression of totipotency in plant cells. Embryogenic cell lines maintain their competence for a long time and provide a necessary medium for the study of the developmental process associated with somatic embryogenesis.

Tissue culture techniques have found wide use in the commercial propagation of plants including forest trees. Somatic embryogenesis has been considered as a potential tool for propagation of conifers for afforestation programmes (Tautorus *et al.*, 1991; Charest *et al.*, 1995; Pullman *et al.*, 2003; Von Arnold, 2003) mainly because:

- It offers an inexpensive, large scale propagation system for superior genotypes
- Production of somatic embryos throughout the year, reducing the risk where seed production is limited and uncertain
- Other applications which have been foreseen for somatic embryogenesis include provision of establishing cell lines for genetic engineering and

somatic hybridization, long term gene storage, and also use in research to gain an understanding of somatic genetics and development

Somatic embryogenesis is the process of embryo formation from somatic cells to give rise to whole plants without the fusion of gametes. Somatic embryogenesis may be *direct*, when the embryo develops directly from the somatic or vegetative cells of the explant without the intervening callus phase. It may be *indirect*, when the embryos develop from an undifferentiated mass of cells. Embryogenesis can be induced very easily in some cells like cells of zygotic embryos and these are called *competent* or *pre-embryonic determined* cells. However, there are also a wide variety of cells which behave as embryos only when they are subjected to major manipulations, like the somatic cells in culture. These cells are called as the induced embryogenic determined cells or *potentially competent* cells. Besides, there are some cells which are so highly differentiated that it is almost impossible to induce embryogenesis in them and are designated as the *non-competent* cells. There are two kinds of growth in tissue culture, *unorganised growth* (forming undifferentiated tissues) and *organised growth* (charactarised by development into embryos) are mediated by the exposure of responsive explants to critical concentration of exogenously applied plant growth regulators during the initial culture phase (Gupta and Grob, 1995; Bozhkov *et al.*, 2002). In the absence of auxin, cells grow in an organised way and develop into embryos. Presence of exogeous auxin stimulates cell

enlargement, which disrupts the cellular organisation in the developing embryos and results in the proliferation of callus (Steward *et al.*, 1964).

It has however been observed that the developmental genetic programme in the embryogenically competent somatic cells under the influence of specific phytohormones, proceeds in the same or closely similar fashion as would in a zygote for the development of a zygotic embryo. But somatic embryos are not very precisely organised like zygotic embryo. The suspensor region may consist of loosely associated cells, and many embryos may share a common suspensor system (Hakman and Fowke, 1987b). In contrast, organogenesis shows sequential shoot and root differentiation on different media.

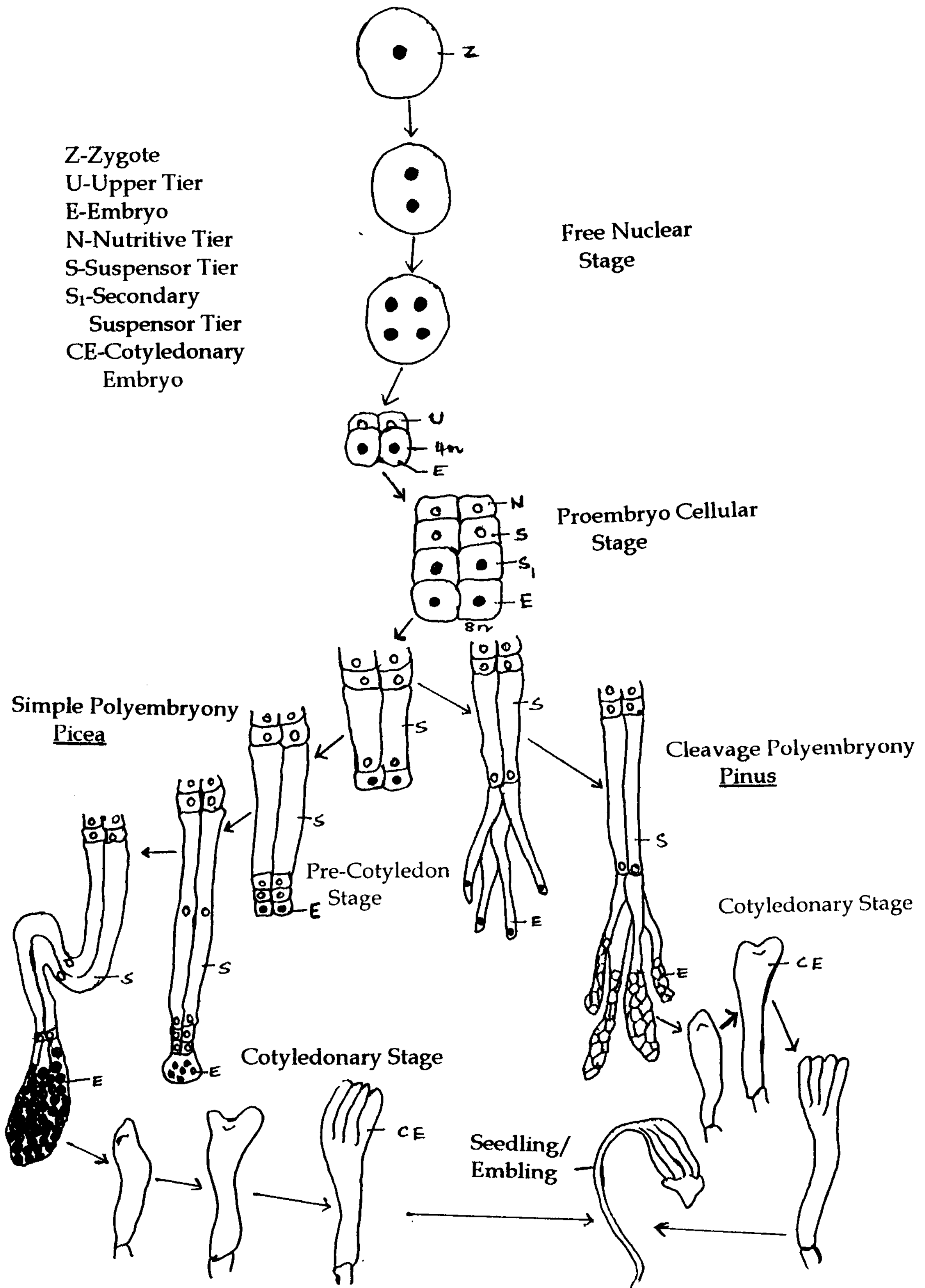
There are three different methods for initiation of embryogenic cultures in conifers:

a) through the continuation of natural cleavage polyembryony of embryonal heads of explanted immature embryos (Durzan and Gupta, 1986b).
b) through cell division in the epidermal and subepidermal layers of hypocotyl, cotyledons or needles resulting in calli which then rapidly organise to form embryonal suspensor masses (Nagmani *et al.*, 1987).
c) through cell division of small cells within the suspensor system of explanted immature embryo (Gupta and Durzan, 1987).

Somatic embryogenesis involves development of embryos from somatic tissues of the plant by redirecting the morphogenetic fate of the cells in the explant to form embryogenic cellular masses or embryoids. Most conifers undergo one of the two types of polyembryony which is a common and natural phenomenon in conifers, and could be either *simple* or *cleavage* (Fig.1). In *simple polyembryony*, as in *Picea* and *Pinus* (Singh, 1978) proembryo is a result of fertilization of more than one egg per ovule by gametes from separate pollen grains, and therefore every proembryo is genetically different. One proembryo usually dominates and continues development while the others abort. *Cleavage polyembryony* is common in pines, where the cleavage embryos result from a separation of the apical tier cells of an individual proembryo into four files of cells, each of which may develop into a separate embryo. The resulting embryos are therefore genetically identical. One of these embryos becomes dominant and the other embryos cease development. The mechanism by which the successful embryo inhibits the growth of other embryos is unknown, although factors such as mechanical, nutritional and growth inhibiting influences of the dominating embryo, probably have some role to play (Dogra, 1967; Owens & Blake, 1985; Tautorus *et al.*, 1991; Von Aderkas *et al.*, 1991).

Of the conifers, pines constitute the most divergent, and economically important group of species. They provide valuable natural resources and though they are known to be pioneer species and active colonizers of

Figure 1. Simple and cleavage polyembryony in conifers



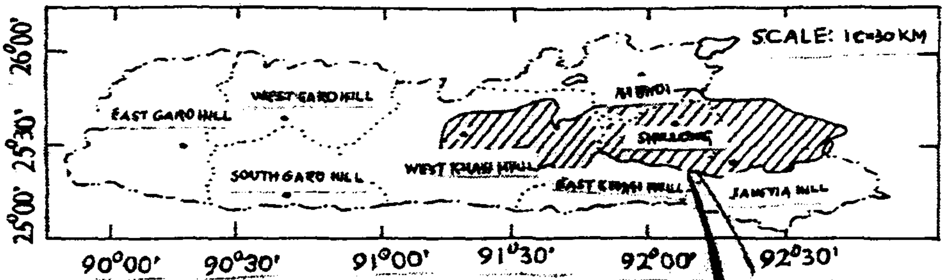
Z-Zygote
U-Upper Tier
E-Embryo
N-Nutritive Tier
S-Suspensor Tier
S1-Secondary Suspensor Tier
CE-Cotyledonary Embryo
Free Nuclear Stage
Proembryo Cellular Stage
Simple Polyembryony
Picea
Cleavage Polyembryony
Pinus
Pre-Cotyledon Stage
Cotyledonary Stage
Cotyledonary Stage
Seedling/ Embling

degraded sites, their population in the recent years have been fast depleting. The fact that they protect watersheds and sustain and regulate water supply in the Himalayan river basin in India, should be reason enough for us to try and sustain their diminishing/ dwindling population.

Ninety species or more of the pine population (genus: *Pinus*, family: Pinaceae, order: Coniferales, Gymnosperms) are known to be distributed in the Northern Hemisphere from the polar region to Guatemala, North Africa and Indonesia. Of these, six pine species are indigenous to India and are found scattered in the Himalayan region from Jammu to the North-east. These species are *P. roxburghii* Sarg. (syn. *P. longifolia* Roxb., *P. serenagensis* Maddeu, chirpine) *P. wallichiana* A.B. Jacks (syn. *P. excelsa*, Blue pine, Kail), *P. gerardiana* Wall. Ex Lamb. (chilgoza pine, Neoza pine), *P. insularis* Endl. (sys. *P. kesiya* Royle ex Gord., khasi pine), *P. merkusii* Jungh (Merkus pine) and *P. armandii* Franchlet (Armandi's pine). Of these *Pinus kesiya* is the most widespread in the north eastern states of India (Fig. 2).

Pinus kesiya (Royle ex Gord.) commonly known as the khasi pine is an economically important timber-yielding tree found in the North-east India and extends up to Myanmar, Phillipines and Vietnam. It grows mostly on the hills at an elevation of 750m to 2000m thriving best at 1350-1500m in sub-temperate and fairly moist regions, while it is distributed in Phillipines at elevations of 450m to 2450m above sea level, in upper Burma between 800m to 1900m above sea level, Yunnan and North Thailand and Malay

Figure 2. Distribution of subtropical pine forests in Meghalaya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KILOMETER
EAST GARO HILL
WEST GARO HILL
SOUTH GARO HILL
WEST KHASI HILL
EAST KHASI HILL
JAINTIA HILL
26°00'
25°30'
25°00'
90°00'
90°30'
91°00'
91°30'
92°00'
92°30'
SCALE: 1c=8 KM
8 0 8
KILOMETER
RI BHOI
WEST KHASI HILL
East Khasi Hills
JAINTIA HILL
26°00'
25°30'
25°00'
91°00'
91°30'
92°00'
92°30'

Archipelago. In Meghalaya, *P. kesiya* is confined to higher reaches (800m to 2000m above sea level) of the Shillong plateau in Khasi and Jaintia hills, in a narrow belt running in east-west direction. Due to variation in altitude from 60m to 2000m above sea level and associated changes in rainfall and temperature, moist tropical forests occur below 1000m and the subtropical semi evergreen forests are found between 1000m and 2000m elevation in the state. In the upper limits it forms pure stand (Fig. 3) while lower down it is mixed with broad leafed species like *Quercus spp., Schima wallichii, Myrica esculenta, Rhododendron arboretum* and *Exbucklandia populnea.* Age old practice of shifting cultivation and other anthropogenic activities such as cutting trees for timber and construction of building, collection of fuelwood during the past several decades have destroyed the climax subtropical broadleafed forests at higher elevations in Meghalaya and elsewhere in the North-east India paving the way for invasion and successful growth of *P. kesiya* (Puri *et al.*, 1989) and according to them, khasi pine was introduced in this region in prehistoric times. The shrub (including small trees) species growing in the forest strands include *Rubus ellipticus, R. khasianus, Myrsine semiserrata, Osbeckia crinita, Nellia thyrsiflora, Eupatorium sp., Lantana camara, Artemesia spp., Viburnum foetidum, Leptodermis spp.,* besides others. During the monsoon the forest floor is covered with a profuse herbaceous undergrowth of annuals and perennial flowering plants and ferns, including *Lindenbergia hispida, L. racemosa, Paspalam sp., Ophiopogon wallichii, Hedychium coccineum, Eurya*

Figure 3. A *Pinus kesiya* strand Shillong, Meghalaya

Figure 4. Decrease in girth of pine trees

3

4

acuminate, Rubus monogynus, Senecio cappa, Galinsoga parviflora and Melastoma malabathricum. However, much of the ground flora lies dormant during winter, giving a barren look to the forest floor.

The wood of this tree is moderately hard, pale brown to red in colour and is used for building of houses because of its high resin content. The resinous wood is much used as firewood, the production of which cause considerable injury to the trees. This and deforestation to meet the ever increasing demand for timber, slash and burn cultivation and unplanned developmental activities such as new townships, road and dams, and clearing of land for permanent agriculture are the main reasons for the dwindling populations of *Pinus kesiya* in the recent years. Even the girth of the trees now shows reduction in many areas (Fig. 4). The problem has attained such a magnitude that Supreme Court of India has banned felling of all trees in the North-eastern region.

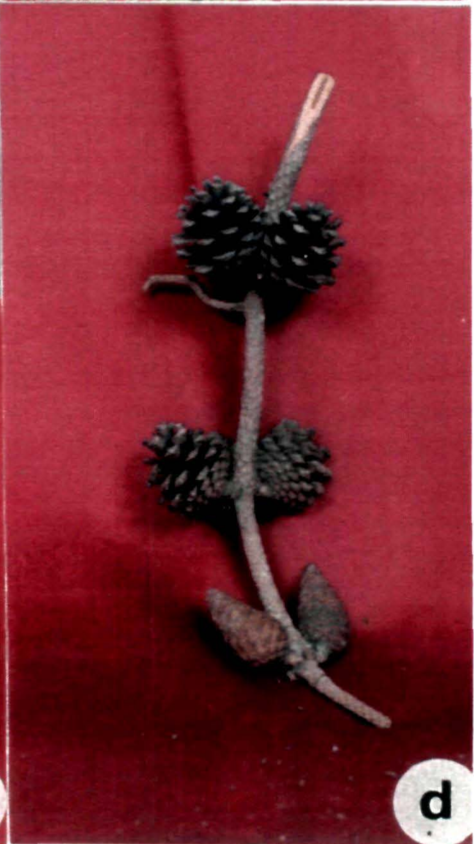
The trees are evergreen and monoecious, generally 25-30m tall with straight cylindrical bole and 2m girth bearing branches in pseudowhorls, crown on mature trees are broad (Fig.5). Bark is 2-4 cm thick, fissured and scale like in younger trees while dark brown in mature trees variously furrowed and plated. Shoots are light brownish, and are of two kinds, dwarf or spur shoot of limited growth, and long shoot of unlimited growth. Leaves are dimorphic, the small, thin, brown scale leaves which occur on long as well as dwarf shoots, from the axis of these scaly bracts arise the needle like

Figure 5. Morphology of *Pinus kesiya*

a. Complete tree
b. A branch with immature male and female cones
c. Mature female cones (before seed dispersal)
d. Mature female cones (showing dehiscence)

a

b

c

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green foliage leaves 15-20 cm long which develop in cluster of three at the apex of the dwarf shoot. The tree generally flowers in April-May and bears ovulate cones after two years. Staminate cones are numerous and small, a mature cone is about 20-30 cm long, mostly ovoid to cylindrical in shape, yellow, orange or light red in colour and composed of many spirally arranged microsporophylls with two pollen sacs, seen as a dense cluster around the base of the current year growth. Pollen grains are shed and dispersed by wind during February to April. The female cones are purplish to deep green in colour and borne solitary or in clusters of two, three or five on the apices of new shoots. The young cones are erect and ovoid with fertile scales of inflorescence spirally arranged, with a bract scale above each of them. The scales on the cone close immediately after pollination and the cones turn inverted or horizontal on the branch to hibernate for the cold winter. The following season the cones again show active growth, turn green, fertilization occurs during this time, a year after pollination, and the cones are pushed to the lateral position on the branch due to the growth of new shoots. The following winter the cones gradually turn hard and brown, and attain their full development. In the third season, ie., 24 - 26 months after appearance of the female cones, they open during the last week of December and dispersal of seeds continue up to March, the dry weather aids in the dispersal of the winged seeds, two in number attached at the base of each

woody scale. On an average, seeds are 5.7mm long and 3.9mm wide, the wing being about 18–20 mm long and the cotyledons being 3.5-4.0mm long.

Most pines are fire adapted, so in *P. kesiya*, the seedlings are fairly resistant to fire once over 3m tall, and mature trees are immune to fire damage owing to their extremely thick outer bark. However short cycles of shifting cultivation and regular annual fires prevent satisfactory regeneration and can lead to the elimination of pine. The pines remain dominant so long as fires occur at intervals of about 5 to 20 years. Pines are a frost tolerant species and can grow on well drained nutrient-poor soils on moderately sloping hillsides and landslide effected areas, all of which they colonize as typical pioneers. Its natural regeneration is best on mineral soils as well as porous soils with partially exposed to fully exposed rocks, primarily limestones. Soil development is minimal where recurrent fire has consumed litter and under growth vegetation. There being very little organic matter left on the surface, which sometimes is a bare rock, the underlying rocks are overlaid with a thin layer of poor soil, which serves as the rooting medium for pine. The species is preferred by foresters and the Forest departments of the North-eastern states have been involved in forest plantations over the years particularly for regeneration of areas affected by shifting cultivation. However, all these plantation programmes are achieved through conventional methods that appear to be inadequate to keep pace with the

ever increasing demand for timber and also lack of uniformity of planting material with desired characteristics.

Propagation of this tree by conventional methods faces constraints as natural regeneration occurs through seeds and the seed orchards show great variations and at times seed germination is very poor. Factors influencing natural regeneration of pines include, seed viability, light, moisture, soil condition, undergrowth, fire, grazing. A large number of seedlings are destroyed by fire, low winter temperature, heavy rainfall, cattle grazing, caterpillars and moths. Moreover, pine trees bear seeds in a cyclic manner and every third year is a good seed year and in between the trees bear only small crop reducing the seed reserves considerably. On the other hand multiplication through seeds gives rise to plants which are not true to type. Weed infestation is another factor adding to the cause of seedling mortality. Progress in large scale improved forest production through conventional methods is usually very slow and time consuming. The vegetative propagation methods used for multiplication from economically favoured genotypes through stem cuttings is rather difficult as this pine reaches sexual maturity at an early stage after which rooting ability of cutting decreases resulting in poor regeneration. In addition, as has been reported in other pine species, with increase in the age of mother tree, the rooting ability of cuttings is reduced considerably. It has been recommended that acreage under forest cover needs to be doubled. But *P. kesiya* is also susceptible to

different types of infection by pathogens at various stages of growth and development i.e., seedlings, nursery, plantations, and in natural strands. Many fungi are responsible for diseases like stem rot, needle rust and seed borne diseases, which results in tremendous loss to annual yield of timber in plantation as well as in natural pine stands. To meet the predicted requirements of timber and to conserve forests, there is an urgent need for mass propagation of *P. kesiya* using unconventional methods of propagation. Somatic embryogenesis through tissue culture has developed into an important tool through which plantation programmes could be implemented, produce plantlets on mass scale in a short period of time, besides establishing a system where the developmental mechanism of the plant under tissue culture can be studied.

Somatic polyembryogenesis has been described by many workers and have termed the embryogenic tissues as 'embryonal suspensor masses' (ESMs) due to their high degree of organization. Most embryogenic cultures of conifers have been induced from members of family Pinaceae and are similar in appearance. An embryo from a seed is placed on a medium containing appropriate nutrients, hormones etc. First a callus culture grows then becomes embryogenic and made of ESMs which are usually white, transluscent in appearance and appears to glister due to production of mucilage when cultured on semi-solidified medium. They anatomically consist of a variable mixture of elongated cells, early stage embryos, which

have embryonal head and a suspensor system and sometimes later stage embryos (Gupta and Durzan, 1987a, and b; Finer *et al.*, 1989; Laine and David, 1990). On the other hand non embryogenic callus appears opaque, friable and green when exposed to light. This type of callus may or may not have an anatomical organization. The somatic embryos differentiate on the callus, these embryos can then be germinated and grown into somatic emblings.

The embryogenic tissues induced in pines are generally glossy, translucent, white, mucilaginous cellular mass containing a mixture of elongated densely cytoplasmic clumped cells, embryo initials and sometimes older stage embryos (Gupta and Durzan, 1987b; Finer *et al.*, 1998; Laine and David, 1990).

Considerable efforts have been directed towards somatic embryogenesis of conifers for many years. Studies have been conducted on the growth, metabolism and developmental patterns that charactarize callus and cell suspension of conifers (Durzan and Steward, 1968; Durzan *et al.*, 1976; Durzan 1980). Somatic embryogenesis and plant regeneration in conifers was first reported in *Picea abies* (Chalupa, 1985 and Hakman *et al.*, 1985). The development of somatic embryogenesis in conifers has shown much progress since then (Steward *et al.*, 1964; Attree and Fowke, 1993; Jain *et al.*, 1995; Bozhkov *et al.*, 2002). Somatic embryogenesis or organogenesis of tissues in *in vitro* cultures are influenced by environmental conditions of the

culture medium (Williams and Maheswaran, 1986) and besides basal medium, induction and development of somatic embryogenesis is influenced by various other factors like light, temperature, relative humidity, pH of the medium and organic carbon source.

The proper explant selection is critical for successful induction of somatic embryogenesis *in vitro*. Various tissues from the same plant or even tissues at different development stages have shown difference in their response when cultured *in vitro* (Attree and Fowke, 1991; Attree *et al.*, 1991, Deb and Tandon, 2002; 2004). Mostly zygotic explants have so far been used to initiate embryogenic cultures in conifers. These tissues include megagametophytes containing developing zygotic embryos (immature embryos), mature zygotic embryos dissected from stored seeds, tissues from hypocotyls, cotyledons, leaf needles, apical domes and also recycled cotyledonary somatic embryos. Explants from various conifers that have been induced to form somatic embryos include explants from genera like *Abies, Larix, Picea, Pinus, Pseudotsuga* and *Sequoia*. *Pinus* is by far the largest and most important genus of conifers comprising approximately 95 spp. widely distributed over the Northern Hemisphere (Preston, 1989). While the process of somatic embryogenesis and its technique has been sufficiently well refined for commercial application in many species of spruce and larch, it is still not available for all conifers. In pine species, it was found much

more difficult to obtain somatic embryogenesis and even mass propagation of a large number of clonal lines is not yet possible.

Induction of somatic embryos in conifers have been obtained from excised tissues from seedlings, mature and immature zygotic embryos (Jain *et al.*, 1989; Laine and David, 1990). In addition, megagametophytes of several conifers have been cultured with varying success. Large scale production of somatic embryos through embryonal suspensor masses by cleavage polyembryony in liquid culture with high concentration of hormones than that used for induction has been reported in conifers (Boulay *et al.*, 1988; Gupta *et al.*, 1991). In pines, somatic embryogenesis could be induced from explants at early stages of zygotic embryo development (Gupta and Durzan, 1987b), while Laine and David (1990) reported from early stages of polyembryony. Finer *et al.* (1989) found response from immature zygotic embryos just prior to cotyledon development in *Pinus strobus,* with increase in induction frequency with increase in age of zygotic embryos i.e., before cotyledonary primordia appeared. In contrast Jones and van Staden (1995) found response and embryogenesis at all stages of embryo development except at very young and cotyledonary stage in *P. patula.*

Many physiochemical factors have been reported to influence the initiation of embryogenic callus initiation in pines species. These factors include: basal media composition (Liao and Amerson, 1995a; Li and Huang, 1996; Deb and Tandon, 2002; 2004) plant growth regulators composition

(Becwar *et al.*, 1988a; Nagmani *et al.*, 1993) gelling agents (Li *et al.*, 1997a, b, 1998) meso-inositol and silver nitrate concentration (Li and Huang, 1996), organic carbon source (von Arnold, 1987; Becwar *et al.*, 1988a, b), organic nitrogen (Barett *et al.*, 1997), and pH of the medium. Generally pH value 5.5 to 6.0 was found to be effective. Besides basal media composition and its supplements, induction and development of somatic embryogenesis has been found to be influenced by some other factors like the cultural conditions including pretreatment, light, temperature and the relative humidity.

Culture conditions play a vital role in tissue culture raised plants and initiation of somatic embryogenesis in cultures is largely regulated by the cultural conditions. Many environmental factors have been reported to which plant cells respond, and hence divide, elongate, polarize and differentiate. Light was found to be inhibitory for the induction of somatic embryogenesis in conifers and cultures incubated in the dark showed profuse callusing. Cultures grown in light tend to turn green and instead showed organogenesis. At the same time temperature effected the rate of embryogenesis in cultures.

Modification of basal medium components and culture conditions can significantly affect induction of embryogenic tissues and play a major role in enhancing initiation for more explants (von Arnold, 1987; Attree *et al.*, 1990a; Tautorus *et al.*, 1990a). Several media have been used by the workers in the

original as well as in various modified forms. Media requirements have thus not been very specific. It has been observed, however, that a certain basal media was best suited for the species of a particular genera. Thus embryonal suspensor masses have been initiated on several media such as LV (Litvay *et al.*, 1981), DCR (Gupta and Durzan, 1985), DCR_1 (Becwar *et al.*, 1995), modified Murashige and Skooge (mMS) (Gupta and Durzan, 1986b), BLG (Verhagen and Wann, 1989), P_6 (Gupta and Pullman, 1990), BM_1 (Gupta and Pullman, 1991), LP (Quoirin and Lepoivre, 1977), WTC (Gupta and Pullman, 1991) besides others. In all these cases it was found that the main modification appeared in the nitrate salt concentration especially ammonium nitrate and potassium nitrate. Barrett *et al.* (1997) has reported that removal of organic nitrogen sources like casein hydrolysate (CH) and L - glutamine is beneficial for *P. glauca.*

Saccharides are known to serve as carbon and energy sources, osmotic agents, stress protectants and signal molecules in plants. A few reviews have focused on deeper studies of carbohydrate metabolism, enabling insight into the physiological background of the crucial effects of carbohydrates by collecting and critically discussing the experimental data on exogenous saccharide applied, resulting endogenous levels and key enzyme activities obtained. The most thoroughly described genus in conifers is *Picea* (Lipavska and Kondradova, 2004). Low percentage of sucrose (1-2%) resulted in more ESMs formation (von Arnold, 1987; Becwar *et al.*, 1988a, b). In the

maintenance medium too, concentration of the carbon source effected the formation and development of the proembryonal masses in media supplemented with 6% sucrose along with 10mgl^{-1} abscisic acid (ABA). Again with decrease of sucrose concentration from 20gl^{-1} to 5gl^{-1}, there was a decrease in proembryo formation and loss of embryogenic potential in *P. caribaea* (Laine and David, 1990). The physiological and osmotic roles of sucrose has been investigated by Trembley and Trembley (1995) in black spruce (*P. mariana*), while a comparative study was carried out to understand the role of maltose and sucrose on the total number of mature somatic embryo formation in *P. strobes* (Garin *et al.*, 2000).

L-Glutamine has been used in the maintainance medium in concentrations lower than in the induction medium for the development of sugar pine (Gupta and Durzan, 1986a, b) and loblolly pine somatic embryos (Gupta and Durzan, 1987). Besides, role of polyethylene glycol (PEG) in the maturation medium was reported by Li *et al.* (1997, 1998) in *P. taeda* cultures which developed into mature somatic embryos successfully.

In most conifers, somatic embryos undergo morphologically similar development to zygotic embryos (Misra, 1994). Conifer somatic embryo usually has to be stimulated by ABA. A combination of ABA and a suitable osmoticum (for eg., non permeating osmoticum like PEG or permeating like higher percentage of carbohydrates like sucrose, maltose, mannitol)

promotes the normal development of somatic embryos in conifers and are essential for their gene expression.

Among the gelling agents gelrite was reported to be a superior compared to agar for initiation of ESMs in *P. strobes* cultures (Finer *et al.*, 1989; Puchooa *et al.*, 1999), while phytagel was effective for *P. taeda* (Li *et al.*, 1998).

Plant growth regulators appear to control all the main developmental events in somatic embryogenesis starting from induction of embryogenic cultures to germination of somatic embryos, and majority of the workers have unambiguously consented to the crucial role of plant growth regulators in the regulation of somatic embryogenesis. The effect of exogenously added growth regulators in media was extensively studied by Vagner *et al.* (1998), but about endogenous state of the growth regulators, very little is known (Dunstan *et al.*, 1995). Usually both auxins and cytokinins are necessary for somatic embryogenesis and amongst the various auxins, 2,4-dichlorophenoxy acetic acid (2,4-D) was the most preferred for the initiation of ESMs in most conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). α-naphthaleneacetic acid (NAA) also has been used for ESMs induction in *P. abies* (Verhagen and Wann, 1989) while no significant difference was reported in ESMs or development of embryos in conifers with NAA versus 2,4-D as the sole auxin source (Gupta and Grob, 1995). Again with 2,4-D as the sole auxin source, somatic embryos were induced in *P. taeda* (Li *et al.*,

1998), and incorporation of either 6-benzylaminopurine (BAP) or kinetin in the medium was found to be beneficial in most cases. Auxin proved to be inhibitory for the initiation of embryogenic cultures in *A. nordmanniana* (Norgaard and Krogstrup, 1991; Norgaard *et al.*, 1992). Development of ESMs was observed by application of different concentrations of hormones, like 2,4-D (2-10mgl^{-1}) and BA (0.5-5.0mgl^{-1}) in *Larix* (Cornu and Goeffrion, 1990; Bonga *et al.*, 1995), NAA and BAP (2mgl^{-1} each) and 2,4-D (10-110mgl^{-1}) in *P. abies* (Gupta *et al.*, 1991), and 2,4-D (2-10mgl^{-1}) along with BAP (0.5-2.5mgl^{-1}) in *P. palustris* (Nagmani *et al.*, 1993). A higher frequency of embryogenic callus was reported at lower concentrations of phytohormones in *P. serotina* (Becwar *et al.*, 1988) and *P. taeda* (Li *et al.*, 1998). Nagmani *et al.* (1993) on the contrary reported an increased frequency at higher level of plant growth regulators in *P. palustris*.

Embryo maturation and plant development have been significantly influenced by different concentration of ABA in the medium. Cleavage polyembryony is inhibited by ABA allowing singulation and continued growth of individual embryos. Several studies resulted in improved embryo maturation after treatment with ABA prior to transfer to phytohormone free medium for final germination (Backs and Reinert, 1970; Durzan and Gupta, 1987; Roberts *et al.*, 1990a; Dunstan *et al.*, 1994). Promotive effect of ABA on maturation of somatic embryos was reported in *P. sylvestris* and *P. pinaster* (Lelu *et al.*, 1999). Different species and genotypes show different frequency

of proliferation of embryogenic cultures in the medium. While some species and genotypes proliferate readily into embryogenic cultures on the induction medium, in some others, reformulation of medium is required (Gupta and Grob, 1995). Maintenance and proliferation of embryogenic culture is important to increase its availability for regeneration and genetic manipulation. Proliferation is usually done on medium with lower concentrations of growth regulators. The final step in conifer somatic embryogenesis is the successful germination of embryoids into emblings which is generally achieved on media free of any growth regulators. There has been limited report of successful establishment of regenerants in conifers and so far, successful germination and subsequent transfer of somatic embryos in *Pinus* is restricted to a few species, viz. *P. patula, P. taeda* (Gupta and Durzan, 1987; Gupta and Pullman, 1990; Tang *et al.*, 1998a,b), *P. caribaea* (Laine *et al.*, 1992), *P. patula* (Jones *et al.*, 1993; Jones and Van Staden, 1995; Ford *et al.*, 2000), *P. sylvestris* (Lelu *et al.*, 1999), *P. elliottii* (Tang *et al.*, 1997), *P. strobes* (Klimaszewska and Smith, 1997; Garin *et al.*, 1998; Klimaszewska *et al.*, 2000), *P. nigra* (Salajova *et al.*, 1999) and *P. pinaster* (Lelu *et al.*, 1999).

In spite of constantly growing knowledge, there is a lack of proper understanding of the biochemical and physiological events involved in somatic embryogenesis. To develop a deeper understanding of the regulation of embryogenesis, it is necessary to conduct detailed biochemical

studies of the complex, highly conserved developmental events which lead to the formation of somatic embryos.

Somatic embryogenesis was first clearly described in domestic carrot (*Daucus carota* L.) and till date the carrot system is the most comprehensively studied with respect to culture conditions and developmental physiology and biochemistry of somatic embryogenesis. Hence, it has been a useful model for investigation of the mechanisms controlling somatic embryogenesis. It was way back in 1970 that the study of biochemical aspects of somatic embryogenesis began to be studied and till date a lot of work has been done in this aspect.

The synthetic auxin 2,4-D has been shown to be the most efficient inducer of embryogenic pathway (Ammirato, 1983; Sung *et al.*, 1984). Embryo specific genes and proteins have been intensely searched for and studied (Backs and Reinert, 1970; Mc William *et al.*, 1974; Nomura and Komamine, 1985; Zimmerman, 1993; Donga and Dunstan, 1994; Paques, 1993). From an ultrastructural study of embryogenesis in carrot cell suspension, it was concluded that embryogenic induction probably occurs during isolation and growth of tissues in auxin containing medium, although formation of more organised structures reminiscent of zygotic embryos is prevented as long as auxin is present in the medium. At the same time, the profile of newly synthesized proteins of nonembryogenic and embryogenic cultures of carrot was made by two-dimensional gel electrophoresis (Sung

and Okimoto, 1981). In this work using ^{3}H-methionine, to label the proteins, the workers have identified two proteins in 12 day old embryogenic cells. The surprising finding was that regardless of the presence or absence of 2,4-D in the medium, these proteins were synthesized by cells during the early days of their growth in fresh medium, but in the presence of 2,4-D, the proteins gradually diminished and completely disappeared after 12 days. Hence, it seems that synthesis of embryogenic proteins is triggered by auxin, its very presence in the medium also prevents the continued synthesis of these proteins necessary for embryogenesis coming to fruitation.

The mechanism of the regulation of somatic embryo development of broad leafed woody flora remained largely unknown. Nevertheless, these systems have begun to be investigated (Gavish *et al.*, 1991, 1992; Puupponen *et al.*, 1993). Gene expression during seed development, maturation and germination has been examined in several angiosperm species and distinct subsets of developmental regulated genes that respond to distinct regulatory signals have been identified (Goldberg *et al.*, 1989); much less is known about conifer somatic embryogenesis. The study has been limited due to the long reproductive cycle and inaccessibility of developing seeds from conifer species. A biochemical marker may be useful for early identification of embryogenic cultures before any morphological changes occur. Its use would help to optimize culture conditions necessary for embryogenesis and to discriminate cultures following the multiplication process. Some of the

reasons for the lack of information was unavailability of data on the isolation and charactarization of markers for embryogenic cells. Efforts were made to find specific molecular markers for somatic embryos and several biochemical variables have been shown to discriminate between embryogenic and non embryogenic tissues in cultures (Sung and Okimoto, 1981; Choi and Sung, 1984; Nomura and Komamine, 1986). Somatic embryogenesis in *Abies alba* Mill was even induced using SH medium (Vooková et al., 1998), though other conifers did not respond in this medium. Macromolecule accumulation and synthesis (proteins, polysaccharides and nucleic acids) are indicators of cell growth and physiological change (De Vries *et al.*, 1988; Neilson *et al.*, 1992; Coutos-Thevenot *et al.*, 1993; Uchiyama *et al.*, 1993). Proteins as indicators of differentiation have been investigated as markers (Sung and Okimoto, 1981; Komamine *et al.*, 1992; Paques *et al.*, 1993; Kormut *et al.*, 2003) while they could be useful to identify specific stages of development of somatic embryos (Menendez *et al.*, 1994). Enzyme patterns also change during developmental stages and peroxidase patterns have been known to indicate embryogenecity of cultures (Kochba *et al.*, 1977; Hrubcova *et al.*, 1994; Egertsdotter, 1998; Bagnoli *et al.*, 1998; Kormut *et al.*, 2003).

In carrot, results from two dimensional gel electrophoresis detected three embryogenic proteins 'a', 'b', 'c', throughout the process of totipotency i.e., from single competent cells to the globular embryo stage via., heart shaped and torpedo shaped embryos, but disappeared during the process of

losing totipotency. Two mRNAs showed the same pattern as the proteins. Additionally, protein 'd' disappeared during the single cell stage. In stages of cell clusters and in globular stage embryos, the pattern of *in vitro* translated products of mRNA extracted from embryogenic and nonembryogenic cultures (in the presence of auxin) were exactly similar except for four proteins, two appeared and two disappeared during these phases. This indicates that a few proteins accumulate during maturation and can identify somatic embryos that have completed the phase of embryogenesis. Donga and Dunstan (1994) observed that intracellular protein content is maximum during the early stage (at day 9) of culture and decreased at a later stage of culture. Against the general assumption that soluble storage proteins are similar in zygotic and somatic embryos of conifers (Hakman *et al.*, 1990; Hakman, 1993). Kormut *et al.* (2003) indicated the presence of a higher number of storage proteins in somatic embryos of silver fir than in their zygotic counterparts. Among six super-numerary fractions revealed in somatic embryos, the 53 kDa fraction was the most conspicuous that marked the divergent nature of somatic embryos. The 16 and 19 kDa fractions were detected in the embryogenic callus only; not in the nonembryogenic calli. The transition between these tissues represents the initial stage of somatic embryogenesis in conifers. Pâques *et al.* (1993) detected both nonembryogenic callus-specific and embryogenic callus-specific polypeptides to distinguish the two types of calli of *P. abies*.

Smith *et al.* (1988) reported a nuclear protein associated with cell division, an antigen against 21D7 monoclonal antibody. Komamine *et al.* (1992) applied 21D7 to the carrot system and analysed by Western blotting and immunocytochemical method to examine whether antigen 21D7 (21D7 protein) can be a candidate for molecular marker of totipotency. 21D7 could be detected throughout the process of expression of totipotency, which disappeared within 48 hrs during the process of losing the totipotency, i.e., when the single cell were cultured in the absence of auxin. Furthermore, when the single cells were microinjected with 21D7, they elongated and no longer divided nor differentiated even if cultured in the presence of auxin. These results indicate that expression of 21D7 protein may be essential for expression of totipotency.

In zygotic embryo development, secretion of extracellular proteins has been considered a physiological event regulated by embryo specific genes. These extracellular proteins have various functions during embryo differentiation and development, such as metabolism, nutrient storage, phytohormone synthesis and transportation. There is increasing evidence to indicate that the secretion of extracellular proteins *in vitro* in embryogenic suspension cultures is also developmentally regulated by genes, reflective of normal requirements of the embryo development.

In one instance, an extracellular protein (EP1) that is only secreted by nonembryogenic cells (Van Engelen *et al.*, 1991). Sterk *et al.* (1991) reported

that another extracellular protein (EP2) identified as lipid transfer protein, was only synthesized by embryogenic cells and somatic embryos. From these and other extracellular proteins described (Satoh and Fujii, 1988), it emerges that the developmental state of carrot suspension cells is reflected in the type of secreted proteins synthesized by these cells.

Extracellular proteins have various functions during embryo differentiation and development such as metabolism, nutrient storage, phytohormone synthesis and transportation. There is increasing evidence to indicate that genes reflective of normal requirements of embryo development also developmentally regulate the secretion of extracellular proteins *in vitro* in embryogenic suspension cultures (Van Engelen *et al.*, 1991), they also identified an extracellular protein which is only secreted by nonembryogenic cells. Sterk *et al.* (1991) have identified a second protein which acts as lipid transfer protein and is synthesized by only nonembryogenic cells and somatic embryos. Egertsdotter *et al.* (1993) observed that the extracellular protein profiles from *P. abies* embryogenic suspension cultures were different between those cultures from which cotyledonary somatic embryos could be matured and those not capable of maturation. All embryogenic cultures consisting of somatic embryos with densely packed cells in their embryogenic region secrete proteins of 28 and 85 kD. Besides, concentrated extracellular proteins from embryogenic cell line stimulate another nonembryogenic cell line to develop further

(Egertsdotter *et al.*, 1993). And since extracellular proteins of 28, 66 and 85 kD are also secreted by the induced cell line once they have attained embryogenic potential, it is assumed that these proteins, which are not normally secreted by this cell line, are involved in the stimulation of this induction process. This protein 28 kD is a member of protein family that appear to act by changing the membrane permeability.

The gene of a similar protein zeamatin isolated from *Zea mays* has been charactarised (Malehorn *et al.*, 1994) the polyclonal antiserum of which recognizes two extracellular proteins of 18 and 28 kD which is absent in the induced tissue. Zeamatin- like proteins, whose only known functions in the plant is to inhibit fungal growth, are stored in high concentrations in seeds (Roberts *et al.*, 1990).

SDS-PAGE of soluble proteins from extracts of harvested tissues of sandalwood revealed the presence of 15 major bands ranging in molecular weight from 14–18 kD. From the profile study it was observed that two low molecular weight proteins of 15 and 30 kD, which could not be detected in the callus stage appeared as major bands in extracts from tissues of later stages. There was a consistent increase in the peak area of the polypeptides with progressive embryogenesis. Polypeptide pattern alteration analysed in the course of somatic embryogenesis of carrot (Sung and Okimoto, 1981; Choi and Sung, 1984; De Vries *et al.*, 1988; Komamine *et al.*, 1992), *Nicotiana plumbaginifolia* and *Digitalis lanata* (Reinbothe *et al.*, 1992), pea cultivars (Stirn

and Jacobson, 1987) and *Cinchorium intybus* (Bayer *et al.*, 1993) similarly revealed only minor adjustments to the pre-existing gene expression programme, and it is likely that many of the molecular processes of embryogenesis are already established in polyembryonal masses (PEMs) in the presence of auxin. Komamine *et al.* (1992) suggested that only a few proteins play an important role during embryogenesis, and these proteins are stage specific.

Using sandalwood somatic embryo cultures, a 48-50 kD intracellular glycoprotein was detected in extracts from tissues of PEMs and globular embryo stage, in gels stained for glycoproteins. The level of protein apparently decreased with transition of embryos to bipolar embryos. However, when globular embryos were cultures in 2,4-D containing MS medium, they disorganized into spheroidal and elongated cells, and the level of glycoproteins was higher than in the first two stages (Shankara Rao *et al.*, 1996).

Endochitinases have been identified (De Jong *et al.*, 1993) that has been able to rescue the development of somatic embryos of *D. carota* in a temperature-sensitive mutant, the main function being formation of the embryo protoderm. An assay system was presented based on the observation that the phenocritical period in temperature sensitive (ts) arrest at globular stage in carrot cell mutant ts11 coincided with the period of sensitivity to replacement of the conditioned medium by fresh growth

medium. When the medium conditioned by the wild type cell line was added to the ts11 culture medium, arrest at the globular stage under nonpermissive temperatures was lifted and embryo development in ts11 was completed up to tornedo stage, resulting in the formation of plantlets. This effect was found to be protease sensitive, suggesting that the secreted proteins were the causative component of the conditioned medium (Lo Schiavo *et al.*, 1990; Anke *et al.*, 1992). Purification of the secreted protein and partial protein sequences obtained from it, as well as biochemical characterization, identified this extracellular protein, designated EP3, as a 32 kD glycosylated acidic endochitinase. These results indicate that, apart from their postulated role in the plant defense response, at least the family of plant proteins with chitinase activity has a function in somatic embryo development. Bacterial signal proteins have been shown to have the same effect on embryo development in *D. carota* as the 32 kD endochitinase (Goldberg *et al.*, 1989). In that study it was concluded that embryogenic cell lines of *P. abies* contain somatic embryos which have reached different stages of development reflected in the presence of extracellular proteins.

Peroxidase activity in embryogenic cells and its somatic embryos have been studied by many workers. Egertsdotter (1998) working on peroxidases and chitinase activity of *P. abies* has shown them to differ between developmental stages of somatic embryogenesis (Kormut *et al.*, 2003). According to Bagnoli *et al.* (1998) reported that the antioxidant enzymes

superoxide dismutase and catalase could be convenient markers to define the developmental stages in *Aesculus hippo-castanum* somatic and zygotic embryogenesis. The same role was also postulated for peroxidase and esterase, whose isoenzyme patterns were shown to reflect the embryogenic potential of *Medicago sativa* and *Dactylis glomerata* suspension cultures (Hrubcová *et al.,* 1994). The peroxidase activity of mature somatic embryos was triple the corresponding enzyme activity of dormant zygotic embryos of silver fir. Starting with the early cotyledonary stage, a decline in peroxidase activity was registered throughout zygotic embryogenesis, and the situation was similar during somatic embryogenesis. However, peroxidase activity changed abruptly during two stages of somatic embryogenesis. The first stage was the transition of nonembryogenic to embryogenic callus, accompanied by a conspicuous decline in specific enzyme activity. The second stage was that of regenerated seedlings, which had seven times higher peroxidase activity than mature somatic embryos (Kormut *et al.,* 2003). Tunicamycin, a fungal antibiotic, which prevents N-glycosylation of proteins was found to inhibit somatic embryo development at an early preglobular stage in *D. carota.* This inhibition could be overcome by simultaneous addition of correctly glycosylated proteins to the culture medium (De Vries *et al.,* 1988). This glycoprotein was purified and identified as a cationic peroxidase of horse radish (Cordewener *et al.,* 1991). Thus, they have isolated and purified a cationic peroxidase that actively prevented cell

expansion in the preglobular stage embryos. The enzyme has been shown to be responsible for development of somatic embryos in carrot cell cultures. Based on the observed expansion of small embryogenic cells in the presence of tunicamycin and the identification of a peroxidase activity that prevents this expansion, a model was presented that identifies the peroxidase - mediated restriction of cell size as an important prerequisite for successful somatic embryogenesis to occur (Van Engelen and De Vries, 1992). It has also been observed that peroxidase activity is significantly higher in areas surrounding wounds and necrosis followed by rise in soluble protein content (Johansson *et al.*, 2004) besides playing a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic compounds cross-link (Saroop *et al.*, 2002).

Ethylene is a phytohormone that plays an important role in every phase of plant growth and development (Abeles *et al.*, 1992). Its biosynthetic pathway has been well established (reviewed by Yang and Hoffman, 1984; Kende, 1993). In higher plants, ethylene is synthesized from methionine through S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC). In plant cell, tissue and organ culture, the influence of ethylene on the regulation of different physiological processes occurring in *in vitro* culture, particularly during somatic embryogenesis, is not fully understood. This led to studies on the effects of ethylene on different steps of somatic embryogenesis and have yielded conflicting results and conclusions

(Gahagan *et al.*, 1968), in conifers (Yang and Hoffman, 1984; Tan and Thimann 1989; Li and Huang, 1996; Selby *et al.*, 1996; El Meskaoui and Tremblay, 1999; El Meskaoui *et al.*, 2000). Silver nitrate being an ethylene antagonist was used to investigate and determine if the ability of an embryogenic cell line to produce mature embryos, i.e. maturation capacity, could be associated with its patterns of ethylene production (Meskaoui and Tremblay, 2001).

An upward shift in the concentration of calcium (Ca^{2+}) present in the medium during somatic embryogenesis increased the number of embryos produced, approximately to two folds (Jansen *et al.*, 1990). It was found that at elevated concentrations of calcium, the synthetic auxin was not able to completely prevent somatic embryogenesis, suggesting that calcium partially counteracts the inhibitory action of 2,4-D on somatic embryogenesis. On the contrary reducing the concentration of calcium to one fourth in the maturation medium improved embryo development (Pullman *et al.*, 2003), this trend however varied when experimented with differing levels of boron, iron, potassium and copper ion concentrations.

Somatic embryos of carrot contain low levels of abscisic acid during early stages of development, the levels then reach a peak and decline during maturation (Kamada and Harada, 1981; Dunstan *et al.*, 1991). Exogenous application of ABA to immature zygotic embryos of carrot suggests that ABA specifically inhibits precocious germination and promotes maturation

and accumulation of storage proteins (Barret, 1986; Kuhlemeier *et al.*, 1987). Before them, Sung and Okimoto (1981) found that the light treatments that promote maturation (formation of cotyledons) also increase levels of exogenous ABA in carrot somatic embryos, and aberrant embryo structures formation is suppressed (Kamada *et al.*, 1981). Some more recent experiments have suggested that ABA may play an important role in regulating the expression of some classes of embryo specific genes (Goldberg *et al.*, 1989; Kermode, 1990). Besides, the maturation medium of conifers commonly contains ABA which is necessary for embryo maturation (Jain *et al.*, 1995). The influence of the levels of ethylene in the embryogenic cultures and maturation of somatic embryos in experiments on black spruce embryogenic cell lines (El Meskaoui and Tremblay, 2001) can be significantly observed and thus, it may be that somatic embryogenesis is regulated by the interaction between endogenous ABA and ethylene metabolism. A link between ethylene and ABA has been reported in oat leaves and in apple slices, where ABA decreases ethylene production through a decrease in ACC synthesis (Tan and Thimann, 1989). Moreover, the endogenous ACC level in embryogenic tissues of black spruce and white spruce growing on maintenance medium, without ABA but with 2,4-D and BA, was very high compared to those in the embryogenic tissues cultivated on maturation medium (El Meskaoui and Tremblay, 2001). In white spruce, ABA reduced ethylene production and promoted maturation (Kong and Yeung, 1994), and

it is important to note that ABA has been found to be both promotive and inhibitory on somatic embryos maturation depending on its concentration, besides auxin/cytokinin ratio that may be responsible for reduced ACC synthesis in cultures during maturation. It has also been indicated that ethylene can negatively affect the quality of white spruce somatic embryos by forming large intercellular spaces in the shoot apex that can be partly responsible for the low conversion rate of the somatic embryos into plants (Kong and Yeung, 1994). However, ethylene did not affect the quality of somatic embryos in the study of El Meskaoui *et al.* (2000) as in the previous study (Kong and Yeung, 1994). From these studies, it may be observed that the optimal somatic embryo maturation capacity does not depend only on the sensitivity of the embryogenic cell line to ABA but many other factors, and this requires more studies.

Keeping in mind the importance of *Pinus kesiya* need for its mass multiplication through somatic embryogenesis and the complex interplay of physiological and biochemical processes during somatic embryogenesis, the following studies were carried out resulting into the current doctoral work:

- Effect of culture conditions, media composition and plant growth regulators on the induction of somatic embryogenesis.
- Quantitative and qualitative estimations of intra- and extra cellular proteins in nonembryogenic and embryogenic cultures.

- Profile study of stage specific soluble proteins (i.e., glycoproteins) if present and their role during embryogenesis.
- Qualitative and quantitative assay of peroxidase activity and its relationship with different stages of somatic embryo induction and development.
- Relation and effect of abscisic acid and calcium to the proteins formed during somatic embryogenesis.

Chapter II

Material and Methods

Somatic embryogenesis in conifers, is considered an efficient method of vegetative propagation that has been used in the recent years in forestry research and commercial propagation. The development of techniques for producing somatic embryos provide a means to propagate conifers clonally and also offers an *in vitro* experimental system that can enhance our basic understanding of the developmental processes underlying the formation of somatic and zygotic embryos. Various workers (Salaj *et al.*, 2004; Sreedhar and Bewley, 1998; Trembley and Trembley, 1991) have studied the different factors involved in establishing somatic embryogenesis in conifers.

A biochemical marker might be useful for early identification of embryogenic cultures before any morphological changes occur. Its use would help to optimise culture conditions necessary for embryogenesis and to discriminate cultures to follow the multiplication process. Several biochemical variables have been shown to discriminate between embryogenic and nonembryogenic tissues in culture. Among these are proteins, isozymes and ethylene. Proteins are valuable indicators of differentiation and have been used in taxonomy as genetic markers and could be useful to identify specific stages of development of somatic

embryos. Storage proteins were the first compounds used as markers in comparing the developmental programs of both somatic and zygotic embryogenesis (Hakman *et al.*, 1990; Hakman, 1993). Enzyme patterns also change with change in the developmental stages. Peroxidase patterns are known to indicate the embryogenecity of the cultures and hence can act as valuable marker in the study of somatic embryogenesis.

Plant materials

Success in the field of somatic embryogenesis in conifer is to a large extent dependent on the proper selection of the explant, collection period, storage, priming (pretreatment), media composition, exogenously applied plant growth regulators and the culture conditions.

Collection of explants

Mature zygotic embryo: Seeds (Fig. 6a) were extracted from mature cones collected during January to March from '+' trees identified by the Forest Department, Government of Meghalaya. The cones were air-dried at 25-35^{0}C to obtain the seeds that were stored at 4^{0}C in sealed polythene bags till used.

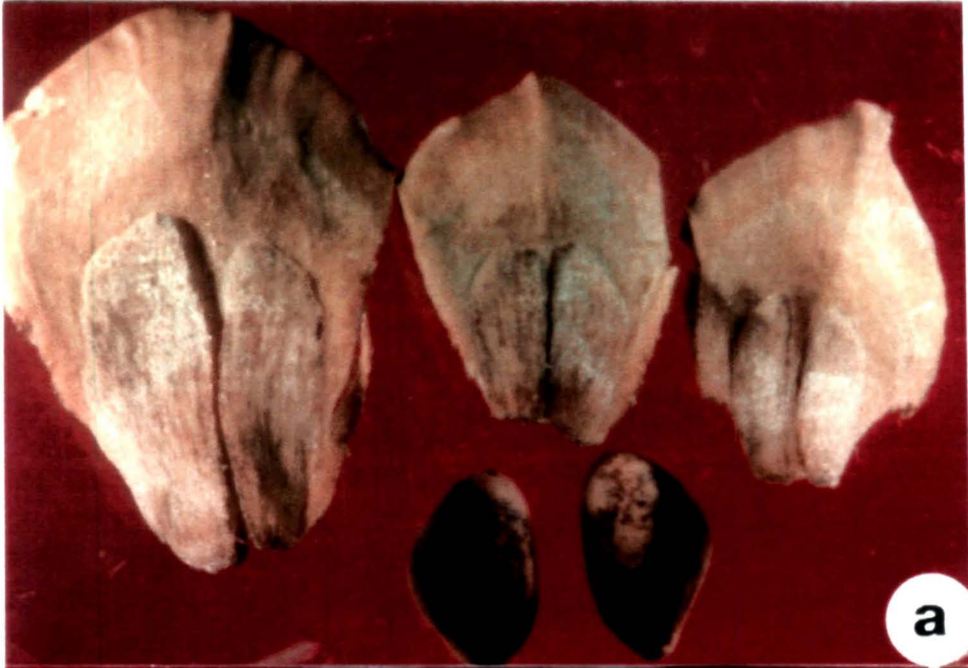
Female gametophyte: Immature second year green cones from open pollinated '+' trees were collected from I.C.A.R. Barapani, Meghalaya during the months of May to October and stored for a maximum of 15 days at 4^{0}C.

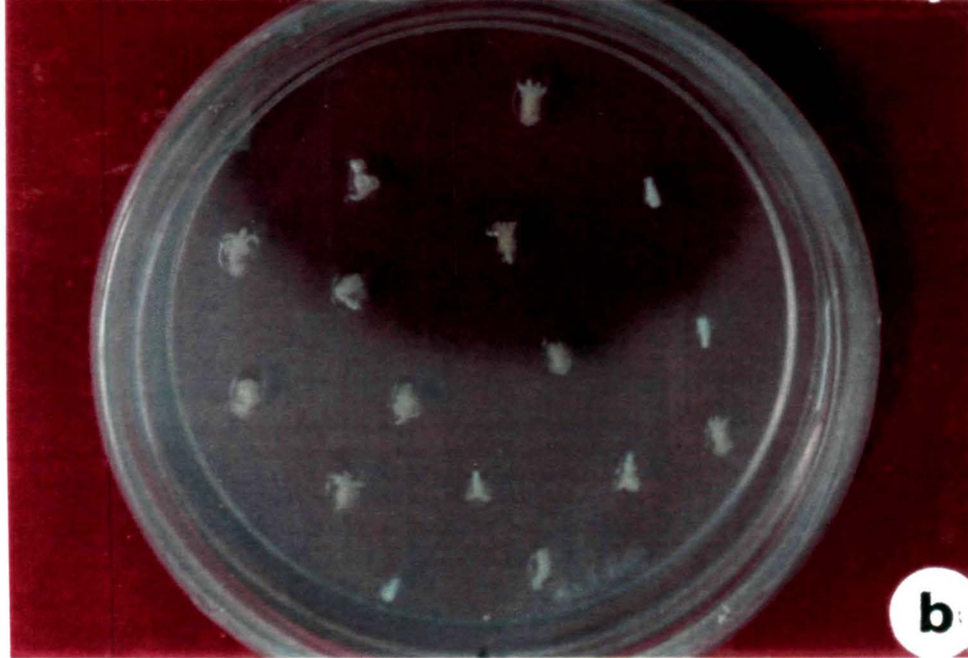
Pretreatment

Sterilization of mature zygotic embryos: Stored seeds were surface cleansed with Labklin (1% v/v commercially available laboratory detergent) for 10

Figure 6. Explant

a. Winged and isolated of *Pinus kesiya* seeds
b. Mature zygotic embryos in culture


a


b

min and washed under running tap water for 10-15 min. Thereafter, the seeds were sterilized with 15% (v/v) commercially available sodium hypochlorite (4-6% available chlorine, Qualigens) in a beaker on magnetic stirrer for 15 min and then thoroughly washed with sterilized ultra pure water (Mili Q water purification system). The seeds were further treated with 6% (v/v) hydrogen peroxide solution for 10 min and rinsed with sterilized ultra pure water for 4 to 5 times.

Stratification of seeds: The treated seeds were then kept for stratification at varying temperatures of 4, 8, 12, 24, 36^{0}C and for different time intervals of 6, 12, 24, 48, 72 hrs. Prior to dissection of the embryos, the seeds were shifted to the laminar air flow chamber and again sterilized with 0.5% (w/v) mercuric chloride ($HgCl_2$) for 3 to 5 min and washed with sterilized ultra pure water 4 to 5 times. The zygotic embryos were dissected out carefully with the help of sterilized forceps and needles.

Sterilization of female gametophyte: The green cones were surface cleansed with Labklin (1% v/v) for 10 min and washed under running tap water for 10 to 15 min followed by rinsing with ultra pure water. The cones were then cut into halves and sterilized aseptically in the laminar flow cabinet with 0.1% $HgCl_2$ for 10 min, washed repeatedly with sterilized ultra pure water and immature seed removed carefully.

Tissue culture

Initiation of cultures on semi solid medium using mature zygotic embryos:

Zygotic embryos, 12 in number were cultured in 90x20 mm diameter disposable petriplates (Tarson) (Fig. 6b). Each petriplate contained 20ml medium and was sealed with parafilm (American National Can). The cultures were incubated in the dark at 25±2^{0}C unless indicated otherwise. All the experiments were repeated three times. Each treatment had two sets of experiments each with 15 replicates (1 replicate = 1 petriplate). The data obtained were statistically analyzed and standard error calculated.

The initiation medium was formulated on completion of the following studies.

Effect of Media: The mature zygotic embryos were inoculated onto nutrient media for somatic embryogenesis. The various basal media used were MS (Murashige and Skoog, 1962), Litvay's (Litvay *et al.*, 1981), DCR (Gupta and Durzan, 1985), modified MS (mMS; Gupta and Durzan, 1986; Deb and Tandon, 2000), BM (Gupta and Pullman, 1991), MSG (Gupta and Pullman, 1991) (Table 1). BM medium was found most suitable for somatic embryogenesis and was used for subsequent studies, unless mentioned otherwise.

Effect of pH of the medium: A range of pH from 5.0 to 6.2 was tried out to adjudge the medium pH most suitable for induction of embryogenic

Table 1: Composition of basal media used for initiation of somatic embryogenesis

Constituents	MS	DCR	Litvay's	BM	MSG	mMs
Inorganic salts (mgl^{-1})						
NH_4NO_3	1650.00	400.00	1650.00	603.80	-	550.00
KNO_3	1900.00	340.00	1900.00	909.90	100.00	4460.00
KH_2PO_4	170.00	170.00	340.00	136.10	170.00	170.00
$Ca(NO_3)_2.\ 4H_2O$	-	556.00	-	236.20	-	-
$MgSO_4.\ 7H_2O$	370.00	370.00	1850.00	246.50	16.9	370.00
$Mg(NO_3)_2.\ 6H_2O$	-	-	-	256.50	-	-
$MgCl_2.\ 6H_2O$	-	-	-	50.00	-	-
KI	0.90	0.83	4.15	4.15	0.08	0.90
H_3BO_3	6.20	3.10	31.00	15.50	6.20	6.20
$MnSO_4.4H_2O$	22.30	22.30	21.00	10.50	16.9	22.30
$ZnSO_4.\ 7H_2O$	9.00	8.60	43.00	14.40	8.60	9.00
$Na_2MoO_4.\ 2H_2O$	0.25	0.25	1.25	0.125	0.25	0.25
$CuSO_4.\ 5H_2O$	0.10	0.25	0.50	0.125	0.025	0.10
$CoCl_2.\ 6H_2O$	0.10	0.025	0.125	0.125	0.025	0.10
$FeSO_4.\ 7H_2O$	28.00	27.80	28.00	6.95	28.00	14.00
$Na_2.EDTA.\ 2H_2O$	37.00	37.30	37.20	9.33	37.00	37.00
$CaCl_2.\ 2H_2O$	440.00	85.00	22.00	-	-	440.00
$NiCl_2.\ 2H_2O$	-	0.025	-	-	-	-
Organic nutrients mgl^{-1}						
Thiamine HCl	1.00	1.00	1.00	1.00	1.00	1.00
Nicotinic Acid	0.50	0.50	0.50	0.50	0.50	0.50
Pyridoxine HCl	0.50	0.50	0.50	0.50	0.50	0.50
Glycine	2.00	2.00	2.00	2.00	2.00	2.00
Meso Inositol	1000.00			1000.00		
Casein Hydrolysate	500.00			500.00		
L - Glutamine	500.00			1000.00		
Carbohydrate	*	*	*	*	*	*
Gelling agents gl^{-1}						
Difco bacto agar	7.5	7.5	7.5	7.5	7.5	7.5
PGRs	*	*	*	*	*	*
PVP	0.2	0.2	0.2	0.2	0.2	0.2
pH	5.8	5.8	5.8	5.8	5.8	5.8

cultures. For this, pH of the medium was adjusted using 0.1N HCl/0.1N NaOH before autoclaving the medium.

Effect of carbon source in the medium: Organic carbon source (0-5%) was incorporated in the medium singly in the form of sucrose, maltose and mannitol. Observations were made to record early callus formation and callus proliferation.

Effect of plant growth regulators in the medium: The medium was supplemented with a range of concentrations of plant growth regulators (PGR) i.e., 0-25µM 2,4-D and NAA each singly or in combination with 0-12µM BAP. The plant growth regulators (Hi-Media) were added directly in the medium before adjustment of pH and autoclaving.

Initiation medium

The initiation medium for mature zygotic embryos comprised of BM basal medium having 3% maltose, 2,4-D (22.6µM), NAA (26.8µM), BAP (11.1µM) and 0.1% mesoinositol. 0.75% Difco bacto agar was used as the gelling agent, while 0.02% polyvinyl pyrrolidone (PVP), was used as phenol scavenger. The pH of the medium was adjusted to 5.8, and autoclaved at 121^0C and 1.06 kg cm^{-2} pressure for 15 min.

The cultures were for 4 passages grown at an interval of 12 days in the initiation medium.

The details of study stages are provided in Fig. 5. Callus formation on initiation medium following the first subculture and before the second subculture was designated as stage I callus.

Culture conditions

Effect of light: Cultures were incubated at 20 and 60μM $sec^{-1}m^{-2}$ light intensity and in the dark.

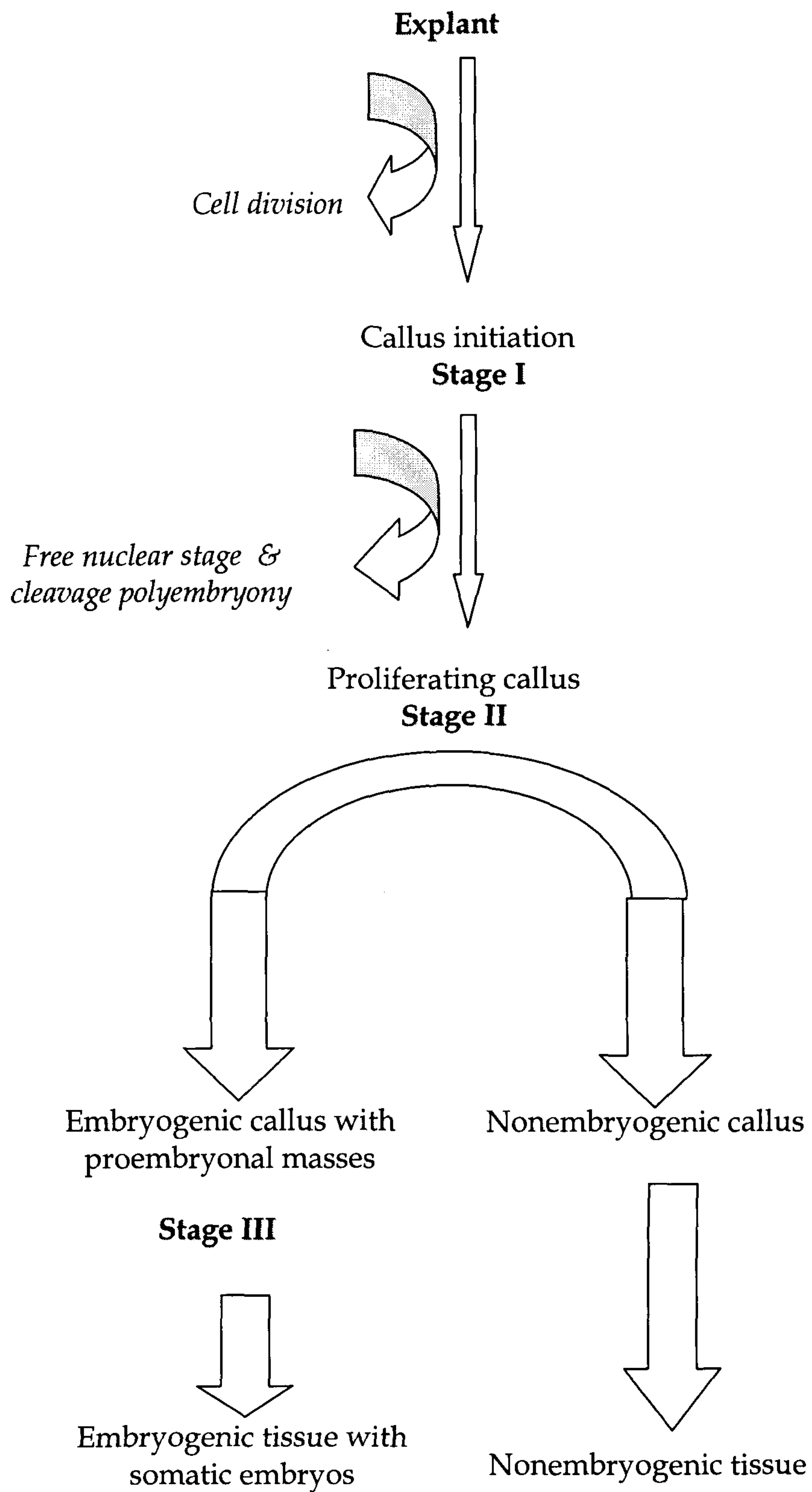
Effect of temperature: The zygotic embryos were cultured at varying temperatures of 4, 8, 12, 24 and 36^{0}C in a BOD.

Addition of $AgNO_3$: Silver nitrate in concentrations of 10, 20, 30, 40 μM, were filter sterilized and added to the initiation medium and 20 ml of the medium was poured into petridishes. Each experiment was set in a randomised design with 5 replicates (1 replicate = 1 petridish) per treatment with 50 mm diameter pieces of callus (12 in each petridish) in the initiation medium.

Initiation of cultures on semi solid medium using female gametophyte:

Surface sterilized female gametophytes of 0.20-1.10 mm size (Table 2) were dissected in the laminar flow chamber and inoculated onto standard ½ Litvay's medium found suitable earlier in our laboratory (Choudhury, 2000). The additional adjuvants were 3% sucrose, 2,4-D (22.6 μM), NAA (13.4 μM), BAP (11.1 μM), 0.1% mesoinositol and 0.2% PVP. The immature zygotic embryos 12 in number were cultured in each of 90x20 mm diameter disposable petriplates respectively, and were incubated under dark at

Figure 7. A schematic diagram of the study stages



Explant
Cell division
Callus initiation
Stage I
Free nuclear stage &
cleavage polyembryony
Proliferating callus
Stage II
Embryogenic callus with
proembryonal masses
Nonembryogenic callus
Stage III
Embryogenic tissue with
somatic embryos
Nonembryogenic tissue

Table 2: Developmental stages of female gametophyte for initiation of somatic embryogenesis

Stage of immature zygotic embryo as defined	Size of embryonal head	Approximate no. of weeks after fertilization
Stage a	8-16 celled	5-6
Stage b	0.05-0.20 mm	9-12
Stage c	0.20-1.10 mm*	13-17
Stage d & e	1.30-3.50 mm	19-26
Mature zygotic embryo	4.00-5.00 mm	30-33

* Most appropriate stage for embryogenic response

25±2^{0}C. Subsequent subcultures were done at an interval of 12 days for four passages.

Stage II sampling stage was the proliferated callus following four subcultures in the initiation medium.

Maturation of embryogenic cultures in semi solid medium:

The soft and creamish white proliferating stage II embryogenic calli harvested during the fourth subculture were inoculated on BM maturation medium supplemented with 1/10 PGR, 3% maltose and 30 µM ABA. ABA was filter sterilized and added in the lukewarm autoclaved medium in a laminar flow table. Observations were made following next two subcultures in same medium to record percentage formation of stage III calli that contained a mixed population of proembryonal masses and early cotyledonary embryos.

Some cultures did not turn friable following 2-4 subculture on maturation medium. Rather they converted into mass of cells, opaque and were clumped together in contrast to easily separating out friable callus. These were the nonembryogenic cultures used for comparative studies along with the stage III cultures.

Establishment of suspension cultures:

The stage II soft, white and transluscent embryogenic calli from both mature zygotic embryos and female gametophytes that were grown for 4 passages on semi-solid medium were harvested and transferred to the

respective liquid media of same composition for development of suspension cultures. For this, 25 ml sterilized liquid medium was dispensed in 100 ml conical flasks and suspension cultures were established using 1 g of rapidly proliferating embryogenic calli. The cultures were maintained in the dark at 25 ± 2^0C on an orbital shaker (Certomat U, B. Braun) at 100 rpm. The embryogenic suspensions were subcultured at 1 week interval by adjusting the sedimentation culture volume (SCV) to 10±1% (v/v) with fresh medium. To adjust the SCV the cultures were aseptically poured into 50 ml sterilized measuring cylinders in laminar flow chamber. After 30 min of sedimentation, the supernatant was discarded leaving 10 ml of sedimented cells. Fresh medium was added to a total volume of 25 ml with autoclaved pipette connected to a sterile rubber bulb. The suspensions were poured into the 100 ml conical flasks and transferred to the orbital shaker and incubated aseptically in the dark.

Maintenance and multiplication of suspension cultures

Mature zygotic embryos:

The embryogenic suspension culture raised was transferred to BM liquid multiplication medium having same additional adjuvants of 3% maltose, 0.1% mesoinositol and 0.02% PVP for maintenance and multiplication of suspension cultures. Varying concentrations of sucrose and maltose singly (0-5%) and a range of growth regulators (0, 1/5, 1/10 and

normal concentrations to that of initiation medium) were used for further multiplication and development.

Observations on embryogenic response were made following the next two subcultures (at 1 week interval each) in the same medium. The spent media were used for the estimation of extracellular proteins.

Female gametophyte:

The cultures established in liquid medium, were transferred to 1/2 Litvay's liquid multiplication medium having same additional adjuvants except for $1/10^{th}$ concentration of plant growth regulators i.e., 2.26 µM 2,4-D, 1.34 µM NAA, 1.11 µM BAP for multiplication of suspension cultures. The effect of a range of growth regulators (0, 1/5, 1/10 and normal concentrations to that of initiation medium) and effect of different concentrations of sucrose and maltose (0-5% singly) were studied.

Observations on embryogenic response were made following the next two subcultures (at 1 week interval each) in the same medium. The spent media were used for the estimation of extracellular proteins.

Stage III calli were harvested following 2-4 subcultures on maintenance medium supplemented with $1/10^{th}$ concentration of plant growth regulators i.e., 2.26 µM 2,4-D, 2.68/1.34 µM NAA and 1.11 µM BAP. Subsequent subcultures were done using the medium supplemented with ABA termed as maturation medium, which resulted in the formation of

creamish, white friable cell mass containing a mixed population of proembryonal embryos and early cotyledonary embryos.

Embryo development and maturation in suspension cultures

Mature zygotic embryos:

Embryogenic cultures proliferating in maintenance suspension medium (in orbital shaker in the dark) were transferred to the liquid maturation medium supplemented with 0-30μM ABA and 0-5% polyethylene glycol-4000. Additional adjuvants were 3% maltose, 0.1% mesoinositol, a range of growth regulators (0, 1/5, 1/10 and normal concentrations to that of initiation medium).

Subcultures were done at 1 week interval (agitated on orbital shaker at 60 rpm, incubated in dark at 25±2^{0}C) to obtain cultures having proembryonal masses. Filter paper base method was used to obtain cultures with mature embryos. For this, embryogenic suspension cultures were collected in respective liquid growth regulator-free media, shaken for a few min by hand. Around 3 ml of this suspension was distributed on sterile filter paper (Whatman 1) and the medium drained for 10 sec. The filter papers with the attached cells were then placed in petri dishes containing maturation medium. The petri dishes were sealed with parafilm. Subculture was done four weeks later, when the filter paper with the tissue was transferred to fresh maturation medium.

The spent media were used for the estimation of extracellular proteins.

Female gametophyte:

Embryogenic calli proliferating in liquid maintanence medium were transferred to ½ Litvay's maturation medium supplemented with 3% sucrose, 2.26 μM 2,4-D, 1.34 μM NAA, 1.11 μM BAP, 0.1% mesoinositol, 30μM ABA with 1/10 PGR (to that of the initiation medium) in the medium following the earlier carried out experiments in our laboratory (Choudhury, 2002). Subculture was done at 1 week interval and cultures were agitated on orbital shaker at 60 rpm and incubated in dark at $25 \pm 2^{0}C$ to obtain cultures having proembryonal masses and early cotyledonary embryos.

Addition of $AgNO_3$: A range of concentrations of silver nitrate (0, 10, 20, 30, 40 μM), was filter sterilized and incorporated singly in 25 ml of the maturation medium (containing 30 μM ABA with 1/10 PGR of the initiation medium) in a 100 ml conical flask. Each experiment followed a randomised design as mentioned earlier.

Addition of calcium: To study the effect of calcium on the maturation of embryogenic calli, in two separate experiments, the calcium source in the maturation medium of mature zygotic embryo (calcium nitrate in BM) and female gametophyte (calcium chloride in ½ Litvay's) were varied.

Observations were made over three weeks study period to record the percentage conversion of suspension cultures containing proembryonal masses to cultures forming early stage somatic embryos.

Microscopic observations:

Cultures were periodically observed under stereo-zoom (Leica Wild M3Z) and inverted microscope (Leitz Fluovert FU) to study the embryogenic and nonembryogenic cultures, their cellular organization and development.

Protein estimations:

Tissues used for protein estimations:

The proteins were estimated in cultures stages I, II and III grown under the influence media composition, pH, carbohydrate source, light and temperature; as mentioned earlier.

Extraction and estimation of intracellular proteins:

Intracellular proteins were extracted as described by Dong and Dunstan (1994). 100mg tissue was homogenized using sonicator (Vibra cell, Sonics& Materials, Danberry) in microcentrifuge tube in liquid nitrogen and extracted in 400 µl chilled extraction buffer [composition- 100 ml Tris HCl, pH 7.0, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1mM ethylene glycol-bis (β-aminoethyl ether) N,N,N',N' tetraacetic acid (EGTA), 10mM magnesium chloride, 100m dithiothreitol (DTT), 10µg ml^{-1} phenyl methyl sulfonyl floride (PMSF), 0.1% (v/v) Triton X-100, 5% (w/v) PVP]. After a brief vortexing the extract was centrifuged at 15,000 rpm for 10 min at 4^0C to pellet out the insoluble materials. The supernatant was transferred to a fresh tube and the pellet was resuspended in extraction

buffer and reextracted once again and centrifuged as stated above. The supernatants were pooled and used for estimations.

Quantification of protein was carried out using Bradford's method (Bradford, 1976). Required amount of the sample was taken in a test tube and made up to 1 ml with distilled water. To this, 4 ml Bradford's reagent was added and incubated at room temperature for 40 min.

The OD was recorded at 595nm using Shimadzu UV-265 Spectrophotometer. Protein was estimated with the help of a standard curve prepared with known concentration of BSA stock solution. Protein content was expressed as mg protein/g fresh weight of tissue.

Extraction and estimation of extracellular proteins:

For extraction of extracellular proteins, 20 ml of the spent liquid medium was collected and concentrated to 80% by ammonium sulphate precipitation, with constant stirring at 4^0C, for 4 hrs following the method of Jayaraman, 1983). The pellet was then suspended in Tris EDTA buffer (pH 8.0) containing 50 mM EDTA, and poured into a dialysis bag. The dialysis bag (Sigma, sieve size > 2 kDa) was sealed with clips and suspended in a beaker of distilled water (having 50 mM EDTA) and dialysed for 72 hrs with change of distilled water at 12 hrs, 6 hrs and 6 hrs interval. The dialysed extract contained total proteins which was then lyophilized and resuspended in distilled water for protein estimation, or in sample buffer for SDS-PAGE run.

Quantification of protein was carried out by Bradford's method as described above.

SDS-PAGE of Proteins:

Gel preparation: Vertical slab gels were prepared in vertically placed glass plates fixed with clamps and sealed with grease to make it leak proof. The acrylamide resolving solution (prepared from stock solutions of 30% acrylamide and 0.8% bis acrylamide, Tris Buffer, pH 8.8, 0.4% sodium dodecyl sulphate (SDS), 10% ammonium persulphate, N,N,N',N'-tetramethylethylenediamine, TEMED) was poured into the gap between the plates while leaving sufficient space to load the stacking gel solution. The gel was overlaid with a layer of butanol (commercially available, Merck). The setup was kept for 45 min under a lighted table lamp. After polymerization, the gel surface was rinsed with ultra pure water and the water was dried with blotting paper. The stacking gel (prepared from stock solutions of 30% acrylamide and 0.8% bis acrylamide, Tris Buffer, pH 6.8, 0.04% SDS, 10% ammonium persulphate, TEMED) was poured over it. A Teflon comb was immediately inserted into the gel solution, being careful to avoid trapping air bubbles. The assembly was kept at room temperature till polymerization was complete. The gel was placed in the electrophoretic tank containing the Electrode buffer (comprising 0.025 M Tris Buffer pH 8.3, 0.192 M Glycine, 0.1% SDS).

The extracted sample was mixed 1:1 with 1x Laemmli or loading buffer (composed of 0.0625 M Tris HCl, pH 6.8, 2% SDS, 1% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue), vortexed for 20 sec and heated at 100^{0}C for 3 min. This was then loaded into 12% gels prepared earlier. The gels were run at 120 constant Voltage and 40mA initially and when the dye reached the running gel, and later run at 65V and 40 mA for 4-5 hrs. Fixing was done in 40 : 10 : 50 of methanol, glacial acetic acid, ultra pure water for 30 min·. Staining was done in 0.25% Coomassie Brilliant Blue R 250 (in 40% methanol and 10 % glacial acetic acid) overnight. Destaining of the gels was done on a shaker with 40 : 10 : 50 of methanol, glacial acetic acid, ultra pure water for 2 hrs and then in reduced concentration i.e., 10 : 5 : 85 of methanol, glacial acetic acid, ultra pure water till the gel background cleared out.

Staining for glycoproteins: Following the procedure of Gradilone *et al.* (1997), the gels were fixed in 10% acetic acid (v/v) and 30% ethanol (v/v) overnight and submitted to the protocol of PAS-Silver stain as described below.

The gel was treated with 7.5% acetic acid for 10min with constant stirring, followed by incubation for 15 min in 1% periodic acid at 4^{0}C. The gel was then rinsed at room temperature with ultra pure water 6 times each of 5 min duration. Then the gel was incubated for 15 min in Schiff's reagent at 4^{0}C. The gel was next treated with 0.5% sodium metabisulphite (w/v) for 3 times each of 10 min duration at room temperature. The gel was again rinsed

with ultra pure water 6 times each of 5 min duration, followed by treatment with a solution containing 0.2% silver nitrate, 0.03% formaldehyde (v/v) for 30 min at room temperature. Following a rapid rinsing (5 sec) with ultra pure water the gel was then treated for 10 min with a solution containing 2.3% sodium carbonate, 0.01% formaldehyde (v/v) and 0.001% sodium thiosulphate (w/v). The gel was shaken well in this solution till the bands appeared. To stop the reaction, the gel was incubated for 30 min at room temperature in 2.5% acetic acid. Finally the gel was rinsed in ultra pure water at room temperature for 30 min.

Except for incubation in periodic acid and Schiff's reagent all the treatments were carried out with constant stirring.

Peroxidase assay:

Tissue extraction: 100 mg of tissue was homogenised using sonicator (Vibra Cell, Sonics & Materials, Danberry) in eppendorf tube on ice, with 400 µl 0.1 M phosphate buffer (pH 6.0) containing 10% PVP. The homogenate was then centrifuged in a cooling centrifuge (Beckman Coulter, USA) at 10,000 rpm for 30 min at $4^{0}C$. The supernatent was used for enzyme assay.

Enzyme assay: The enzyme assay was carried out using the method of Mahadevan and Sridhar (1982). In a 6 ml reaction mixture, 2.5 ml of 0.1 M pyragallol, 2.98 ml of phosphate buffer (0.1 M, pH 6.0) and 20 µl of enzyme was taken and absorption calibrated to zero at 420 nm. To this 0.5 ml of hydrogen peroxide (1% v/v) was added and the tube inverted immediately

to mix the contents. Three replicates of each study sample were taken and the assay carried out.

The change in absorbance was recorded at 20 sec interval for 3 min at 420 nm using the Spectrophotometer. The activity was expressed as Δ OD min^{-1} per g fresh wt. tissue. Specific activity of peroxidase was calculated Δ OD min^{-1} mg^{-1} protein.

PAGE of Peroxidase: Vertical polyacrylamide slab gels were prepared as described above, but without SDS. Electrophoretic separation of the extract was carried out in 10% gel and run at 65V, 40 mA for 2 hrs. The assembly was kept inside the refrigerator at 8^0C during gel run.

Staining of gels: Peroxidase gels were stained using method of Pitel *et. al.*, (1984). The isozymes were localized by placing the gel in a solution containing equal amounts of 1.5% benzidine in 25% acetic acid and 1% hydrogen peroxide in distilled water. The bands were denoted as dark, medium and light depending on the intensity of colour. A few bands first became light bluish (charactaristic of peroxidase isozyme) but turned blackish soon afterwards. The gel was photographed as soon as possible since the bands turned black within 15 min.

Chapter III

Results

Pretreatment

Effect of stratification of seeds on the establishment of embryogenic cultures from mature zygotic embryos:

Stratification of seeds for 12 hrs at 24^{0}C resulted in optimum response in initiation of stage I callus from mature zygotic embryos (Table 3). Stratification for 12 and 24 hrs at varying temperatures were found better for callus initiation. However, lower and higher duration of stratification did not result in any response except for 48 hrs at 12^{0}C and 48 and 72 hrs at 24^{0}C.

The stage I callus obtained from seeds soaked for 12 hrs at 4^{0}C exhibited optimum conversion to stage II callus (Table 4). Following three passages of subculture on the initiation medium rapid proliferation of callus resulted in 38% response in forming stage II calli (Table 4).

When seeds were stratified for 12 hrs or more at 12^{0}C and at higher temperatures, there was rapid browning in calli followed by degeneration over subsequent subcultures. When seeds were stratified for longer period 24, 48, 72 hrs at lower temperatures of 4 and 8^{0}C, cultures also showed browning and degeneration (Table 4).

Table 3: Effect of stratification of seeds on initiation of Stage I callus from mature zygotic embryos

Stratification treatment		Type of response	% Response (±SE)[1]
Temp. (^{0}C)	Time (in hrs.)		
4	0	-	-
	6	-	-
	12	Soft callus initiation	22.4 (±1.23)
	24	Soft callus initiation	4.53 (±0.33)
	48	-	-
	72	-	-
8	0	-	-
	6	-	-
	12	Soft callus initiation	3.69 (±0.92)
	24	Soft callus initiation	0.92 (±0.75)
	48	-	-
	72	-	-
12	0	-	-
	6	-	-
	12	Slight callus initiation	7.40 (±1.85)
	24	Slight callus initiation	31.47 (±4.89)
	48	Explants turned brown	-
	72	-	-
24	0	-	-
	6	-	-
	12	Soft callus initiation	39.25 (±0.92)
	24	Callus profuse and soft	33.33 (±1.60)
	48	Callus profuse and soft, turned brown	32.95 (±3.04)
	72	Slight callus initiation, turned soft and brown	18.14 (±1.29)
36	0	-	-
	6	-	-
	12	Slight callus initiation, turned soft and brown	6.31 (±3.65)
	24	Soft callus initiation, turned brown	17.40 (±2.01)
	48	-	-
	72	-	-

[1] Standard error

- No response

Table 4: Effect of stratification of seeds on formation of Stage II callus from mature zygotic embryos

Stratification treatment		Type of response	% Response (± SE)[1]	
Temp. (^{0}C)	Time (hrs.)		Embryogenic	Non embryogenic
4	0	-	-	-
	6	-	-	-
	12	Late initiation of callus, callus soft	38.17 (±2.59)	-
	24	Late initiation of callus, callus soft and brown	26.21 (±2.65)	2.35 (±3.12)
	48	Late initiation of callus, callus soft and brown	12.61 (±1.58)	16.82 (±2.15)
	72	Slight callus initiation, callus turned brown and degenerated	-	3.96 (±0.09)
8	0	-	-	-
	6	-	-	-
	12	Soft transluscent callus formed	9.25 (±2.44)	7.40 (±1.84)
	24	Callus soft, transluscent and brown	4.97 (±1.15)	4.06 (±1.29)
	48	-	-	-
	72	-	-	-
12	0	-	-	-
	6	-	-	-
	12	Slight proliferation of callus	16.66 (±0.55)	12.03 (±4.03)
	24	Callus proliferated, but turned brown	31.47 (±4.89)	35.18 (±0.92)
	48	Explant degenerated without callusing	-	-
	72	-	-	-
24	0	-	-	-
	6	-	-	-
	12	Callus proliferated, creamish and transluscent	21.10 (±1.95)	13.88 (±2.77)
	24	Callus proliferated, creamish and transluscent	24.07 (±0.92)	21.24 (±2.76)
	48	Transluscent callus turned brown	12.22 (±1.60)	32.22 (±0.60)
	72	No further proliferation of callus	-	2.77 (±0.00)
36	0	-	-	-
	6	-	-	-
	12	Callus soft, transluscent and brown	4.18 (±0.184)	8.14 (±0.180)
	24	No further proliferation of callus	-	13.88 (±4.24)
	48	-	-	-
	72	-	-	-

[1] Standard error

- No response

Stratification for 12 hrs at 4^0C resulted in stage III cultures with proembryonal masses (PEMs) and early cotyledonary embryos (ESMs, 32.66%), while seeds stratified for the same duration at 8^0C also exhibited 22.77% response (Table 5). The remaining treatments resulted in browning and degeneration of the calli.

Stage I callus:

Callus harvested following first subculture in the initiation medium formed stage I callus (Fig. 8).

Stage II callus:

Four to five subcultures on initiation medium formed friable callus containing more PEMs (Fig. 9).

Stage III cultures:

Cultures that converted to masses containing early stage cotyledonary embryos were termed stage III cultures (Fig. 10).

Effect of different media on the establishment of embryogenic cultures from mature zygotic embryos:

Mature zygotic embryos when inoculated onto different basal media responded differently (Table 6).

MS:

Mature zygotic embryos showed stage I and stage II callus initiation and proliferation (Table 6 and 7, Fig. 11a) during the first three subcultures on initiation medium. However, during the fourth subculture, the callus

Table 5: Effect of stratification of seeds on formation of Stage III callus from mature zygotic embryos

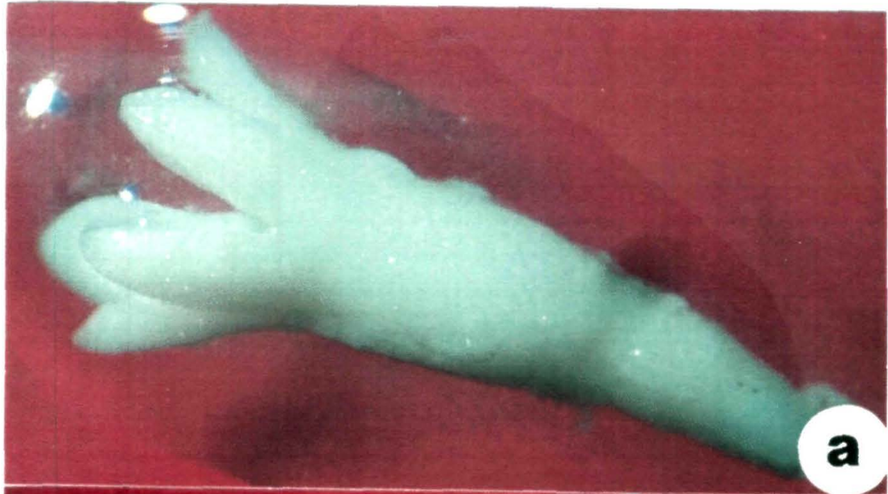
Stratification treatment		Type of response	% Response (± SE)[1]	
Temp. (°C)	Time (in hrs.)		Embryogenic	Non embryogenic
4	0	-	-	-
	6	-	-	-
	12	Callus transluscent and friable	32.66 (±1.92)	42.19 (±0.12)
	24	Callus transluscent and friable	19.96 (±2.07)	32.35 (±3.12)
	48	Callus transluscent, friable but turned brown	5.79 (±1.27)	36.82 (±2.15)
	72	Callus degenerated	0.00	3.96 (±2.09)
8	0	-	-	-
	6	-	-	-
	12	Proliferating callus turned friable and showed presence of proembryonal masses (PEMs)	22.77 (± 1.69)	7.40 (±1.84)
	24	Proliferating callus turned brown and degenerated	-	4.06 (±1.29)
	48	-	-	-
	72	-	-	-
12	0	-	-	-
	6	-	-	-
	12	Slight proliferation of callus, contained PEMs	6.47 (±0.92)	12.03 (±4.03)
	24	Proliferating callus turned brown and degenerated	-	35.18 (±0.92)
	48	Explant degenerated without callusing	-	-
	72	-	-	-
24	0	-	-	-
	6	-	-	-
	12	Callus proliferated, creamish and transluscent	2.77 (±0.95)	13.88 (±2.77)
	24	Callus turned brown and degenerated	-	21.24 (±2.76)
	48	Transluscent callus turned brown and degenerated	-	22.22 (± 1.60)
	72	No further proliferation of callus	-	2.77 (±0.00)
36		-	-	-

[1] Standard error

- No response

Figure 8. Initiation of Stage I callus from mature zygotic embryo in semi solid medium

a. Mature zygotic embryo explant (25X)
b. Callus initiation from micropylar end (10d old, 25X)
c. Stage I callus (25X, after first subculture)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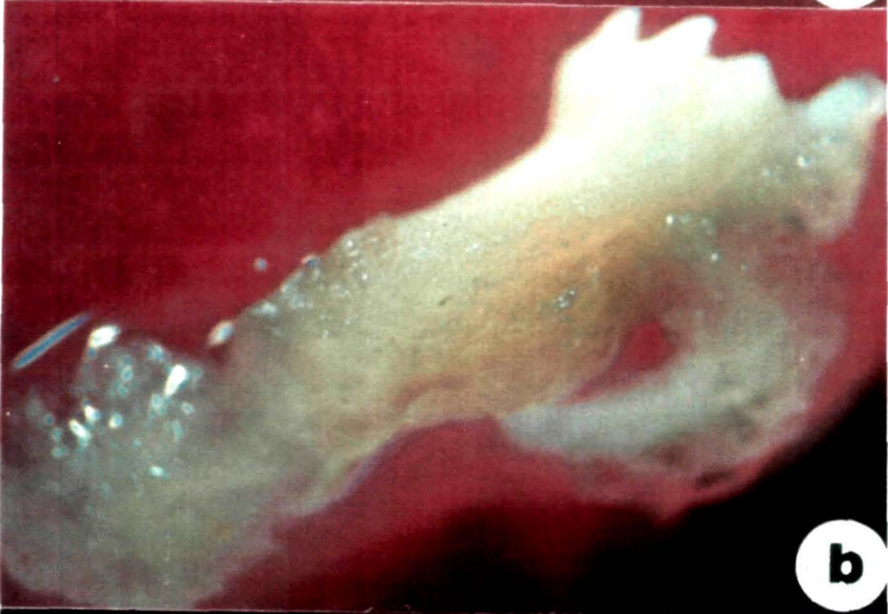
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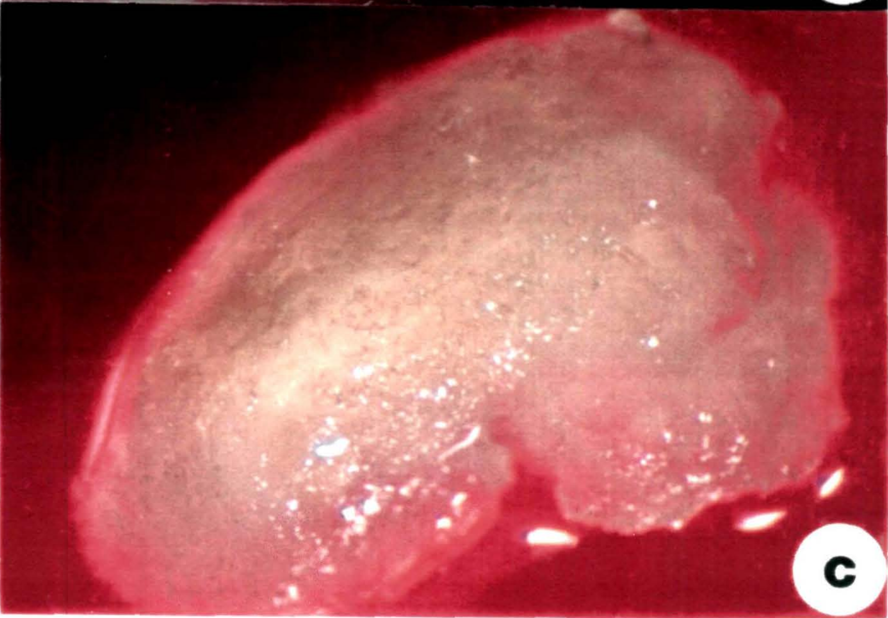
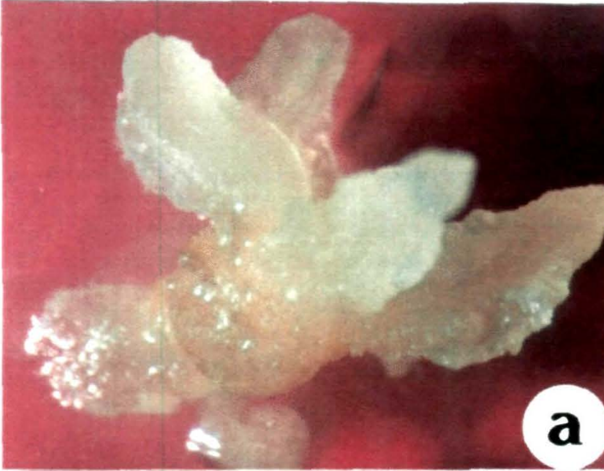

c

Figure 9. Development of Stage II callus from mature zygotic embryo in semi solid medium

a. callus after 2 passages of subculture (10X)
b. callus 6 days after second subculture (25X)
c. callus after three passages of subculture (25X)
d. callus after four passages of subculture (35X)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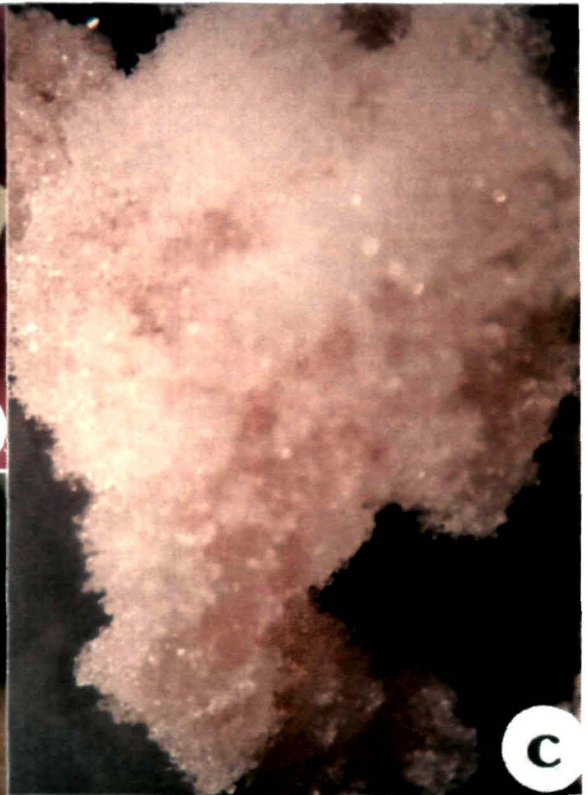
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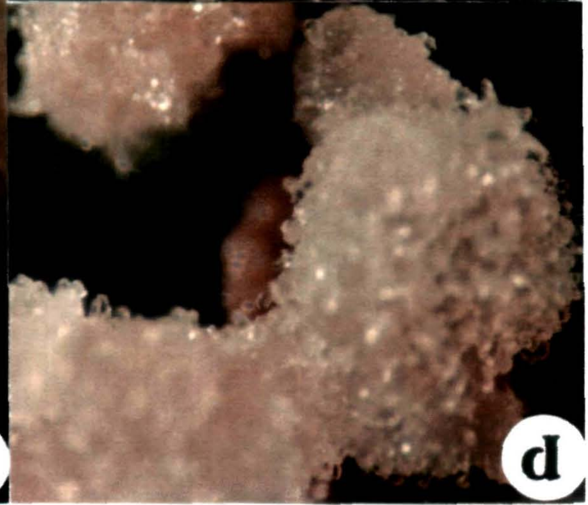
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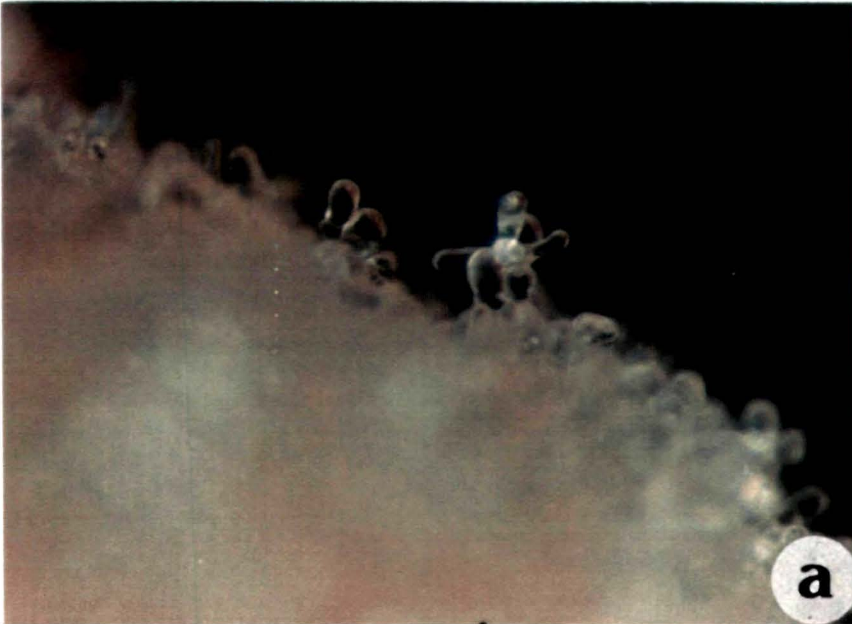
Figure 10. Formation of Stage III callus from mature zygotic embryo in semi solid medium

a. Embryogenic callus with proembryonal masses (25X)
b. Embryogenic callus showing proembryos (35X)
c. Stage III embryogenic callus showing enlarged proembryos (40X)
d. Somatic embryos turning green when kept in light (40X)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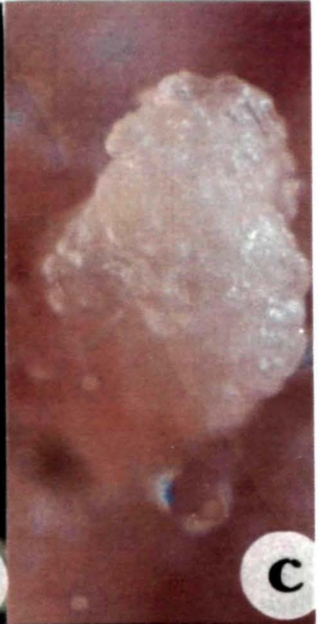
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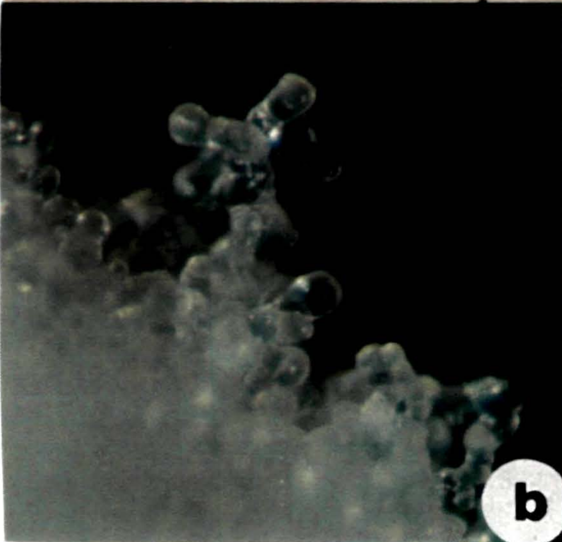
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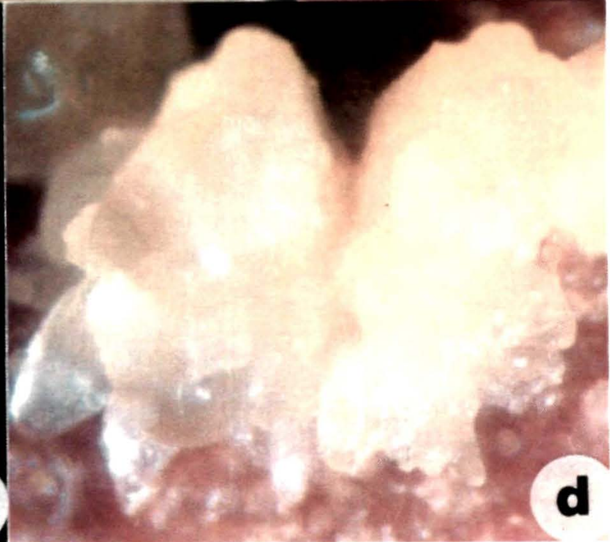
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Table 6: Effect of different media on the initiation of Stage I callus from mature zygotic embryos

Medium	Type of response	% Response (±SE)[1]
MS	Early initiation of callus	32.23 (±0.86)
DCR	Callus soft, transluscent and slightly brown	10.86 (± 0.63)
Litvay's	No callus initiation, but cotyledons opened up	2.37 (±1.21)
BM	Callus initiation, callus soft and creamish	22.81 (±1.53)
MSG	Callus initiation, callus soft and creamish	20.09 (±2.08)
mMS	Callus initiation, callus soft and transluscent	33.3 (±1.67)

[1] Standard error

- No response

Table 7: Effect of different media on the formation of Stage II callus from mature zygotic embryos

Media	Type of response	% Response (± SE)[1]	
		Embryogenic	Non-embryogenic
MS	Proliferation of callus observed at some parts of explant, browning in the rest	22.48 (±2.20)	35.82 (±1.87)
DCR	Proliferation of callus, with simultaneous browning in some areas	20.41 (±2.43)	8.14 (±1.30)
Litvay's	Explants turned brown and shriveled up	-	-
BM	Slow proliferation of callus	34.16 (±1.62)	-
MSG	Proliferated callus sticky, with simultaneous browning in some areas	31.37 (±3.62)	4.12 (±0.62)
mMS	Profuse callus proliferation, but showed browning in some areas	37.14 (±0.82)	-

[1] Standard error

- No response

turned brown with rapid degeneration of stage II calli with decreased embryogenic response. With an increase in non embryogenic cultures, the stage III embryogenic response was very low (Table 8).

DCR:

The trend of the response in this medium was similar to MS medium. There was rapid callusing with simultaneous browning of the callus (Table 7). Following subcultures on to maturation medium, the rapidly proliferating embryogenic cultures turned brown. Also observed was a percentage rise in non embryogenic callus and a very low 3.74% of stage III callus with PEMs seen only on the surface at some points (Table 8, Fig. 11c).

Litvay's:

Explants showed no callus initiation in this medium. However only the cotyledons opened up (Table 6).

BM:

There was early callus initiation in this medium with rapid proliferation of stage I callus following the first subculture (Table 6, Fig. 11d). Though slow proliferation of stage III callus was noticed (Table 6), the optimum (34.16%, Table 7) embryogenic stage III callus developed which formed 29.47% PEMs (Table 8).

MSG:

Slight callus initiation resulted in MSG medium upto first subculture stage (Table 6). Stage II callus was formed that was sticky (Table 7, Fig. 11b)

Table 8: Effect of different media on formation of Stage III callus from mature zygotic embryos

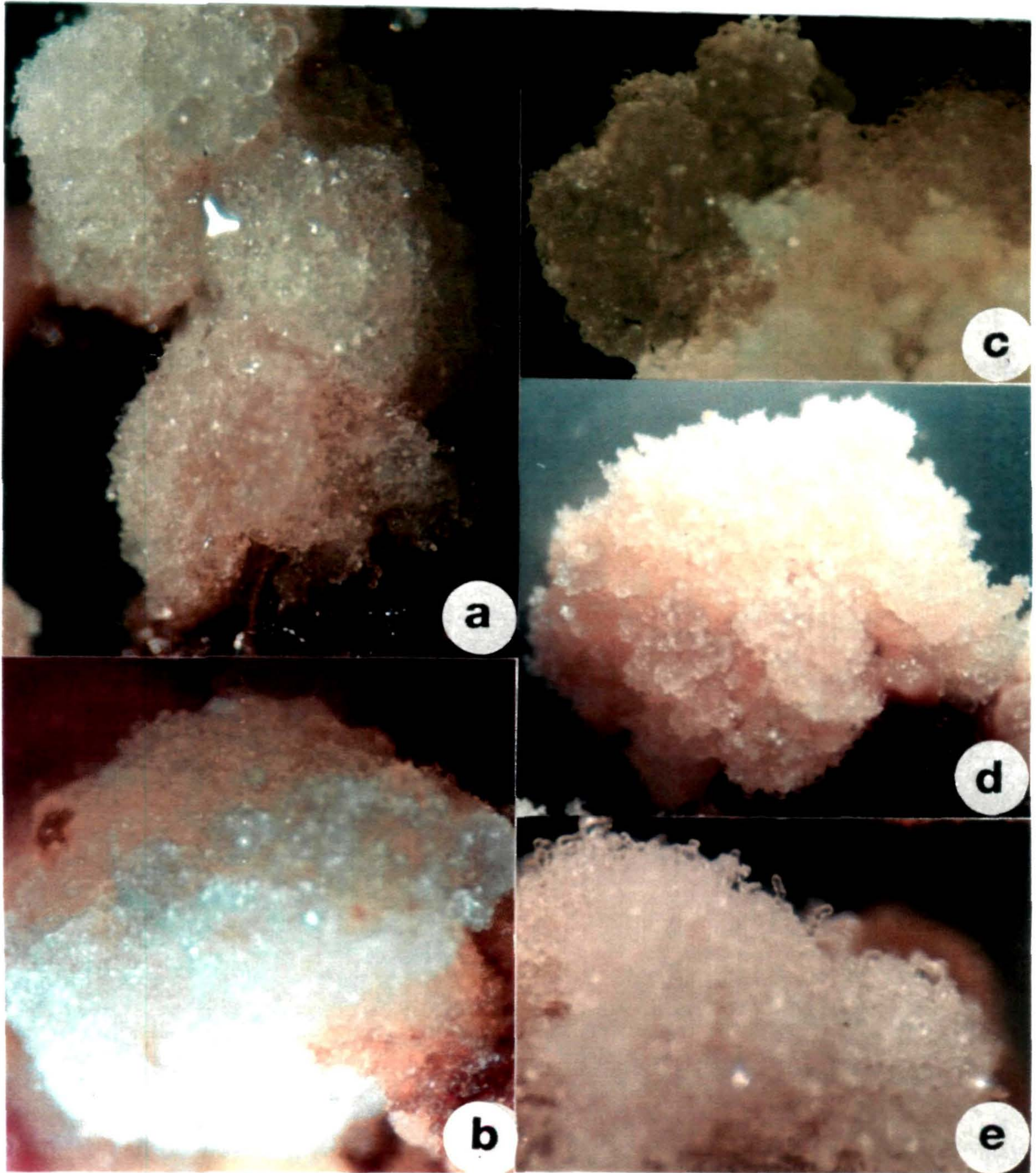
Media	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
MS	Callus soft and sticky, not friable, gradually degenerated	3.41 (±0.99)	26.54 (±0.92)
DCR	Callus turned brown with PEMs seen only on the surface of the callus at some places which could not be sustained	3.74 (±0.95)	17.09 (±3.6)
Litvay's	-	-	-
BM	Callus showed PEMs all over the surface, remained friable, which separated easily with touch	29.47 (±1.35)	18.0 (±3.74)
MSG	No further proliferation, callus degenerated and turned dark brown	4.30 (±0.83)	34.10 (±2.11)
mMS	Callus with PEMs, generally brown in some areas	20.36 (±1.85)	22.76 (±2.98)

[1] Standard error

- No response

Figure 11. Effect of different media on formation of embryogenic callus from mature zygotic embryo (after 2 passages of subculture)

a. Callus on MS medium
b. Callus on MSG medium
c. Callus on DCR medium
d. Callus on BM medium
e. Callus on mMS medium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d
e

that later degenerated and hardly converted to any stage III cultures (Table 8).

mMS:

Cultures on mMS medium were soft, friable and transluscent (Table 6) that showed profuse proliferation following second subculture (Table 7, Fig. 11e). Percentage of browning was higher compared to BM medium while there was a reduction in the formation of stage III callus (Table 8).

Effect of pH of BM medium on the establishment of embryogenic cultures from mature zygotic embryos:

pH 5.0:

The medium gelled at this pH was soft and slightly brown. Soft and transluscent stage I callus initiation was recorded (Table 9). Further subcultures in the initiation medium resulted in formation of callus that was clumped together and soft (Table 10) and later showed a decline in growth and turned non embryogenic (Table 11).

pH 5.4:

At this pH, stage I callus exhibited fast growth initially (Table 9). Subsequent subcultures resulted in rapid proliferation to form 32% of stage II callus (Table 10). However callus also showed rapid degeneration, with no further proliferation during further subcultures in the maturation medium as seen in Table 11.

Table 9: Effect of pH of the medium on the initiation of Stage I callus from mature zygotic embryos

pH	Type of response	% Response (±SE)[1]
5.0	Callus initiation, soft and transluscent	11.08 (±3.19)
5.4	Callus soft, transluscent with fast growth	12.96 (±1.84)
5.8	Callus soft, transluscent with fast growth	22.21 (±3.2)
6.2	Initiation of callus slow, turned creamish	24.0 (±3.68)

[1] Standard error

- No response

Table 10: Effect of pH of the medium on the formation of Stage II callus from mature zygotic embryos

pH	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
5.0	Slow proliferation of callus, callus soft, sticky	20.18 (±0.74)	4.96 (±0.38)
5.4	Very rapid proliferation of callus	31.8 (±0.74)	-
5.8	Rapid proliferation of callus which turned creamish and transluscent	36.29 (±1.61)	-
6.2	Very slow growth of callus	22.96 (±2.42)	-

[1] Standard error

- No response

Table 11: Effect of pH of the medium on formation of Stage III callus from mature zygotic embryos

pH	Type of response	% Response (±SE)[1]	
		Embryogenic	**Non-embryogenic**
5.0	No further proliferation of callus, callus degenerated	-	9.62 (±1.48)
5.4	Proliferation of callus slow, showed no PEMs	5.0 (±0.96)	39.6 (±1.36)
5.8	Callus friable with PEMs all over the surface	27.4 (±0.64)	13.33 (±0.60)
6.2	Callus turned brown, no formation of PEMs	-	27.0 (±2.67)

[1] Standard error

- No response

pH 5.8:

Cultures raised in medium having pH 5.8 resulted in soft, transluscent, fast growing stage I and stage II cultures (Table 9 and 10) that maintained higher embryogenecity (Table 11).

pH 6.2:

Initiation of stage I callus was profuse and found better in the medium at this pH (Table 9). There was however, slow growth of stage II callus (Table 10) and later rapid browning of the callus with no formation of stage III cultures (Table 11). All the cultures turned non embryogenic.

Effect of different carbohydrate sources in varying concentrations in BM medium on the establishment of embryogenic cultures from mature zygotic embryos:

Sucrose:

Concentrations of sucrose at 20 and 30 gl^{-1} resulted in an early initiation of stage I callus at 34.23 and 36.10% respectively (Table 12). Subsequent subcultures in initiation medium showed rapid callus proliferation to form friable and healthy stage II callus (Table 13). In the absence of sucrose in the medium, the explants turned brown with no callus formation. At 10 gl^{-1} concentration of sucrose, the callus was soft and sticky at stage I while at 40 gl^{-1} in the medium, hard and nonproliferating callus was observed. At 50 gl^{-1} of sucrose in the medium the explants did not show any response (Table 13).

Table 12: Effect of different concentrations of sucrose in the medium on the initiation of Stage I callus from mature zygotic embryos

Concentration (gl^{-1})	Type of response	% Response (±SE)[1]
0	Cotyledons opened up, no callus formation	-
10	Late callus initiation, soft and sticky callus	32.63 (±3.19)
20	Callus soft and transluscent	34.23 (±1.99)
30	Callus soft and transluscent, with rapid proliferation	36.1 (±2.89)
40	Callus hard, with slow proliferation	8.72 (±1.28)
50	-	-

[1] Standard error

- No response

Table 13: Effect of different concentrations of sucrose in the medium on formation of Stage II callus from mature zygotic embryos

Concentration (gl^{-1})	Type of response	% Response (±SE)[1]
0	Cotyledons turned brown, no callus formation	-
10	Slow proliferation of callus	16.97 (±1.25)
20	Callus friable and profuse, growth more rapid than above	28.87 (±3.74)
30	Rapid proliferation of callus, callus friable and healthy	37.83 (±1.47)
40	Callus turned hard and brown	-
50	-	-

[1] Standard error
- No response

The optimum response of stage III cultures was recorded in the medium supplemented with 30 gl^{-1} sucrose (Table 14). These cultures could not be sustained during subsequent subcultures reducing the percentage of ESMs formation. Most of the proliferating callus in sucrose containing medium turned brown, eventually converting the small percentage of embryogenic cultures to non embryogenic cultures (Table 14). Figure 12a shows a high percentage of browning of cultures in sucrose containing medium.

Maltose:

In the medium supplemented with maltose, initiation of stage I callus was slow compared to that in the medium containing sucrose. At 20 and 30 gl^{-1} percentage initiation of stage I calli was 19.72 and 22.10% respectively (Table 15). Cultures on medium with 30 gl^{-1} maltose on subsequent subcultures showed rapid proliferation of stage II calli (32.2%) with no browning (Table 16). There was formation of Stage III cultures having both PEMs and ESMs in the medium containing 30 gl^{-1} of maltose (Table 17).

In the absence of maltose in the medium there was no callus initiation while at a lower concentration of 10 gl^{-1} the callus initiation was very low (Table 15 and 16). At higher concentrations of 40 and 50 gl^{-1} the response was poor (Table 15 and 16). On subsequent subcultures, there was gradual degeneration and formation of nonembryogenic calli (Table 17). Browning

Table 14: Effect of different concentrations of sucrose in the medium on the formation of Stage III callus from mature zygotic embryos

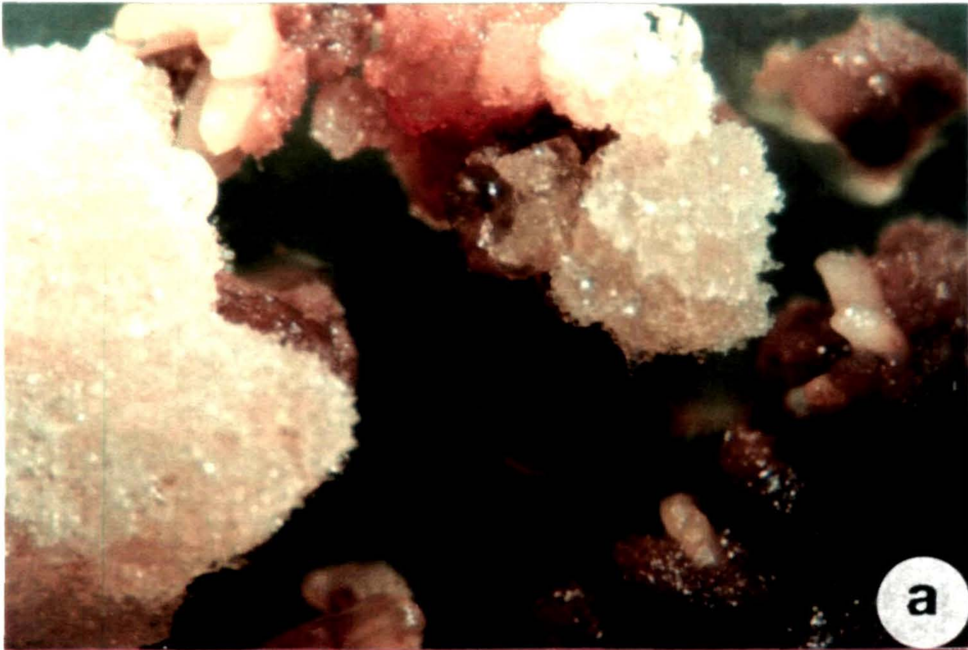
Concentration (gl^{-1})	**Type of response**	**% Response (± SE)[1]**	
		Embryogenic	**Non-embryogenic**
0	-	-	-
10	No further proliferation of callus, callus rapidly turned brown	-	23.13 (±2.40)
20	Slow proliferation with slight browning in callus	14.41 (±0.95)	22.11 (±1.26)
30	Rapid callus proliferation callus soft, friable and detach when touched	24.26 (±1.83)	18.72 (±1.28)
40	Callus hard and brown	-	-
50	-	-	-

[1] Standard error

- No response

Figure 12. Effect of carbohydrate source in medium on formation of embryogenic callus from mature zygotic embryo (after 4 passages of subculture)

a . Callus on 3% sucrose containing medium
b. Callus on 3% maltose containing medium


a

b

Table 15: Effect of different concentrations of maltose in the medium on the initiation of Stage I callus from mature zygotic embryos

Concentration (gl^{-1})	**Type of response**	**% Response (±SE)[1]**
0	Cotyledons opened up, no callus formation	-
10	Cotyledons opened up, callus formed at micropylar end	8.9 (±1.70)
20	Callus soft, growth more rapid than above	19.72 (±0.89)
30	Callus soft, initiated from micropylar end	22.10 (±0.89)
40	Callus slightly brown	6.45 (±1.07)
50	Callus slightly brown	7.21 (±0.76)

[1] Standard error

- No response

Table 16: Effect of different concentrations of maltose in the medium on formation of Stage II callus from mature zygotic embryos

Concentration (gl^{-1})	**Type of response**	**% Response (±SE)[1]**
		Embryogenic
0	-	-
10	Slow proliferation of callus	9.10 (±1.33)
20	Profuse callusing, callus soft and transluscent	22.5 (±1.53)
30	Rapid proliferation of callus, soft and transluscent all over	32.2 (±1.93)
40	Slow proliferation of callus, turned brown	9.5 (±1.37)
50	Callus turned completely brown	8.98 (±1.04)

[1] Standard error

- No response

Table 17: Effect of different concentrations of maltose in the medium on the formation of Stage III callus from mature zygotic embryos

Concentration (gl^{-1})	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
0	-	-	-
10	Callus rapidly turned brown, could not be sustained	-	-
20	Friable callus with PEMs	28.5 (±1.32)	5.0 (±1.15)
30	Rapid proliferation of callus, soft and friable, with PEMs all over	32.93 (±2.91)	13.5 (±1.16)
40	Callus mixture of embryogenic and nonembryogenic areas	2.75 (±1.26)	6.0 (±1.30)
50	Callus hard and brown, white callus at the tips	-	0.98 (±0.09)

[1] Standard error

- No response

was relatively less in cultures with maltose in the medium compared to sucrose (Fig. 12b).

Effect of plant growth regulators on the establishment of embryogenic cultures from mature zygotic embryos:

When plant growth regulators were added to the medium in the combination of 22.6μM 2,4-D, 26.8μM NAA and 11.1μM BAP, the stage I callus proliferated to stage II friable embryogenic callus with PEMs (Table 18). At lower concentrations of auxins the callus was soft but turned brown while at higher concentrations of both auxin and cytokinin the callus turned hard and degenerated.

Effect of light on the establishment of embryogenic cultures from mature zygotic embryos:

It was observed that explants formed 24% stage I callus in the dark while presence of varying light intensities resulted in lower stage I callus initiation (Table 19). Light was found inhibitory to callus initiation and proliferation. The stage I callus initiated in light at 20 μmol m^{-2} sec^{-1} resulted in non embryogenic calli later. At 60 μmol m^{-2} sec^{-1} explants turned green (Fig. 13). In the dark there was rapid stage II callus proliferation (Table 20) that converted to stage III embryogenic calli with PEMs (Table 21). However, non embryogenic cultures were also recorded.

Table 18: Effect of 2,4-D, NAA and BAP on the formation of Stage II callus from mature zygotic embryos

2,4-D (µM)	NAA (µM)	BAP (µM)	Type of response	%Response (±SE)[1]
				Embryogenic
11.3	13.4	11.1	-	-
22.6	13.4	11.1	Callus friable and soft, but no PEMs formation	6.44 (±0.84)
33.9	13.4	11.1	-	-
45.2	13.4	11.1	-	-
11.3	26.8	11.1	Soft callus formed but turned brown by first subculture	12.34 (±0.52)
22.6	26.8	11.1	Callus friable and soft, proliferated into PEMs	30.58 (±1.46)
33.9	26.8	11.1	Callus proliferates initially, then turned hard and brown	12.56 (±0.91)
45.2	26.8	11.1	-	-
11.3	40.2	11.1	Callus soft, turned mucilaginous and nonembryogenic	8.32 (±1.43)
22.6	40.2	11.1	-	-
33.9	40.2	11.1	-	-
45.2	40.2	11.1	-	-
11.3	53.6	11.1	-	-
22.6	53.6	11.1	Callus formed, which turned hard by first subculture	2.32 (±0.65)
33.9	53.6	11.1	-	-
45.2	53.6	11.1	-	-
11.3	13.4	22.2	-	-
22.6	13.4	22.2	-	-
33.9	13.4	22.2	-	-
45.2	13.4	22.2	-	-
11.3	26.8	22.2	-	-
22.6	26.8	22.2	-	-
33.9	26.8	22.2	Callus soft initially and proliferated but turned hard by second subculture	12.26 (±2.14)
45.2	26.8	22.2	-	-

[1] Standard error

- No response

Table 19: Effect of light on the initiation of Stage I callus from mature zygotic embryos

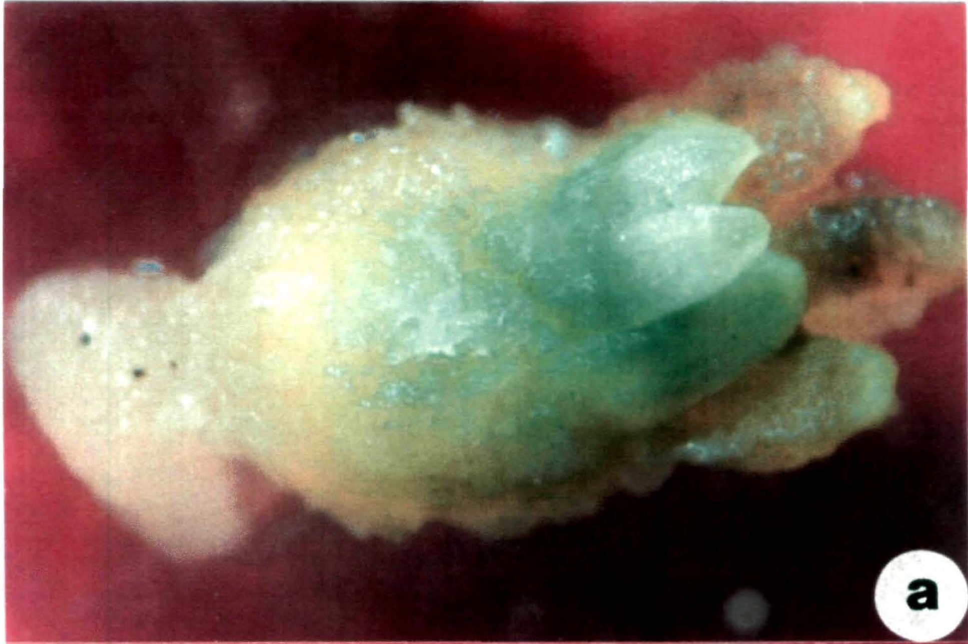
Light intensity ($\mu mol\ m^{-2} sec^{-1}$)	Type of response	% Response (±SE)[1]
Dark	Soft, creamish callus formed from micropylar end	24.19 (±1.26)
20	Slow callus initiation	16.66 (±0.95)
60	Cotyledons turned green, no callus initiation	-

[1] Standard error

- No response

Figure 13. Effect of light intensity on formation of embryogenic callus from mature zygotic embryo

a. at 60 µmol m^{-2} sec^{-1}
b. in dark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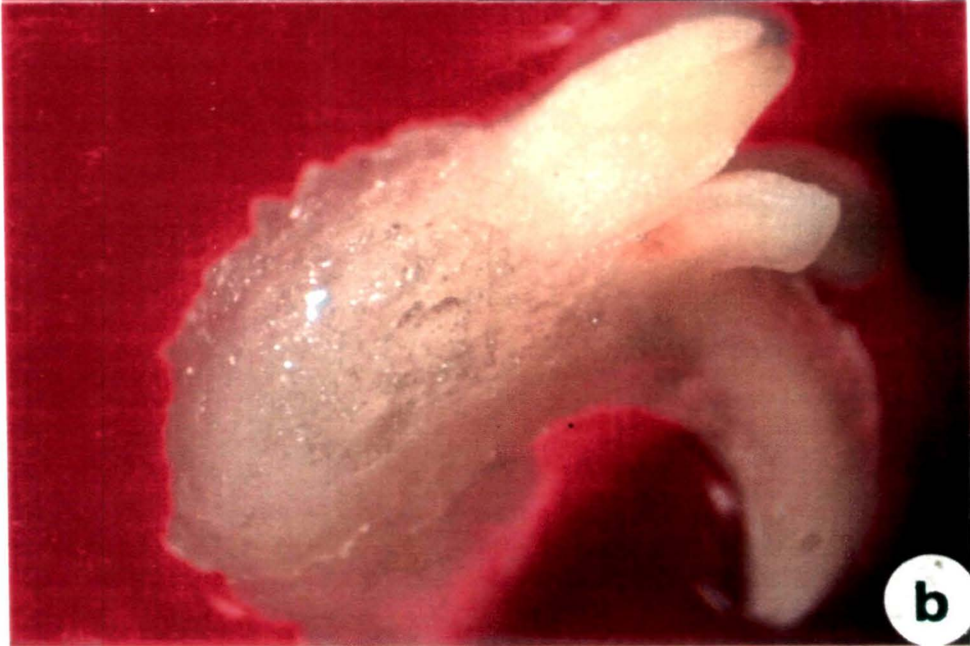

b

Table 20: Effect of light on formation of Stage II callus from mature zygotic embryos

Light intensity ($\mu mol\ m^{-2} sec^{-1}$)	**Type of response**	**% Response (±SE)[1]**	
		Embryogenic	**Non-embryogenic**
Dark	Profuse callusing, callus creamish white and soft	37.21 (±0.55)	-
20	Callus showed no proliferation	-	8.62 (±0.91)
60	Cotyledons showed elongation and turned green	-	-

[1] Standard error

- No response

Table 21: Effect of light on formation of Stage III callus from mature zygotic embryos

Light intensity ($\mu mol\ m^{-2} sec^{-1}$)	**Type of response**	**% Response (± SE)[1]**	
		Embryogenic	**Non-embryogenic**
Dark	Friable callus formed with PEMs all over	27.9 (±0.73)	14.89 (±0.91)
20	Callus degenerated	-	28.62 (±0.91)
60	Cotyledons elongated, turned green	-	-

[1] Standard error

- No response

Effect of culture temperature on the establishment of embryogenic cultures from mature zygotic embryos:

Optimum response was obtained in cultures grown at 24^{0}C exhibiting stages I to III (Table 22, 23 and 24). While there was no response at lower temperatures of 4 and 8^{0}C (Table 22), stage I callus initiated at 36^{0}C shriveled up and turned nonembryogenic (Table 23 and 24).

Effect of $AgNO_3$ on the establishment of embryogenic cultures from mature zygotic embryos:

Incorporation of $AgNO_3$ in the semi-solid medium had an inhibitory effect on callus proliferation (Table 25). $AgNO_3$ in the medium induced oxidative browning of the culture medium that in turn caused browning of the cultures. Higher concentrations of $AgNO_3$ reduced the level of proliferation of stage I callus and formed clumps thus inhibiting formation of PEMs. Lower concentrations were also inhibitory for callus proliferation. Incorporation of $AgNO_3$ was not effective in proliferation and formation of PEMs.

Effect of varying concentrations of plant growth regulators in the maintenance and multiplication of embryogenic suspension cultures from mature zygotic embryos:

Cultures when subcultured onto medium having the same concentration of PGR as in the initiation medium, showed slow proliferation and turned nonembryogenic (Table 26). At 1/10 concentration of PGR, the

Table 22: Effect of different culture temperatures on the initiation of Stage I callus from mature zygotic embryos

Temperature (^0C)	Type of response	% Response (± SE)[1]
4	-	-
8	-	-
12	Slight opening of cotyledons, no callus formation	-
24	Callus formed from micropylar end	31.10 (±1.74)
36	Rapid callus formation from micropylar end	28.01 (±4.18)

[1] Standard error

- No response

Table 23: Effect of different culture temperatures on the formation of Stage II callus from mature zygotic embryos

Temperature (^{0}C)	Type of response	% Response (±SE)[1]	
		Embryogenic	**Non-embryogenic**
4	-	-	-
8	-	-	-
12	-	-	-
24	Callus profuse, soft and transluscent	30.25 (± 0.92)	-
36	Callus dried up and turned brown	-	19.89 (±0.91)

[1] Standard error

- No response

Table 24: Effect of different culture temperatures on the formation of Stage III callus from mature zygotic embryos

Temperature (^0C)	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
4	-	-	-
8	-	-	-
12	Cotyledons did not show further callusing, turned brown	-	-
24	Friable callus formed with PEMs all over	28.82 (±1.95)	16.21 (±0.99)
36	Callus dried up and turned brown	-	19.89 (± 0.91)

[1] Standard error

- No response

Table 25: Effect of different concentrations of silver nitrate in initiation medium on development of Stage II callus*

Stage of callus	**Concentration of silver nitrate (μM)**	**Embryogenic response**
Stage I	0	+++
	10	++
	20	++
	30	+
	40	+

+ Callus initiated but did not turn embryogenic
++ Callus turned embryogenic but did not form PEMs
+++ Callus turned friable with formation of PEMs
*observations were made following next three subcultures on initiation medium

Table 26: Effect of 2,4-D, NAA and BAP on the formation of Stage III embryogenic suspension cultures from mature zygotic embryos

Concentrations	2,4-D (µM)	NAA (µM)	BAP (µM)	Type of response	% Response (± SE)[1]
Full	22.6	13.4	11.1	+	-
1/2	11.3	6.70	5.55	+	-
1/5	4.52	2.68	2.22	++	11.10 (± 1.30)
1/10	2.26	1.34	1.11	+++	20.36 (±0.75)
0	0.00	0.00	0.00	+	-

[1] Standard error

- No response

+ Embryogenic cultures turned nonembryogenic

++ Embryogenic cultures turned brown following first subculture, did not form PEMs

+++ Embryogenic cultures showed formation of PEMs

cultures showed formation PEMs. There was browning of cultures and gradual degeneration in 1/2, 1/5 and absence of PGRs.

Effect of varying concentrations of carbohydrate sources in the maintenance and multiplication of embryogenic suspension cultures from mature zygotic embryos:

Sucrose:

20 and 30 gl^{-1} sucrose in the maintenance medium resulted in growth of embryogenic cultures (Table 27). At 30 gl^{-1} PEMs were formed in the cultures, however there was rapid browning and degeneration of the cultures in subsequent subcultures. In the absence of sucrose, there was no callus proliferation. Higher concentrations of sucrose at 40 and 50 gl^{-1} did not promote callus proliferation.

Maltose:

Incorporation of maltose in the medium showed less browning in the cultures compared to sucrose. 20 gl^{-1} maltose in the medium resulted in formation of stage III callus with PEMs, while with 30 gl^{-1} a higher response of 9.45% was recorded (Table 27). Conversion to PEMs and eventually into cultures having early stage cotyledonary embryos was higher when subcultured in medium with 30 gl^{-1} which was therefore considered the optimum concentration.

The maintenance medium therefore contained 1/10 PGR supplemented with 30 gl^{-1} maltose.

Table 27: Effect of different concentrations of carbohydrates in the medium on the formation of Stage III suspension cultures from mature zygotic embryos

Carbohydrate	Conc (gl^{-1})	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
Sucrose	0	Cultures turned brown, no culture proliferation	-	-
	10	Cultures showed slow growth which rapidly turned brown	3.10 (±0.096)	-
	20	Relatively more growth of culture	5.13 (±1.09)	-
	30	Culture proliferation was rapid and resulted in formation of PEMs	4.20 (±0.34)	-
	40	Slow proliferation of cultures, which turned brown	-	23.20 (±1.22)
	50	Slow proliferation which rapidly turned brown	-	0.98 (±0.09)
Maltose	0	Explants turned brown, no callus formation	-	-
	10	Slow proliferation of cultures	-	8.50 (±0.94)
	20	Slow culture proliferation, but eventually formed PEMs	- 7.50 (±0.78)	36.20 (±1.9)
	30	Satisfactory proliferation of culture, converted to PEMs, showed no browning	9.45 (±2.98)	20.43 (±0.97)
	40	Slow browning of cultures	-	22.50 (±0.53)
	50	Culture formed non embryogenic cell line	-	38.0 (±1.83)

[1] Standard error

- No response

Effect of different concentrations of ABA, PEG and PGR on the maturation of embryogenic suspension cultures obtained from mature zygotic embryos:

The medium with 1/10 PGR, 30 µM ABA and without PEG was most suitable in the maturation of stage III suspension cultures (6.73%, Table 28). Presence of PEG at higher concentrations in the medium singly or in combination with ABA converted the embryogenic cultures to nonembryogenic where the dissociated cell mass clumped and turned brown and hard. Absence of ABA in the medium induced the formation of nonembryogenic cultures. Medium supplemented with full concentrations of PGR with out ABA and PEG resulted in reduced embryogenic response (0.87%) in the cultures.

Effect of different concentrations of $CaNO_3$ on the maturation of embryogenic suspension cultures obtained from mature zygotic embryos:

The optimum concentration of $CaNO_3$ in the maturation medium was 0.90 mM which is the same as used in BM medium (Table 29). The number of suspension cultures that retained embryogenic cell masses along with ESMs i.e., having early stage cotyledonary embryos was found to be inhibited at higher and lower concentrations of $CaNO_3$.

Table 28: Effect of different concentrations of abscisic acid, polyethylene glycol and plant growth regulators on the formation of Stage III embryogenic suspension cultures from mature zygotic embryos

ABA (μM)	PEG-4000 (%)	PGR*	Type of response	%Response (±SE)[1]
0	0	1/2	Cultures turned non embryogenic	0.00
		1/5	Cultures turned non embryogenic	0.00
		1/10	Friable cultures could be maintained, did not turn brown, formed PEMs in suspension	0.81 (±0.07)
0	3.75	1/2	The entire cell mass turned brown and hard	-
		1/5	-do-	-
		1/10	-do-	-
0	5.0	1/2	-do-	-
		1/5	-do-	-
		1/10	-do-	-
30	0	1/2	Cultures could be maintained, only a portion formed ESMs** in suspension	0.083 (±0.037)
		1/5	-do-	2.46 (±0.27)
		1/10	-do-	6.73 (±1.19)
30	3.75	1/2	-do-	1.05 (±0.030)
		1/5	-do-	0.12 (±0.016)
		1/10	-do-	0.097 (±0.04)
30	5.0	1/2	Culture turned brown, no longer embryogenic	-
		1/5	-do-	-
		1/10	-do-	-
0	0	Full PGR	Cultures with PEMs in some areas of the suspension cultures	0.87 ± (0.034)

[1] Standard error; - No response

*PGR - plant growth regulators in the initiation medium - 2,4-D (22.6 μM), NAA (26.8 μM) and BAP (11.1 μM)

**ESMs contained mixture of PEMs and early stage cotyledonary somatic embryos

Table 29: Effect of different concentrations of calcium nitrate in the maturation medium on the development of early stage somatic embryos in suspension cultures from mature zygotic embryos*

Days of observation	**$CaNO_3$ (mM)**	**Type of response**	**No. of cell lines formed**	
			Embryogenic	**Non-embryogenic**
7	0.22	Embryogenic cultures degenerated	21	29
	0.45	Embryogenic cultures showed degeneration	23	27
	0.90	Culture showed proliferation with formation of ESMs	28	22
	1.12	Most of the cells clumped together and turned hard, a portion continued to proliferate in suspension forming ESMs	45	5
	1.35	Suspension with PEMs showed degenerated cells	23	27
	1.80	Embryogenic suspension cultures turned non-embryogenic	19	31
15	0.22	Cultures turned brown and degenerated	2	48
	0.45	Cultures turned brown and degenerated	2	48
	0.90	Entire suspension showed formation of ESMs	11	41
	1.12	A portion of the culture showed ESMs formation	6	44
	1.35	Cultures turned brown and degenerated	-	50
	1.80	Culture turned brown and degenerated	-	50

- No response

* observation made over two weeks after transfer to the maturation medium

50 numbers of cell cultures were used in each case

Effect of $AgNO_3$ in the establishment of stage III embryogenic suspension cultures from mature zygotic embryos:

When stage II cultures were subcultured in the medium containing varying concentrations of $AgNO_3$ supplemented with 1/10 PGR and 30 µM ABA, it resulted in browning of cultures that inhibited the formation of Stage III cultures (Table 30). Addition of $AgNO_3$ did not prove effective in either proliferation or conversion to stage III cultures. Contrary to this, in the absence of $AgNO_3$,optimum formation of Stage III cultures was recorded.

Establishment of embryogenic cultures from female gametophyte:

The cultures were established on 1/2 Litvay's semi solid medium having 30 gl^{-1} sucrose and 22.6µM 2,4-D, 13.4µM NAA and 11.1µM BAP with 1 gl^{-1} mesoinositol and 0.2 gl^{-1} PVP. Stage I (Fig. 14) and stage II (Fig. 15) calli thus formed were used for further studies. Further subcultures on the semi-solid maintenance medium, formed callus with PEMs and early stage cotyledonary embryos (Fig. 16).

Effect of varying concentrations of plant growth regulators on the maintenance and multiplication of embryogenic suspension cultures from female gametophyte:

Using 1/10 PGR in the medium, embryogenic cultures resulted in the formation of PEMs (Table 31), while subculture in the medium with full PGR resulted into a fused hardened mass that gradually degenerated.

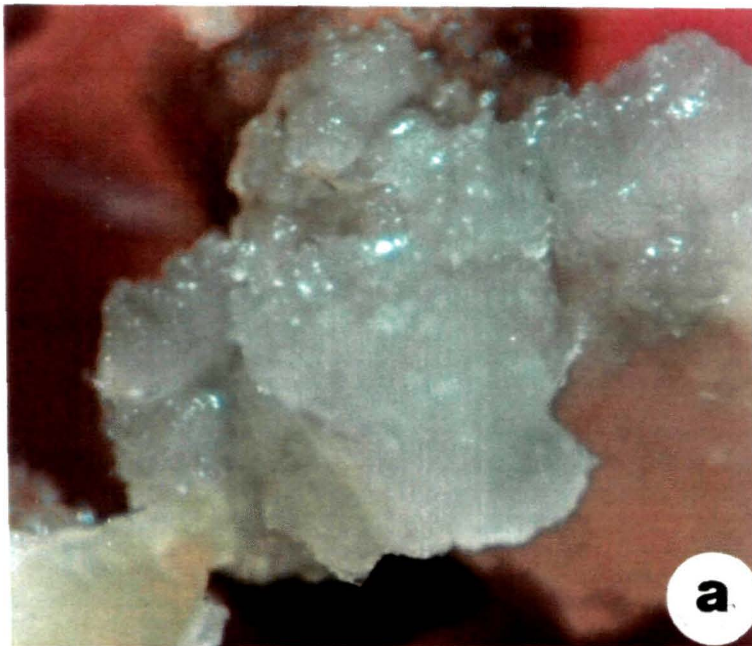
Table 30: Effect of different concentrations of silver nitrate in the maturation medium on development of Stage III embryogenic suspension cultures*

Stage of callus	Concentration of Silver nitrate (μM)	Embryogenic response
Stage II	0	+++
	10	++
	20	+
	30	+
	40	+

\+ Cultures showed multiplication but did not turn embryogenic
++ Cultures turned embryogenic but did not form PEMs
+++ Cultures showed formation of PEMs
* observations made following next two subcultures on maturation medium

Figure 14. Initiation of Stage I callus from female gametophyte in semi solid medium

a. Stage I callus (10X)
b. Embryogenic callus after second subculture (10X)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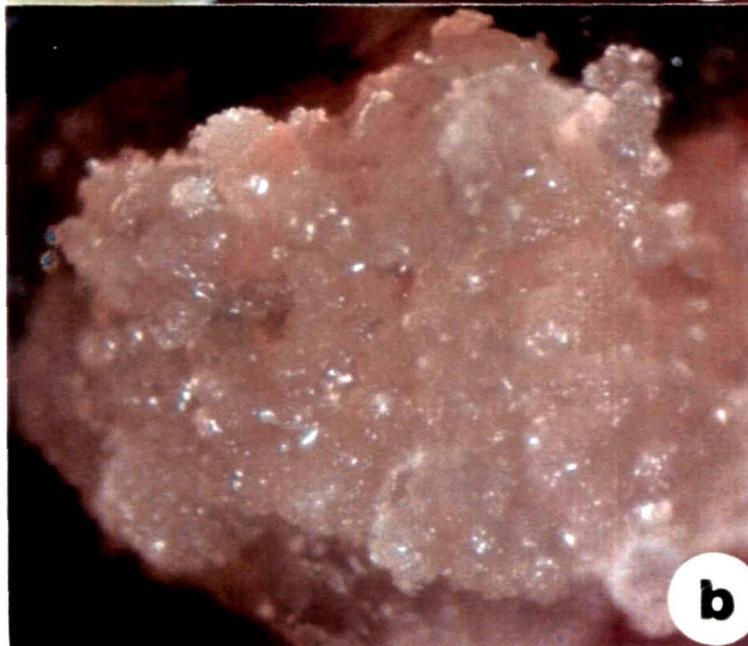
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Figure 15. Development of Stage II callus from female gametophyte in semi solid medium

a. callus six days after second subculture (10X)
b. callus after third subculture(16X)
c. callus after fourth subculture (25X)
d. callus after fourth subculture (35X)

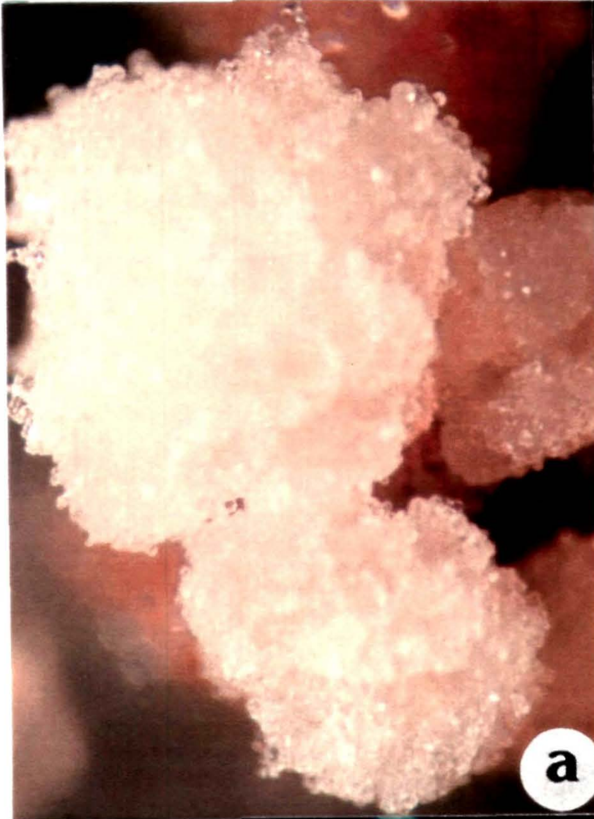
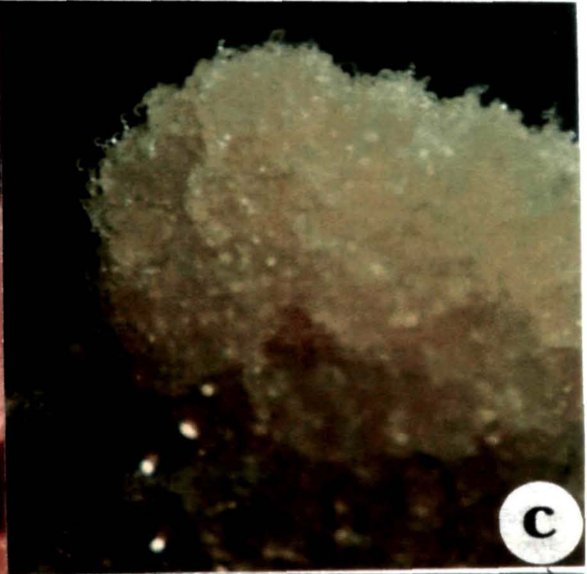
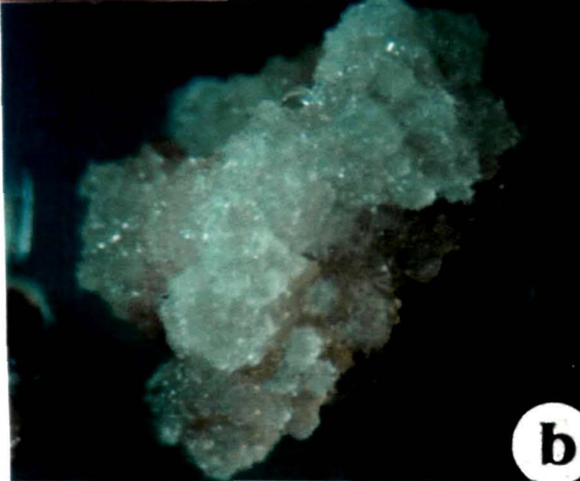
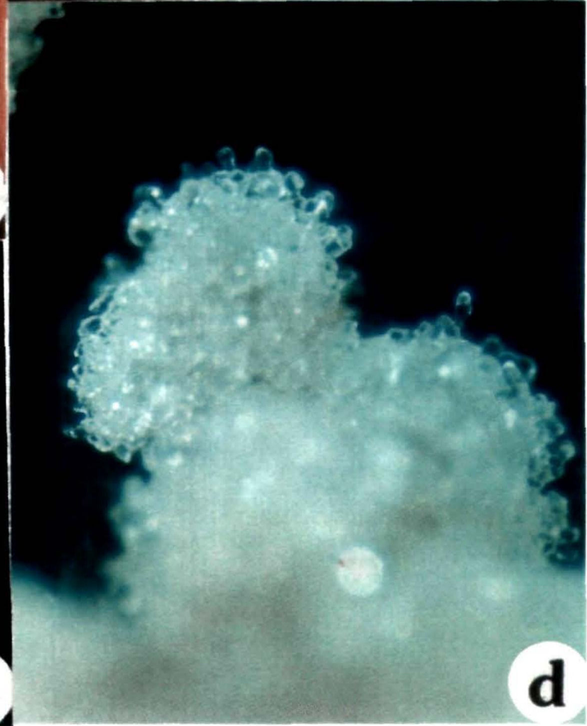
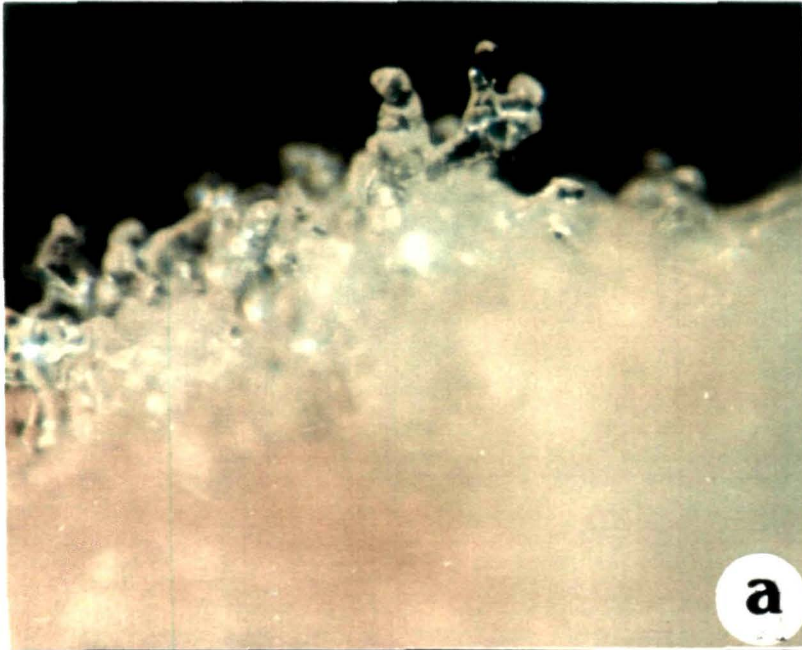





Figure 16. Formation of Stage III callus from female gametophyte in semi solid medium

a. Embryogenic callus showing proembryos (25X)
b. Embryogenic callus showing two proembryos (35X)
c. Stage III embryogenic callus showing a single proembryo (35X)
d. A cotyledonary somatic embryo turning green when kept in light (40X)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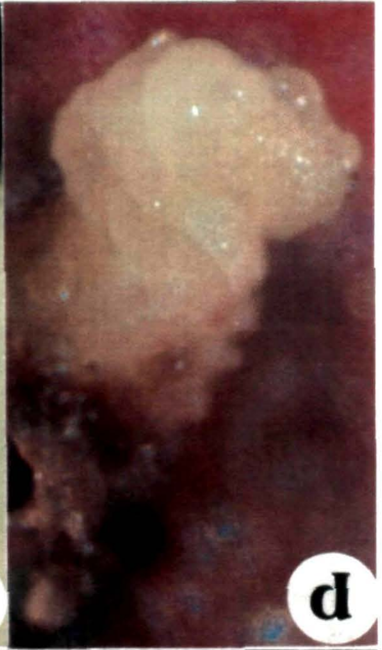
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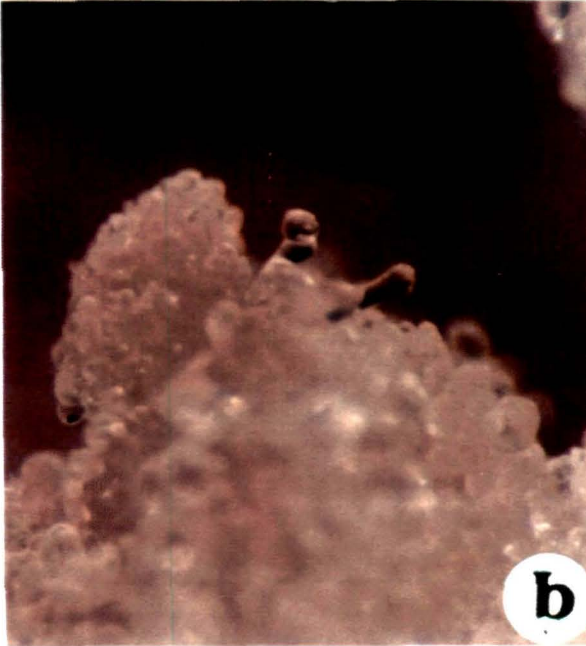
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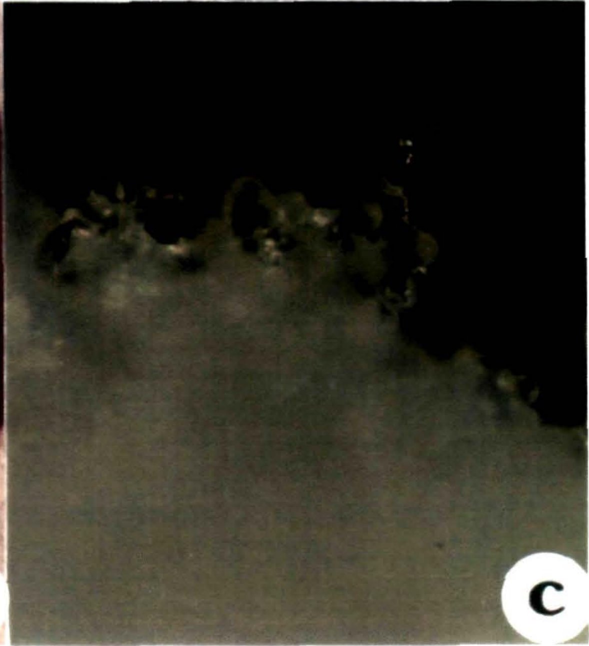
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Effect of varying concentrations of carbohydrate sources on the maintenance and multiplication of embryogenic suspension cultures from female gametophyte:

Sucrose:

In female gametophyte, sucrose at 30 gl^{-1} resulted in rapid proliferation of cultures that formed 16% PEMs (Table 32). The cultures with 20 gl^{-1} sucrose also resulted in proliferation but with more browning in the cultures that turned nonembryogenic. The medium supplemented with 40 gl^{-1} sucrose resulted in browning and gradual degeneration of cultures.

Maltose:

Maltose at 20 and 30 gl^{-1} showed proliferation of stage II cultures forming stage III cultures with PEMs at 13.27 and 12.78% respectively (Table 32). In maltose supplemented media, there was less browning and proliferation of cultures with lower percentage of stage III cultures compared to the cultures supplemented with sucrose.

For female gametophyte, 30 gl^{-1} sucrose was found optimum for maintenance and proliferation. The percentage embryogenic response in sucrose was higher compared to that in maltose supplemented cultures (Table 32).

Table 31: Effect of 2,4-D, NAA and BAP on formation of Stage III embryogenic suspension cultures from female gametophyte

Concentrations	2,4-D (μM)	NAA (μM)	BAP (μM)	Type of response	% Response (±SE)[1]
Full	22.6	13.4	11.1	+	-
1/2	11.3	6.70	5.55	+	-
1/5	4.52	2.68	2.22	++	7.62 (± 0.75)
1/10	2.26	1.34	1.11	+++	15.18 (± 2.72)
0	0.00	0.00	0.00	+	-

[1] Standard error

- No response

\+ Embryogenic cultures turned nonembryogenic

++ Embryogenic cultures turned brown following first subculture, did not form PEMs

+++ Embryogenic cultures turned friable with formation of PEMs

Table 32: Effect of different concentrations of carbohydrates in ½ Litvay's medium on the formation of Stage III suspension cultures from female gametophyte

Carbohydrate	Conc (gl^{-1})	Type of response	% Response (± SE)[1]	
			Embryogenic	Non-embryogenic
Sucrose	0	-	-	-
	10	Cultures showed slow growth	-	13.63 (±0.76)
	20	Rapid proliferation of cultures which eventually turned brown	-	43.16 (±1.56)
	30	Rapid proliferation of cultures which eventually formed PEMs	15.62 (±1.16)	33.33 (±1.50)
	40	Slow proliferation of cultures but turned brown	-	9.11 (±0.77)
	50	-	-	-
Maltose	0	-	-	-
	10	Culture proliferation slow, could not be sustained	-	1.28 (±0.59)
	20	Culture proliferation slow	13.27 (±0.45)	29.12 (±1.76)
	30	Culture proliferated, satisfactory formation of PEMs	12.78 (±1.05)	32.10 (±1.09)
	40	Slow proliferation, cultures turned brown and non embryogenic	-	9.45 (±0.73)
	50	Cultures turned brown and degenerated	-	1.61 (±0.04)

[1]:Standard error

- No response

Effect of different concentrations of ABA, PEG and PGR on the maturation of embryogenic suspension cultures from female gametophyte:

The concentrations of 1/10 PGR supplemented with 30 µM ABA and absence of PEG in the medium was found to be optimum for the maturation of embryogenic cultures with formation of 18.3% of PEMs and ESMs (Table 33). Higher concentrations of ABA in the medium resulted in nonembryogenic cultures. Presence of PEG in the medium was inhibitory to the formation of PEMs and ESMs in cultures. Figure 17 shows embryogenic suspension cultures established from female gametophyte

Effect of different concentrations of $CaCl_2$ on the maturation of embryogenic suspension cultures obtained from female gametophyte:

The optimum concentration of $CaCl_2$ was 0.90 mM as currently used in 1/2 Litvay's medium for maturation (Table 34). It was observed that lower concentration of $CaCl_2$ (0.22 and 0.45 mM) in the medium resulted in degeneration of cultures, while higher concentrations (1.35 and 1.8 mM) converted the cells in suspension into clumps that turned brown and degenerated. The effect of $CaCl_2$ (in concentrations of 0.9 and 1.12 mM) in the maturation medium influenced the conversion percentage of PEMs to early stage cotyledonary embryos.

Nonembryogenic cultures:

Figure 18 shows the nonembryogenic cultures that resulted during different stages of somatic embryogenesis using both mature zygotic

Table 33: Effect of different concentrations of abscisic acid, polyethylene glycol and plant growth regulators on the formation of Stage III embryogenic suspension cultures from female gametophyte

ABA (μM)	PEG-4000 (%)	PGR*	Type of response	%Response (±SE)[1]
0	0	1/2	Cultures turned non embryogenic	0.00
		1/5	Cultures turned non embryogenic	0.00
		1/10	Cultures could be maintained, did not turn brown, formed PEMs	1.10 (±0.07)
0	3.75	1/2	-	-
		1/5	-	-
		1/10	-	-
0	5.0	1/2	-	-
		1/5	-	-
		1/10	-	-
30	0	1/2	Cultures could be maintained, only a small area formed ESMs	7.49 (±0.071)
		1/5	-do-	0.83 (±0.037)
		1/10	-do-	18.3 (±0.064)
30	3.75	1/2	-do-	4.44 (±0.036)
		1/5	-do-	0.83 (±0.009)
		1/10	-do-	4.9 (±0.166)
30	5.0	1/2	Cultures no longer embryogenic	-
		1/5	-do-	-
		1/10	-do-	-
0	0	Full PGR	Cultures formed PEMs in some areas	1.38 ± 0.066

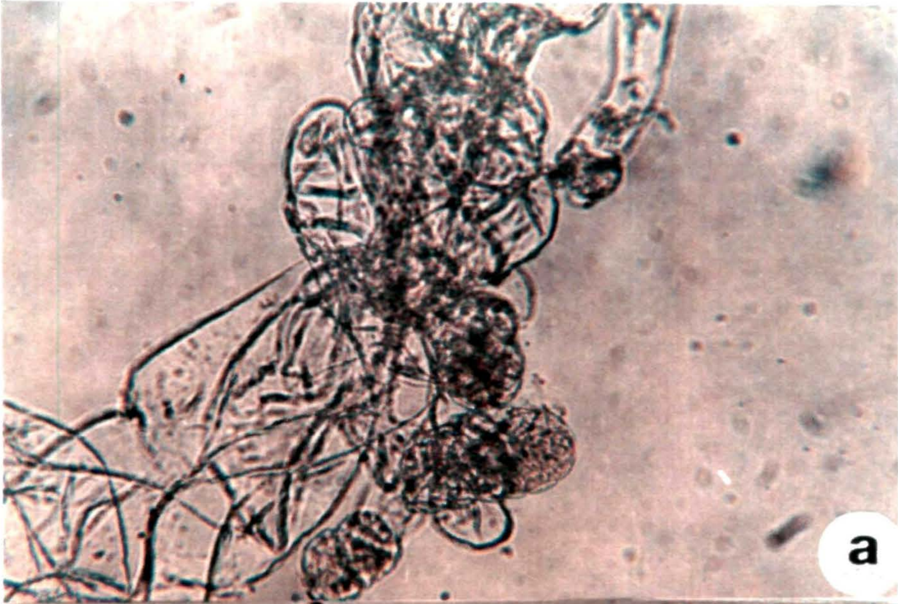
[1] Standard error

- No response

*PGR - plant growth regulators in the initiation medium - 2,4-D (22.6 μM), NAA (13.4 μM) and BAP (11.1 μM)

Figure 17. Embryogenic suspension cultures from female gametophyte

a. Early stage pro-embryos sharing common suspensor like cells (40X)
b. A pro-embryo with 4-8 embryonal head cells and 2 long suspensor like cells (40X)
c. An early stage SE with dense embryonal head and 4 long vacuolated suspensor like cell (40X)
d. A SE sharing common suspensor like cells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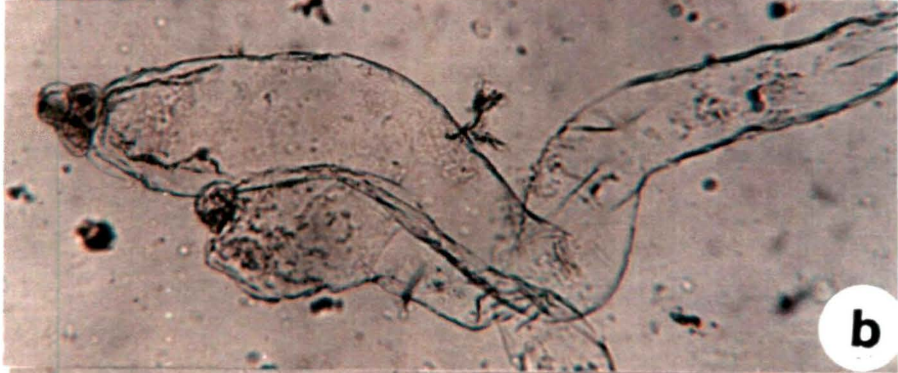
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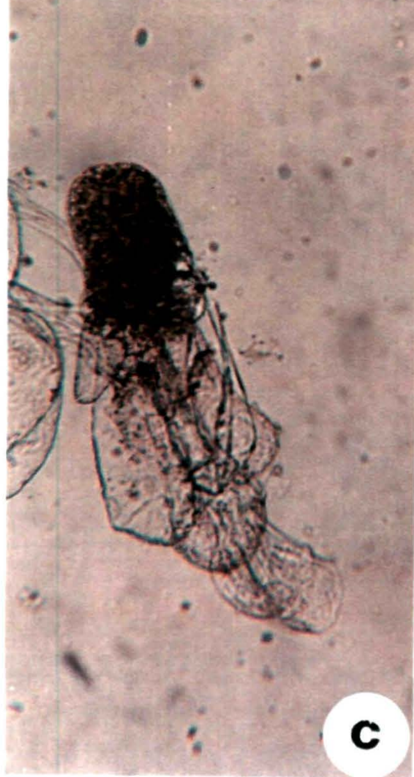
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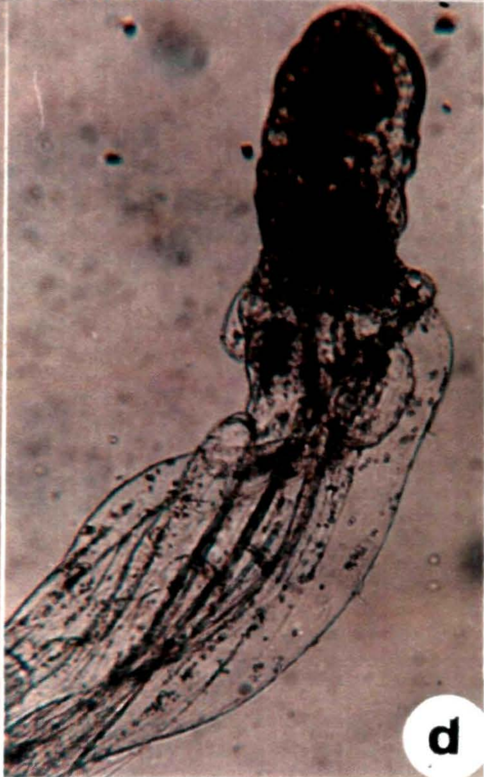
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Table 34: Effect of different concentrations of calcium chloride in ½ Litvay's maturation medium on the development of early stage somatic embryos in suspension cultures from female gametophyte*

Days of observation	$CaCl_2$ (mM)	Type of response	No. of cell lines formed	
			Embryogenic	Non-embryogenic
7	0.22	Degeneration of cultures observed	17	33
	0.45	Cultures with PEMs proliferate, but with gradual browning	16	34
	0.90	Cultures continued to proliferate, showed ESMs	37	13
	1.12	Most of the cells continued to proliferate, showed ESMs	50	-
	1.35	Cultures gradually turned brown	23	27
	1.80	Cultures turned brown	8	42
15	0.22	Cultures turned brown and degenerated	3	47
	0.45	Cultures turned brown and degenerated	2	48
	0.90	Entire area of the suspension cultures filled with ESMs	32	18
	1.12	A portion of the cultures showed ESMs formation	40	10
	1.35	Cultures turned brown and degenerated	2	48
	1.80	Cultures turned brown and degenerated	4	46

- No response

*observation made over two weeks after transfer to the maturation medium

50 numbers of cell cultures were used in each case

Figure 18 Development of non embryogenic cultures

a. Explant after first subculture (10X)
b. Nonembryogenic callus from mature zygotic embryo after 4 passages of subculture,10X)
c. Nonembryogenic callus from female gametophyte after 4 passages of subculture in semi solid medium, 25X)
d. Nonembryogenic suspension culture from female gametophyte after 4 passages of subculture, 40X)
e. Nonembryogenic suspension culture from female gametophyte after 7 passages of subculture, 40X)

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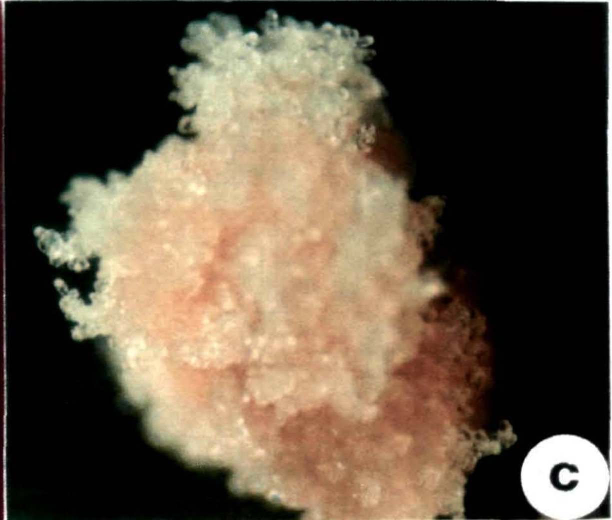
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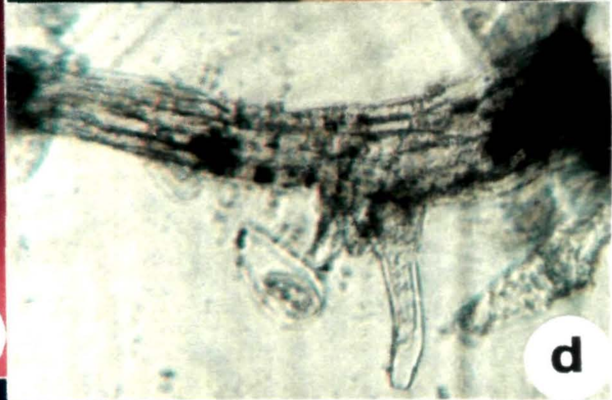
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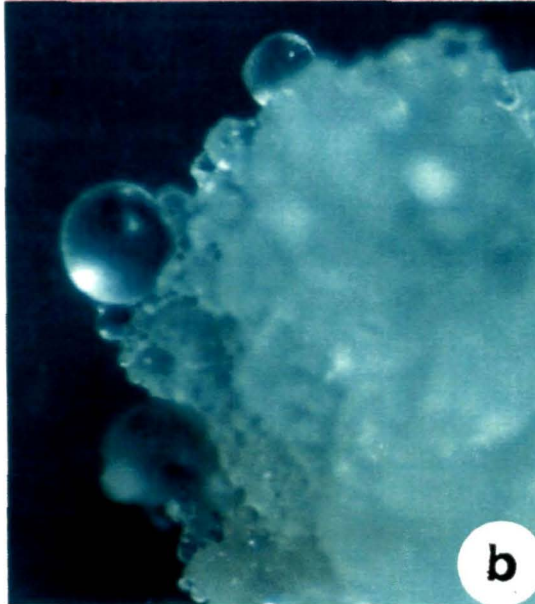
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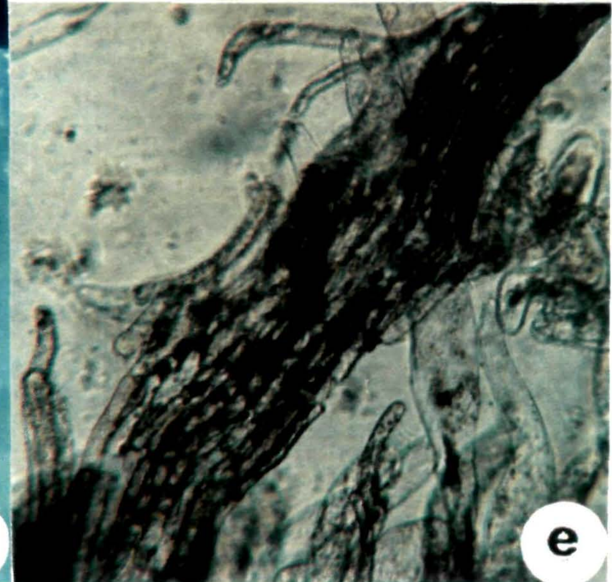
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embryos and female gametophyte and depicts the degenerating callus in semi solid medium.

Microscopic observation:

Comparative embryo morphology at developmental stages in somatic and zygotic embryogenesis

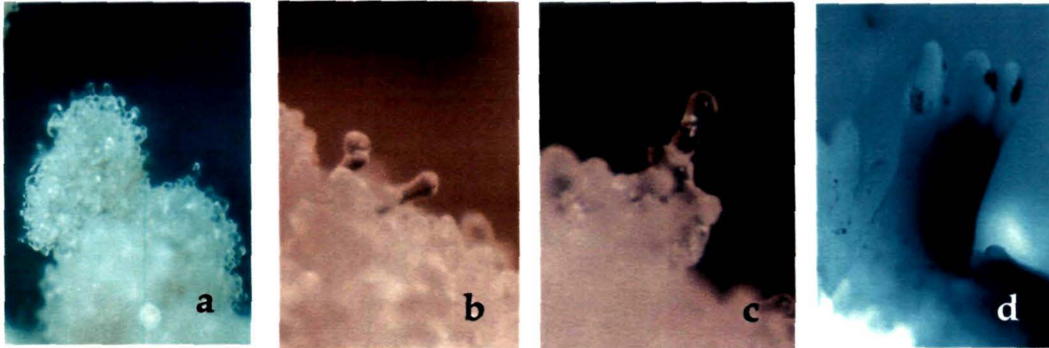
There is a similarity in the developmental stages in zygotic and somatic embryogenesis as seen in the figure 19. There was callus initiation from micropylar end of mature zygotic embryos within 10 days of culture forming stage I callus. From female gametophytes, there was extrusion from micropylar end within 12 days of culture in the initiation medium. The callus was transluscent to white (Fig. 19a).

Proliferating callus was considered established when they were 1-1.5 cm in diameter. In *Pinus*, the proliferating callus generally comprises of suspensor cells with cells having cone shaped heads (Fig. 19b). Early stage somatic embryos (Stage III in the present study) separated out from the embryogenic callus showing embryogenic heads (Fig. 19c).

Estimation of intracellular proteins:

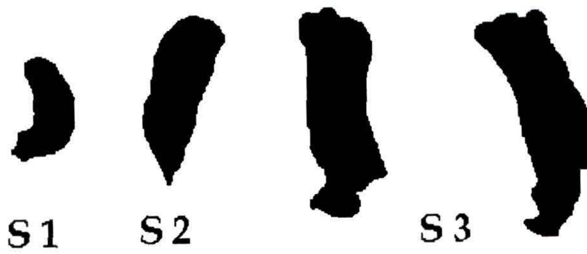
In cultures that turned embryogenic in BM medium, the protein content was 0.3 mg g^{-1} fresh weight in stage I and 2.1 mg g^{-1} fresh weight stage II. The protein content decreased to 0.62 mg g^{-1} fresh weight in stage III callus (Fig. 20). There was increase in protein content from stage I to II and then a decline at stage III except the ones grown in MS, DCR and MSG media

Figure 19. Study stages and comparative embryo development in conifers



S1 S2 S2 S3

Somatic Embryogenesis



Normal embryos

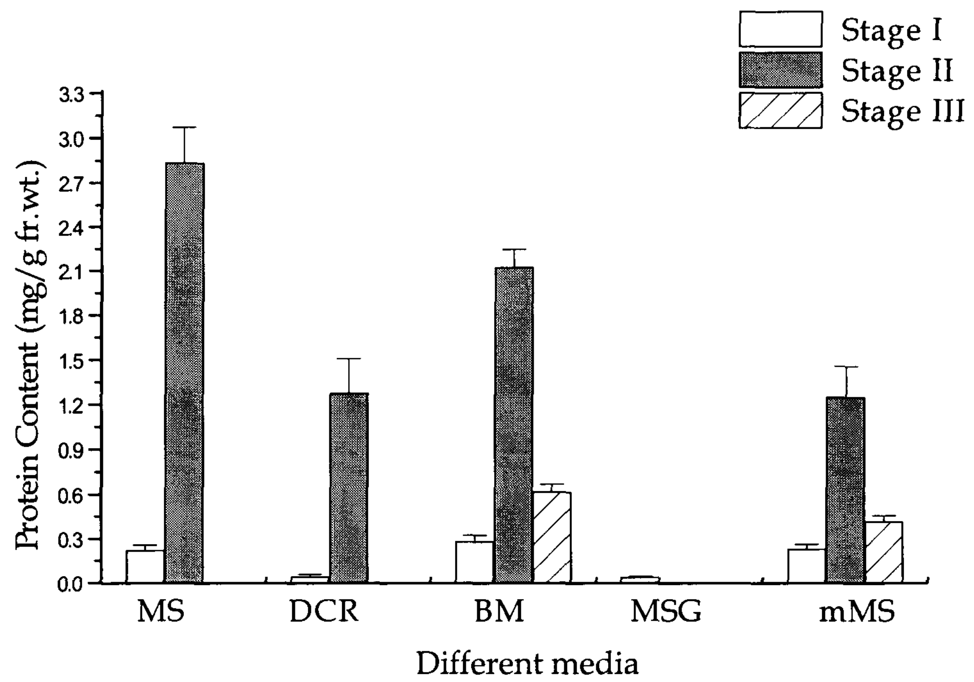
Abnormal embryos

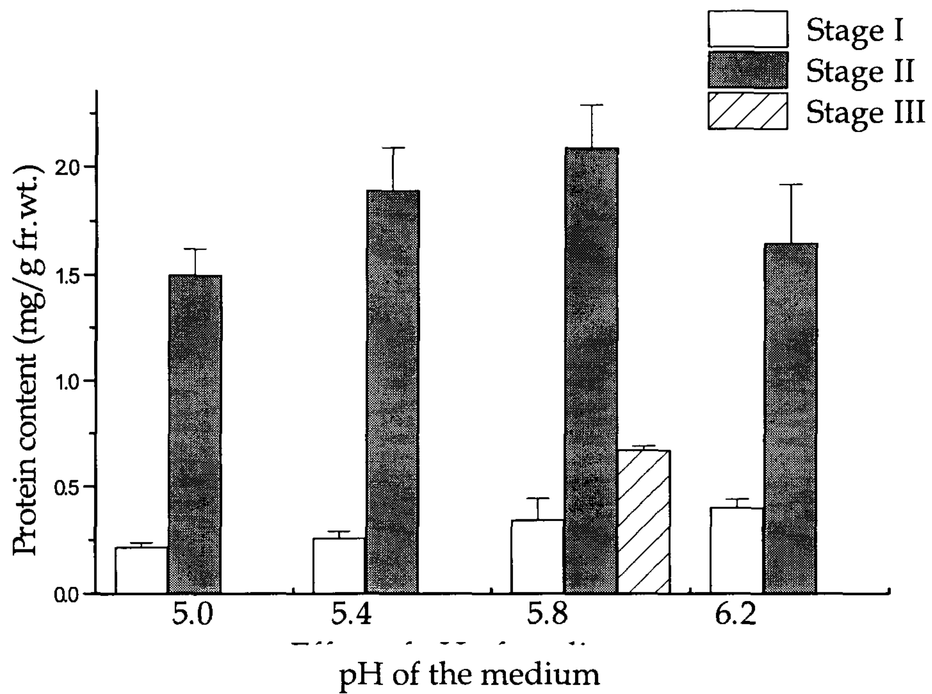
Zygotic Embryogenesis

Comparative embryo morphology at developmental stages
S1- club shaped
S2- cone shaped head
S3-cotyledonary embryos

Figure 20. Effect of different media on the protein content in cultures

Figure 21. Effect of different pH of media on the protein content in cultures


Stage I
Stage II
Stage III
Protein Content (mg/g fr.wt.)
3.3
3.0
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
MS
DCR
BM
MSG
mMS
Different media


Stage I
Stage II
Stage III
Protein content (mg/g fr.wt.)
2.0
1.5
1.0
0.5
0.0
5.0
5.4
5.8
6.2
pH of the medium

(Fig. 20). In cultures that turned nonembryogenic the stage I protein content saw a sharp increase at stage II which however could not be sustained and the cultures degenerated.

At pH 5.8, the stage I callus contained ~0.35 mg protein g^{-1} fresh weight, 2.1 mg protein g^{-1} fresh weight at stage II and stage III callus had ~0.67mg protein g^{-1} fresh weight (Fig. 21). The cultures grown in media with other pH did not show any stage III development.

The protein content increased from stage I to stage II in media containing sucrose (Fig. 22). The content was higher in stage II cultures grown in media containing 20 gl^{-1} of sucrose than in 30 gl^{-1} containing media.

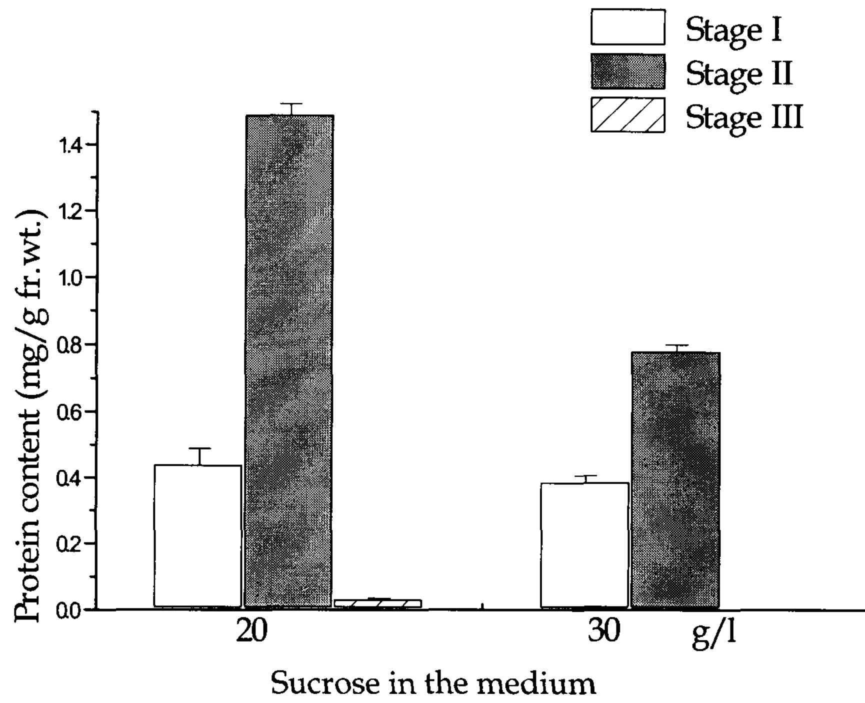
The effect of maltose in the medium on protein content is shown in Fig. 23. With the conversion of cultures from stage I to stage II an increase in protein content was recorded that declined in stage III. The protein contents of the stage II cultures grown in 30 gl^{-1} of maltose in the medium were much higher compared to the ones grown in 20 gl^{-1}.

The protein content of 0.34, 2.1 and 0.64 mg protein g^{-1} fresh weight was recorded at the stage I, II, and III respectively in cultures grown in media with 30 gl^{-1} maltose.

Light inhibited callus initiation while in the dark the stage II protein content was significantly higher compared to those cultured in light (Fig. 24). In dark the protein content at stage I, II, and III were 0.256, 1.95, and 0.85 mg

Figure 22. Effect of different concentrations of sucrose in the medium on the protein content in cultures

Figure 23. Effect of different concentrations of maltose in the medium on the protein content in the cultures


Stage I
Stage II
Stage III
Protein content (mg/g fr.wt.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
g/l
Sucrose in the medium

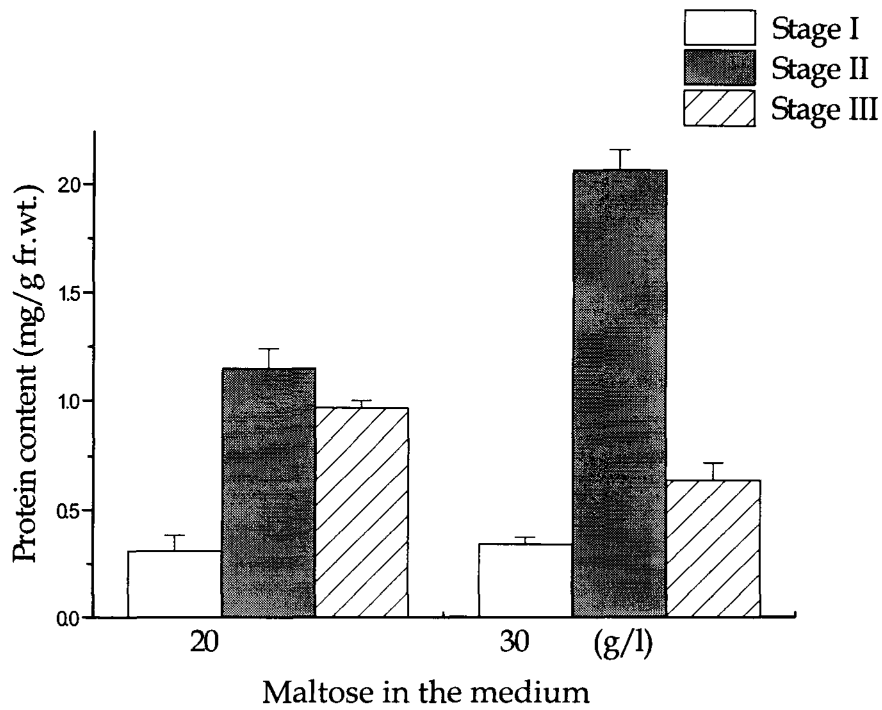
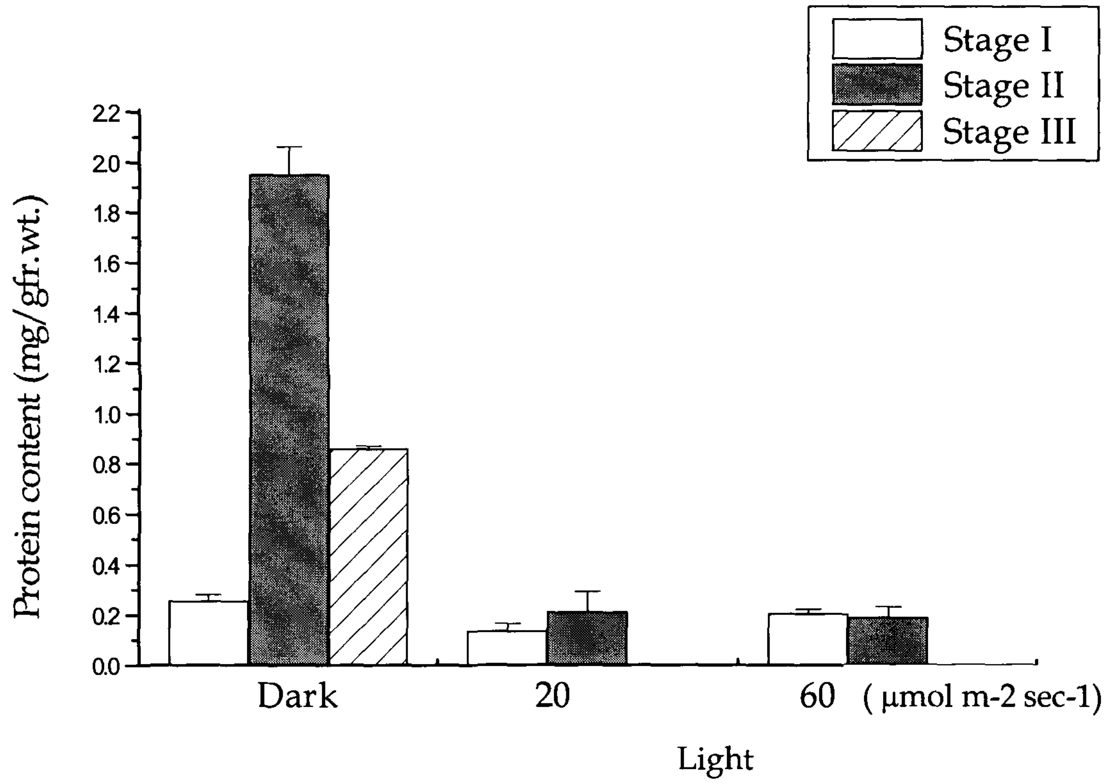
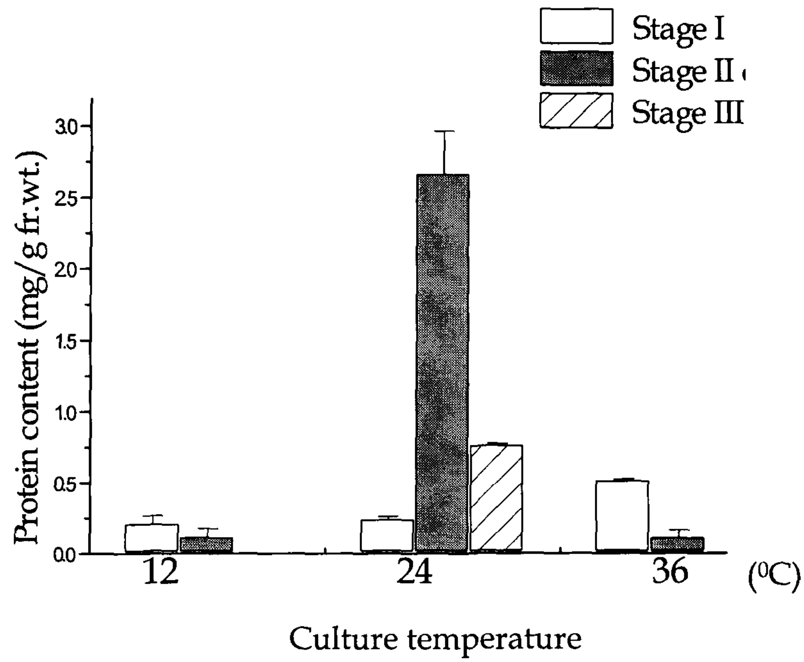

Stage I
Stage II
Stage III
Protein content (mg/g fr.wt.)
2.0
1.5
1.0
0.5
0.0
20
30
(g/l)
Maltose in the medium

Figure 24. Effect of light on the protein content in cultures

Figure 25. Effect of temperature on the protein content in cultures


Stage I
Stage II
Stage III
Protein content (mg/gfr.wt.)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
20
60 (μmol m-2 sec-1)
Light


Stage I
Stage II
Stage III
Protein content (mg/g fr.wt.)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
12
24
36
(⁰C)
Culture temperature

protein g^{-1} fresh weight respectively, compared to 0.206 and 0.192 mg protein g^{-1} fresh weight in callus grown in light at 60 µmol m^{-2} sec^{-1}.

Culture temperature also significantly affected the protein content. At 24^{0}C temperature, the stage I, II, and III cultures contained 0.237, 1.76 and 0.76 mg protein g^{-1} fresh weight respectively (Fig. 25). There was a marked decrease in protein contents in the cultures at lower and higher temperatures.

Protein profile of intracellular proteins:

The protein profile of mature zygotic embryo explants revealed 65, 63, 35, 30, 23 and 22 kDa proteins under reduced conditions (+ β-mercaptoethanol) and 98, 65, 42 and 25 kDa doublets under nonreduced (- β-mercaptoethanol) extraction conditions (Fig. 26).

SDS-PAGE protein profile of callus obtained from mature zygotic embryo and female gametophyte were similar to mature zygotic embryos. Major proteins with apparent molecular weights of 66, 65, 63, 45, 32, 30, 28 kDa, and minor proteins at 37 and 35 kDa were observed (Fig. 27).

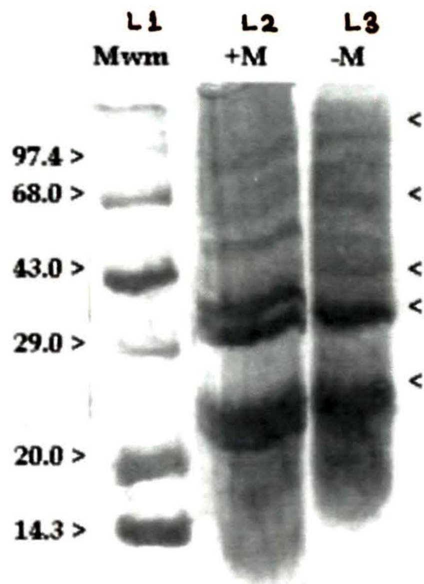
In this study, the majority of the resolved proteins remained constant between unorganised cell clusters (stage I and stage II) but could be differentiated with zygotic embryo by the absence of two bands between 40-42 and one band between 30-32 kDa present in the zygotic embryo and stage I callus from female gametophyte but absent in stage I callus from mature zygotic embryo (Fig 27).

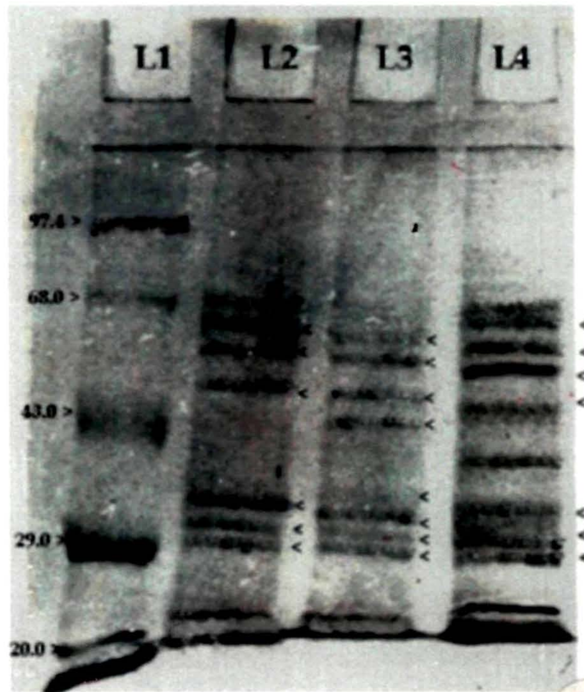
Figure 26. Coomassie blue stained SDS PAGE of zygotic embryo

L1-Molecular weight standards (kDa)
L2-extract under reduced (+ β-mercaptoethanol) conditions
L3-extract under non reduced (-β- mercaptoethanol) conditions

Figure 27. Coomassie blue stained SDS-PAGE of zygotic embryo

L1- Molecular weight standards (kDa)
L2-Stage I callus from mature zygotic embryos
L3-Stage I callus from immature embryos of female gametophyte
L4-extract from mature zygotic embryos


L1
L2
L3
Mwm
+M
-M
97.4 >
68.0 >
43.0 >
29.0 >
20.0 >
14.3 >
26


L1
L2
L3
L4
97.4 >
68.0 >
43.0 >
29.0 >
20.0 >
27

Comparison of protein profile of callus at different stages of somatic embryogenesis from undifferentiated callus (stage I) to callus cultures having proembryonal masses (stage II) showed major proteins of 45, 43-41, 32-35, 30, 28, 25, 18 and 15 kDa molecular weights. These proteins remained constant between the stages. However, intensity of the bands increased with age of callus (Fig. 28).

In the present study, when the intracellular protein patterns of initial zygotic embryo explants was compared with the later stage embryogenic calli (Fig. 29), major difference was in the presence of 22-25, and 25-27 kDa major protein, present in both zygotic embryo and cotyledonary embryos (in maturation medium, minus plant growth regulators, L4). Besides, two very low molecular weight proteins with low intensity bands were observed in stage III callus having bands of 12-13, and 7-9 kDa size. Also a 15-17 kDa band were observed in the stage III callus after 2 subcultures which disappeared when subcultured into medium devoid of plant growth regulators.

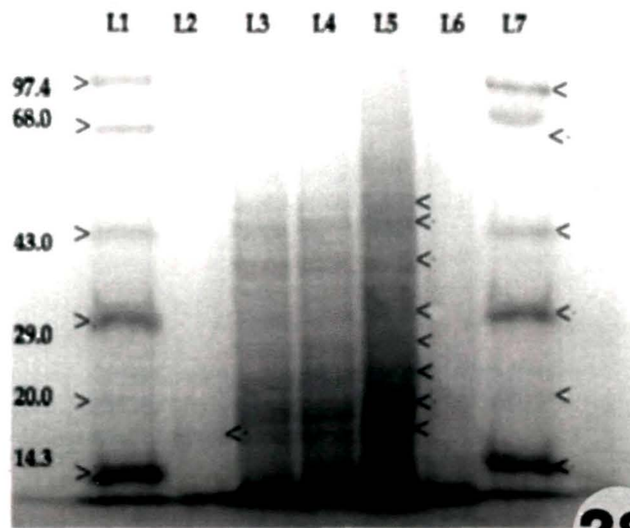
Protein profile of stage I callus grown in different media showed similar banding patterns as compared to extracts of mature zygotic embryos (Fig. 30). Protein profile of stage I and stage III under PGR treatments showed some low molecular weight proteins in 12-13 kDa and 6-7 kDa bands in stage III, that were absent in stage I callus (Fig. 31). With different concentrations of sucrose in the medium the cultures showed (Fig. 32) low

Figure 28. Coomassie blue stained SDS-PAGE of protein at different stages

L1- Molecular weight standards (kDa)
L2 and L6- Stage II nonembryogenic callus
L3- Extract of mature zygotic embryos
L4- Stage I callus
L5- Stage II callus with proembryonal masses

Figure 29. Coomassie blue stained SDS-PAGE of protein at different stages

L1- Extract of mature zygotic embryos
MWM- Molecular weight standards (kDa)
L2- Stage II embryogenic callus with proembryonal masses
L3- Stage III embryogenic callus in maturation medium (after 2 subcultures)
L4- Stage III embryogenic callus in maturation medium (after 4 subcultures)


L1
L2
L3
L4
L5
L6
L7
97.4
68.0
43.0
29.0
20.0
14.3
28

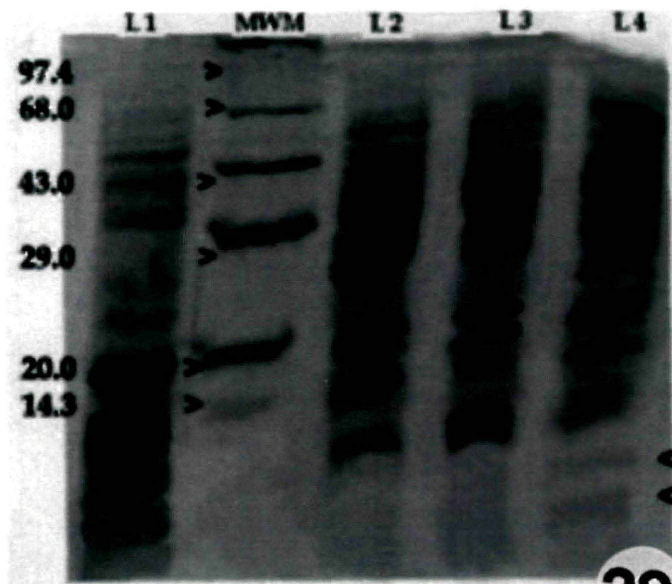
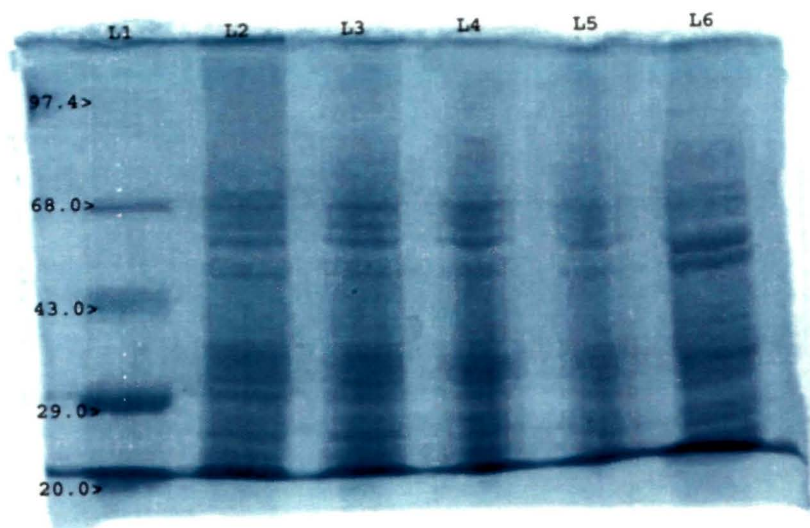

L1
MWM
L2
L3
L4
97.4
68.0
43.0
29.0
20.0
14.3
29

Figure 30. Protein profile of stage I callus in different media

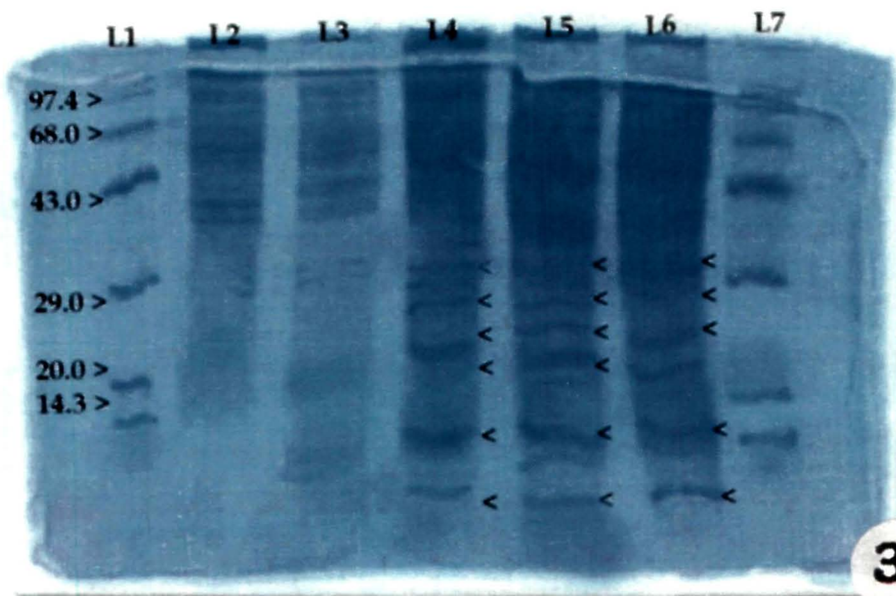
L1-Molecular weight standards (kDa)
L2- extract of mature zygotic embryos
L3- extract from tissue grown in MS medium
L4- extract from tissue grown in DCR medium
L5- extract from tissue grown in mMS medium
L6- extract from tissue grown in BM medium

Figure 31. Protein profile of stage I, II and III cultures grown in medium with plant growth regulators

L1-Molecular weight standards (kDa)
L2- extract of mature zygotic embryos
L3- extract of female gametophyte
L4- Stage II callus
L5- Stage III callus (after 2 subcultures)
L6- Stage III callus (after 4 subcultures)



30



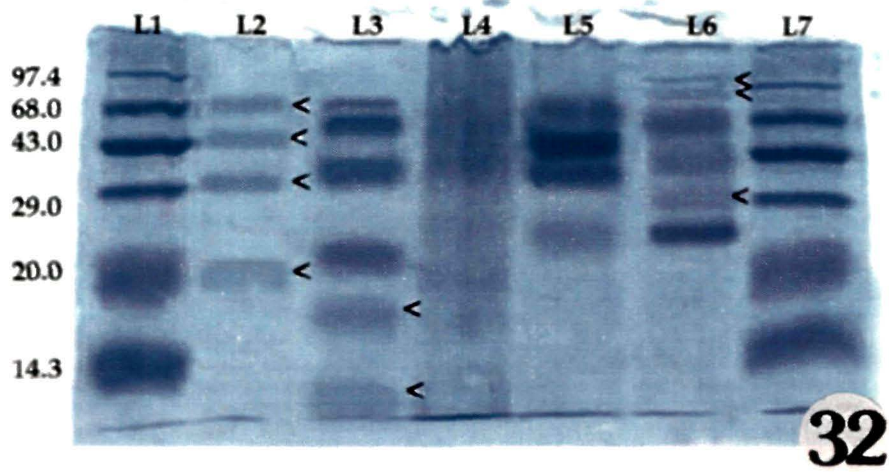
31

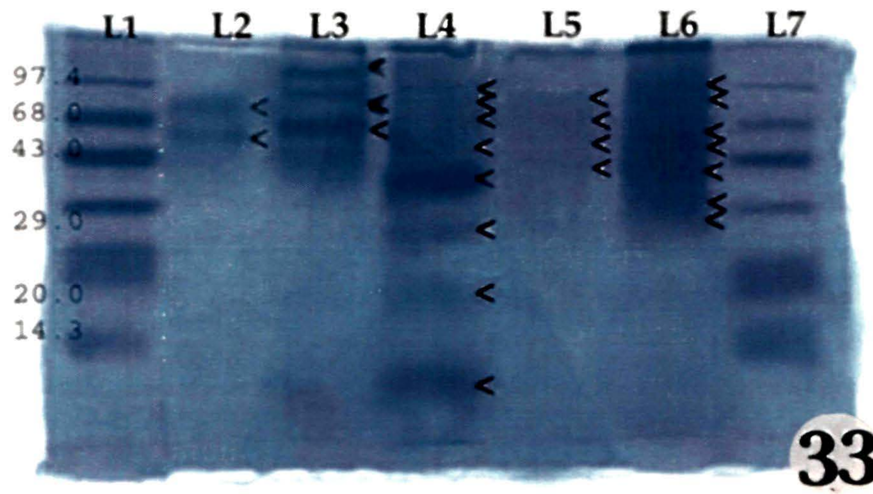
Figure 32. Protein profile of stage III callus in medium with varying sucrose concentration

L1- Molecular weight markers
L2- Extract from tissues grown in medium with 20 gl^{-1} sucrose
L3- Extract from tissues grown in medium with 30 gl^{-1} sucrose
L4- Nonembryogenic callus
L5- Extract from tissues grown in medium with 40 gl^{-1} sucrose
L6- Extract from tissues grown in medium withs 50 gl^{-1} sucrose
L7- Molecular weight markers

Figure 33. Protein profile of Stage III callus in medium with varying maltose concentration

L1- Molecular weight markers
L2- Extract from tissues grown in medium with 10 gl^{-1} maltose
L3- Extract from tissues grown in medium with 20 gl^{-1} maltose
L4- Extract from tissues grown in medium with 30 gl^{-1} maltose
L5- Extract from tissues grown in medium with 40 gl^{-1} maltose
L6- Extract from tissues grown in medium with 50 gl^{-1} maltose


L1 L2 L3 L4 L5 L6 L7
97.4
68.0
43.0
29.0
20.0
14.3
32


L1 L2 L3 L4 L5 L6 L7
97.4
68.0
43.0
29.0
20.0
14.3
33

molecular weight bands in L3 (cultures grown in medium containing 30 gl^{-1} sucrose). Figure 33 shows low molecular weight bands in L4 (callus raised in medium supplemented with 30 gl^{-1} maltose).

When gels were stained for glycoproteins some low molecular weight bands appeared in stages II and III (Fig. 34) with high molecular weight bands in stage I. There were no bands seen in extract of nonembryogenic cultures (L5 in Fig.34 and L2 in Fig. 35). Low molecular weight bands of 25, 18, 13 and 12 kDa were observed in stage III cultures after 4 subcultures in the maturation medium.

Quantification of intracellular proteins at different stages of culture showed an increase in protein content from stage I to III (Fig. 36). However, on formation of cotyledonary somatic embryos (stage III) there was a decrease in the protein content. A similar trend was observed for nonembryogenic cultures. However, the content of protein was markedly lower in the latter.

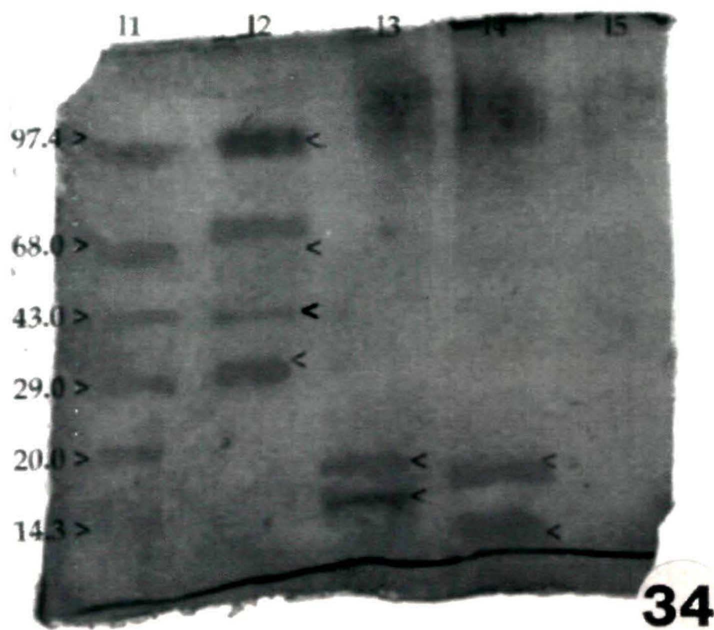
Three phases in the growth was observed study period, an initial steady phase at stage I, followed by a rapid linear increase during the next 2 subcultures (Stage II) followed by a declining stage III phase. Following two subcultures on PGR free medium there was an increase in the protein content (Fig. 36).

Figure 34. Intracellular protein profile at different stages (Pas-Silver stain for glycoproteins)

L1- Molecular weight markers
L2- Stage I
L3- Stage II
L4- Stage III
L5- Nonembryogenic callus

Figure 35. Intracellular protein profile at different stages (Pas-Silver stain for glycoproteins)

L1- Molecular weight markers
L2- Nonembryogenic callus
L3- Stage III (after 4 subcultures in maturation medium)


l1
l2
l3
l4
l5
97.4 >
68.0 >
43.0 >
29.0 >
20.0 >
14.3 >
34

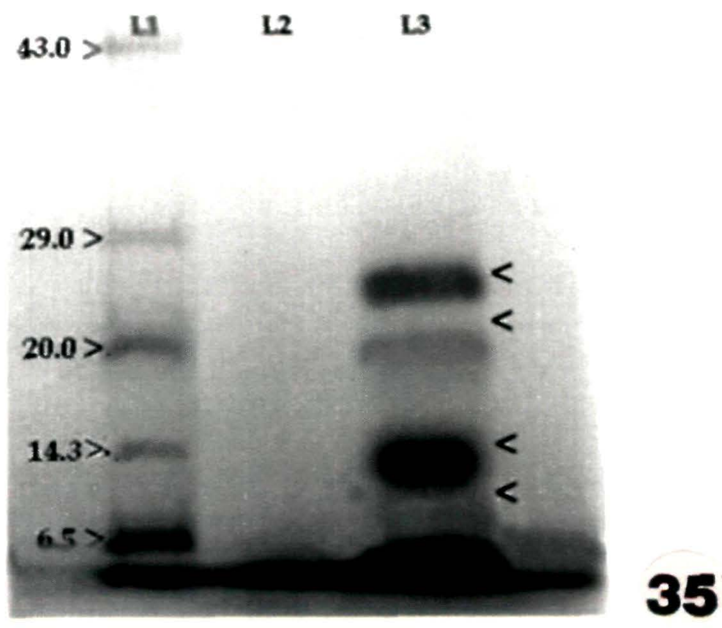
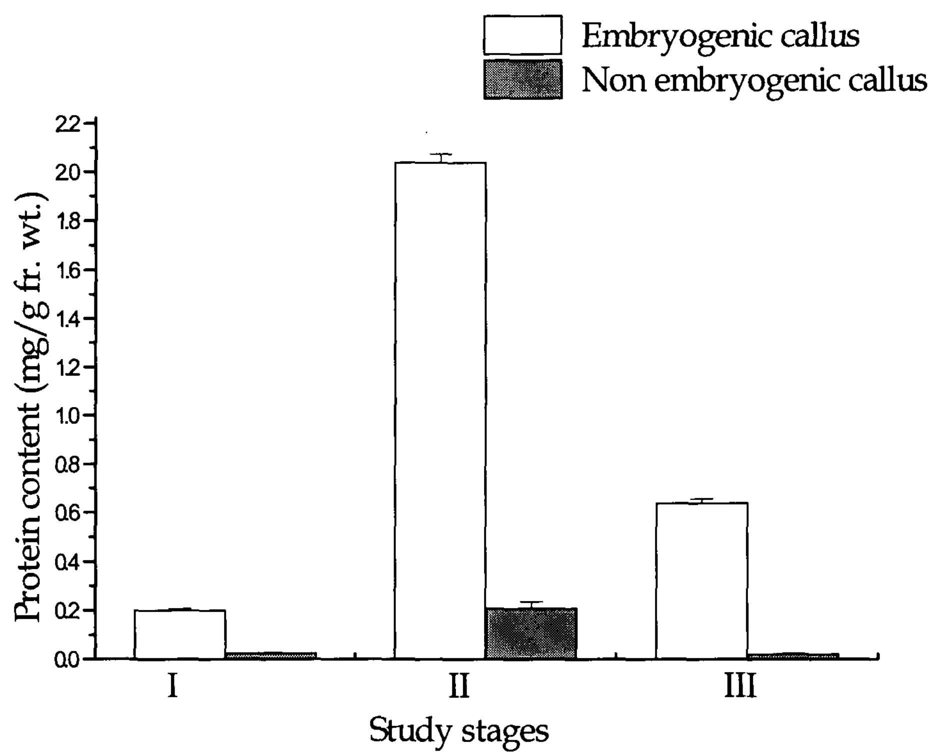

L1
L2
L3
43.0 >
29.0 >
20.0 >
14.3 >
6.5 >
35

Figure 36. Protein content of embryogenic calli at different study stages


Embryogenic callus
Non embryogenic callus
Protein content (mg/g fr. wt.)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
Study stages

Extracellular proteins in the spent medium:

The protein content in the spent media at different study stages showed an increase with maturation of the cultures (Fig. 37).

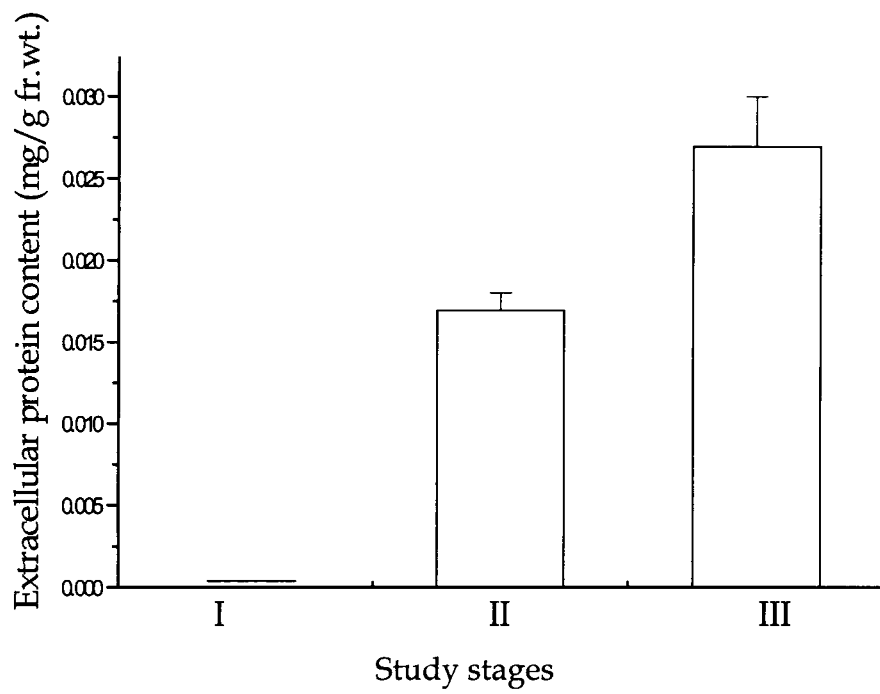
The SDS-PAGE protein profile and staining for glycoprotein revealed low molecular weight protein bands in the profile at different study stages (Fig. 38).

The SDS-PAGE profile revealed a gradual accumulation of some low molecular weight proteins in the spent medium of (cultures from female gametophytes), of 35, 32, 20, 15, 13 kDa size. Spent medium from stage I cultures showed a faint protein band of 15 kDa size while medium collected from cultures following 2 subcultures on maintanence medium showed 15 and 13 kDa protein size. Proteins obtained from spent medium of cultures following 4 subcultures in maintenance medium showed intense bands of 20, 15, and 13 kDa size (Fig. 39).

Peroxidase activity:

Callus raised under different treatments were tested for specific activity of peroxidase (Fig. 40 to 45) and the following general trend was observed. There was a decrease in activity with maturation, thus stage II cultures had lower activity of peroxidase compared to stage I cultures. The specific activity increased with further maturation and was higher in stage III cultures in BM and mMS media.

Figure 37. Protein content of extracellular protein from spent medium at different study stages



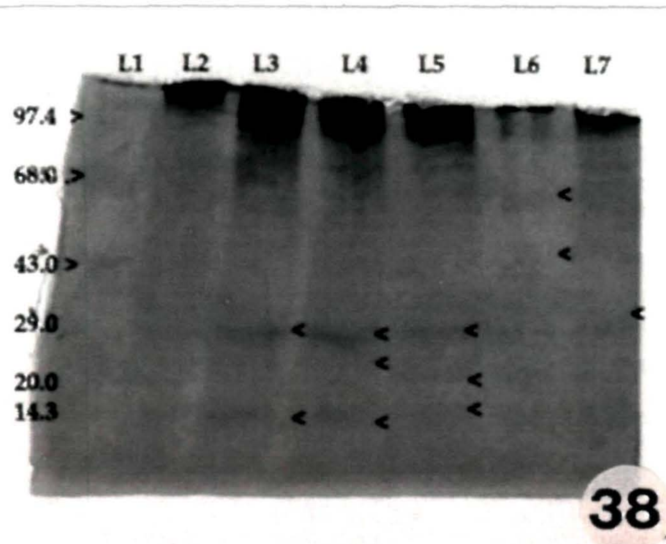
Extracellular protein content (mg/g fr.wt.)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
I
II
III
Study stages

Figure 38. SDS-PAGE protein profile in the spent medium (Pas-Silver stain for glycoproteins)

L1- Molecular weight markers
L2 -Stage I
L3 -Stage II
L4- Stage III
L5- Nonembryogenic callus

Figure 39. SDS-PAGE protein profile in the spent medium

L1- Mol wt. markers
L2- spent medium of nonembryogenic cultures
L3- spent medium of Stage III culture (after 4 subcultures)
L4- spent medium of Stage II culture
L5- spent medium of Stage III culture (after 2 subcultures)


L1
L2
L3
L4
L5
L6
L7
97.4
68.0
43.0
29.0
20.0
14.3
38

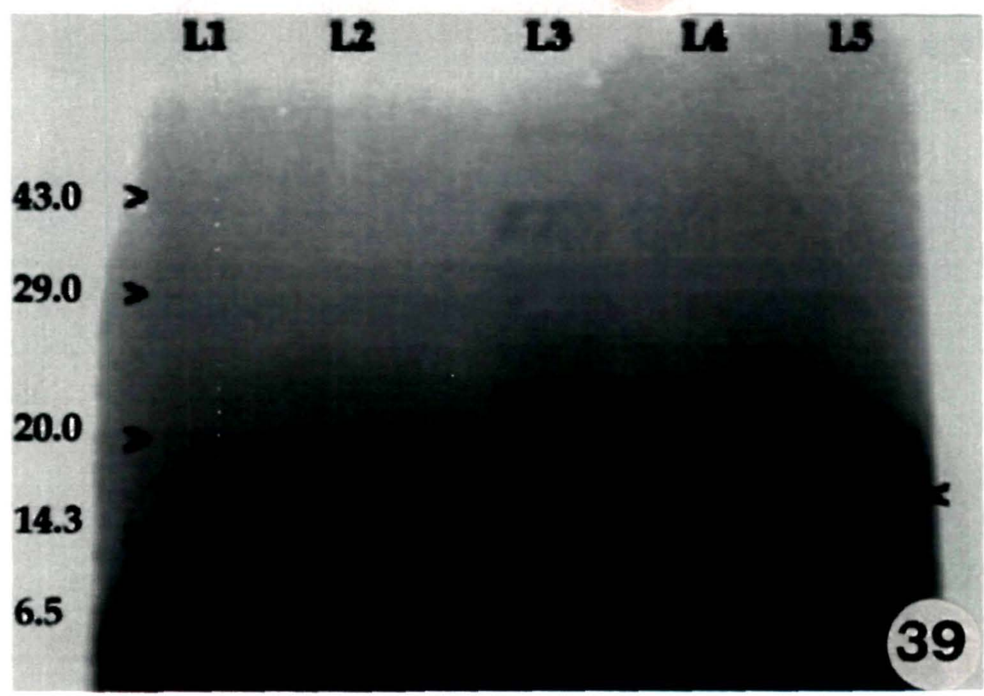
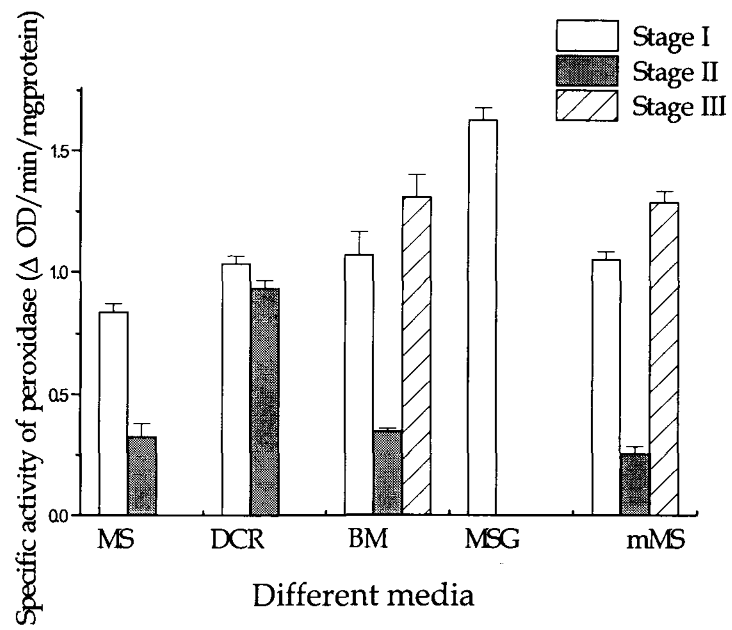
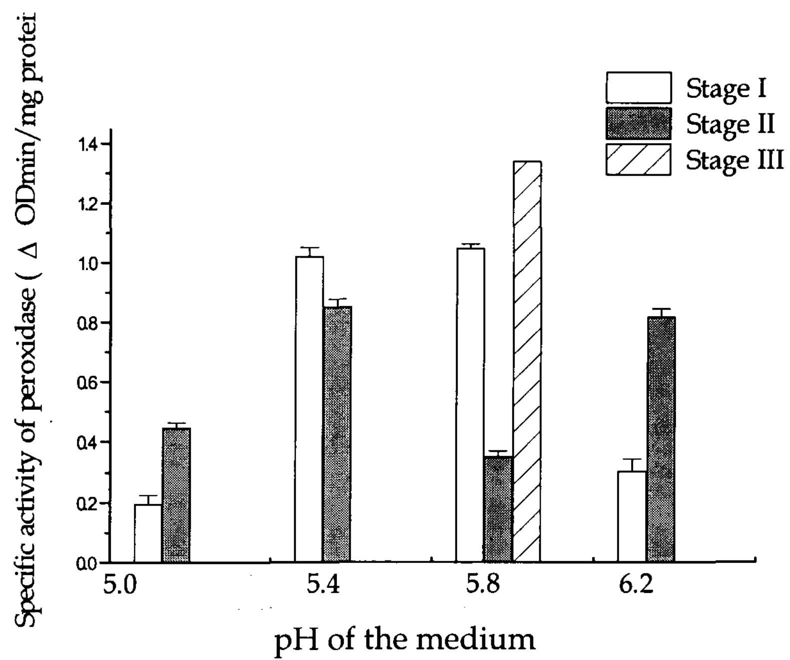

L1
L2
L3
L4
L5
43.0
29.0
20.0
14.3
6.5
39

Figure 40. Specific activity of peroxidase in cultures grown in different media

Figure 41. Specific activity of peroxidase in cultures grown in media having different pH



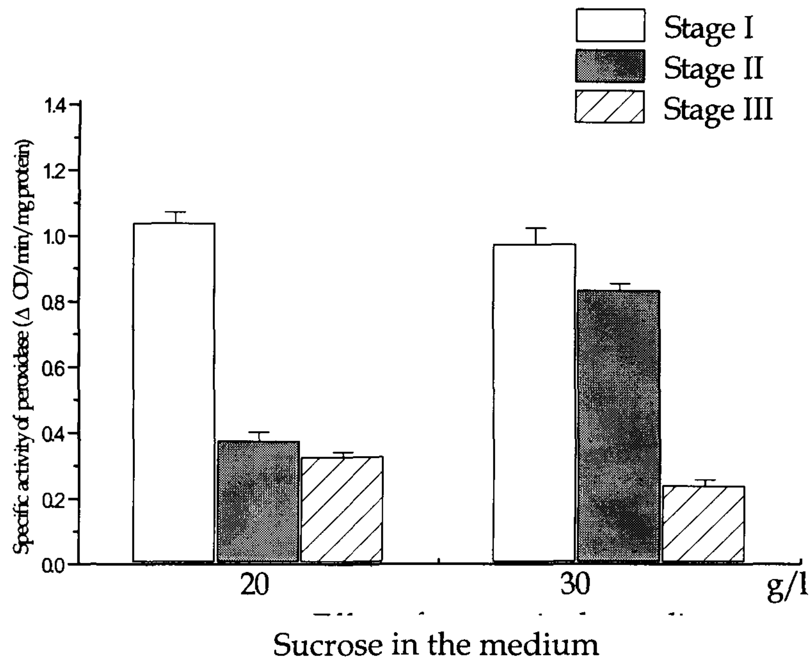
Specific activity of peroxidase (Δ OD/min/mgprotein)
0.0
0.5
1.0
1.5
Stage I
Stage II
Stage III
MS
DCR
BM
MSG
mMS
Different media



Specific activity of peroxidase (Δ ODmin/mg protei
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Stage I
Stage II
Stage III
5.0
5.4
5.8
6.2
pH of the medium

Figure 42. Specific activity of peroxidase in cultures grown in medium with varying concentrations of sucrose

Figure 43. Specific activity of peroxidase in cultures grown in medium with varying concentrations of maltose


Stage I
Stage II
Stage III
Specific activity of peroxidase (Δ OD/min/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
g/l
Sucrose in the medium

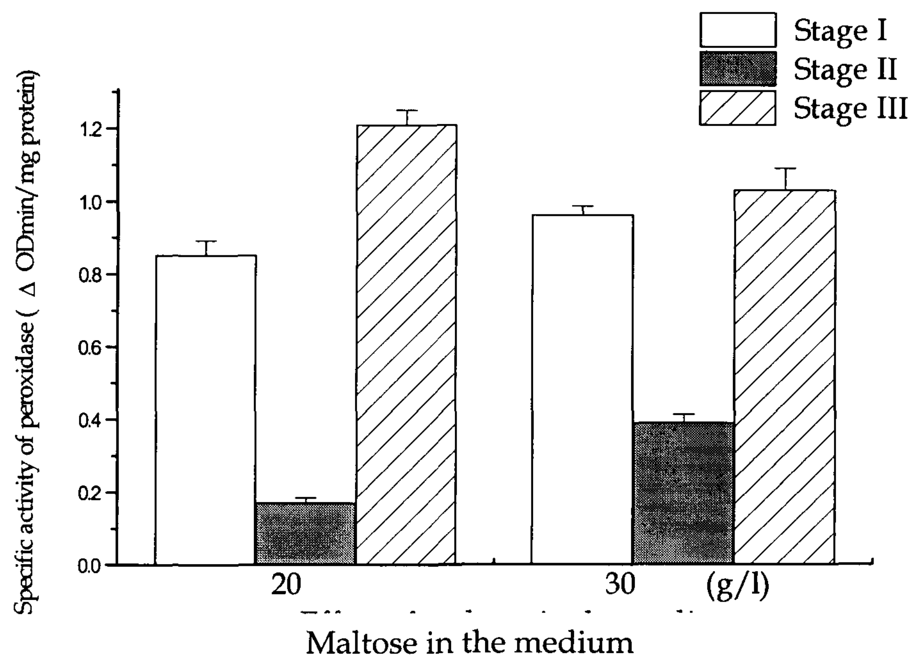
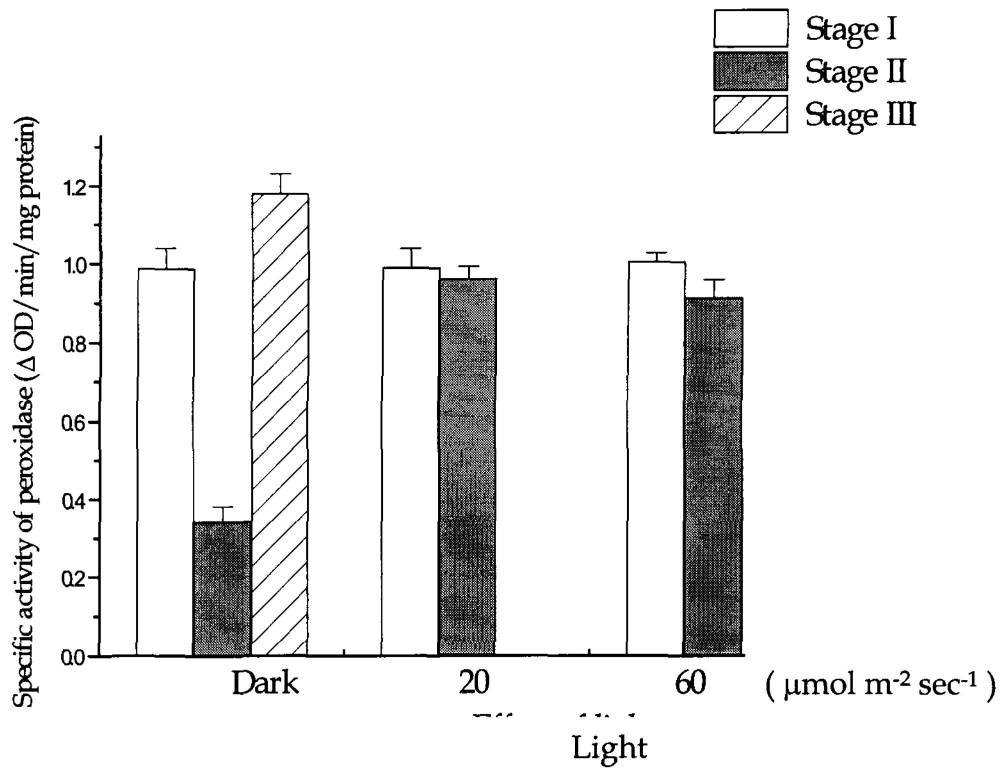
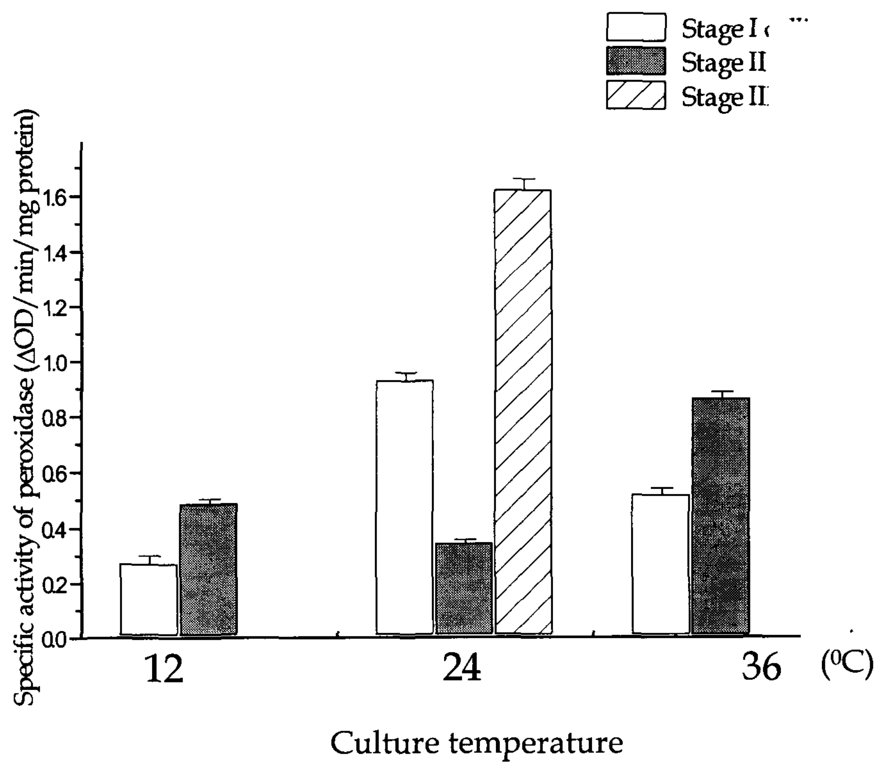

Stage I
Stage II
Stage III
Specific activity of peroxidase (Δ ODmin/mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
30
(g/l)
Maltose in the medium

Figure 44. Specific activity of peroxidase in cultures grown in the dark and in the light

Figure 45. Specific activity of peroxidase in cultures grown at different temperatures


Stage I
Stage II
Stage III
Specific activity of peroxidase (Δ OD/min/mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
20
60
(μmol m-2 sec-1)
Light


Stage I
Stage II
Stage II
Specific activity of peroxidase (ΔOD/min/mg protein)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
12
24
36
(0C)
Culture temperature

Profile study of peroxidase activity:

Profile studies of peroxidase activity in PAGE at different stages of callus showed four significant bands that appeared during stage I and remained in stage III. The observable difference was increase in the intensity of the bands with maturation of cultures and formation of ESMs (Fig. 46 and 47).

Peroxidase content in developing embryogenic cultures:

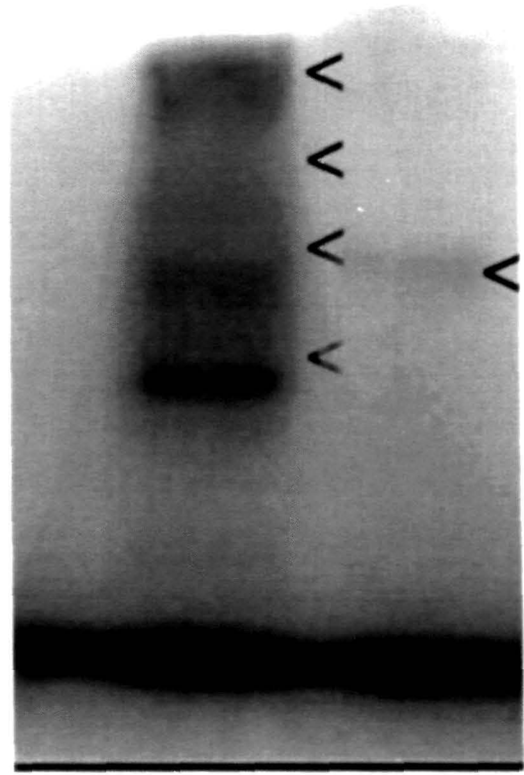
Peroxidase activity in stage I and stage II cultures were generally similar at all sampling durations. However, 3-4 fold increase in the activity was recorded in stage III cultures (Fig. 48).

Figure 46. Peroxidase activity profile of stage III callus

L1- Callus from female gametophyte
L2- Callus from mature zygotic embryo

Figure 47. Peroxidase activity profile at different stages

L1- Stage I
L2- Stage II
L3- Stage III


L1
L2
46

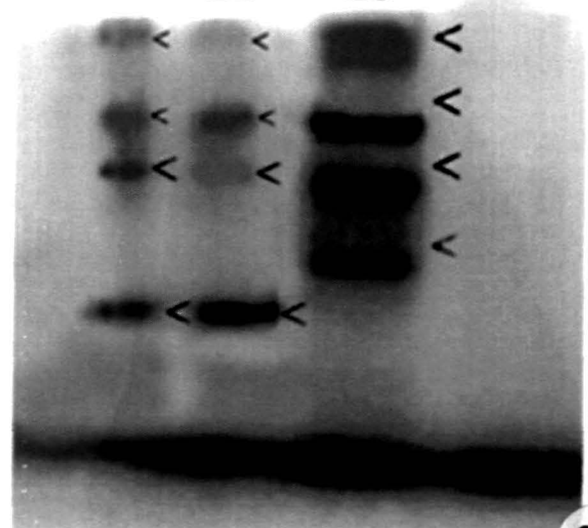
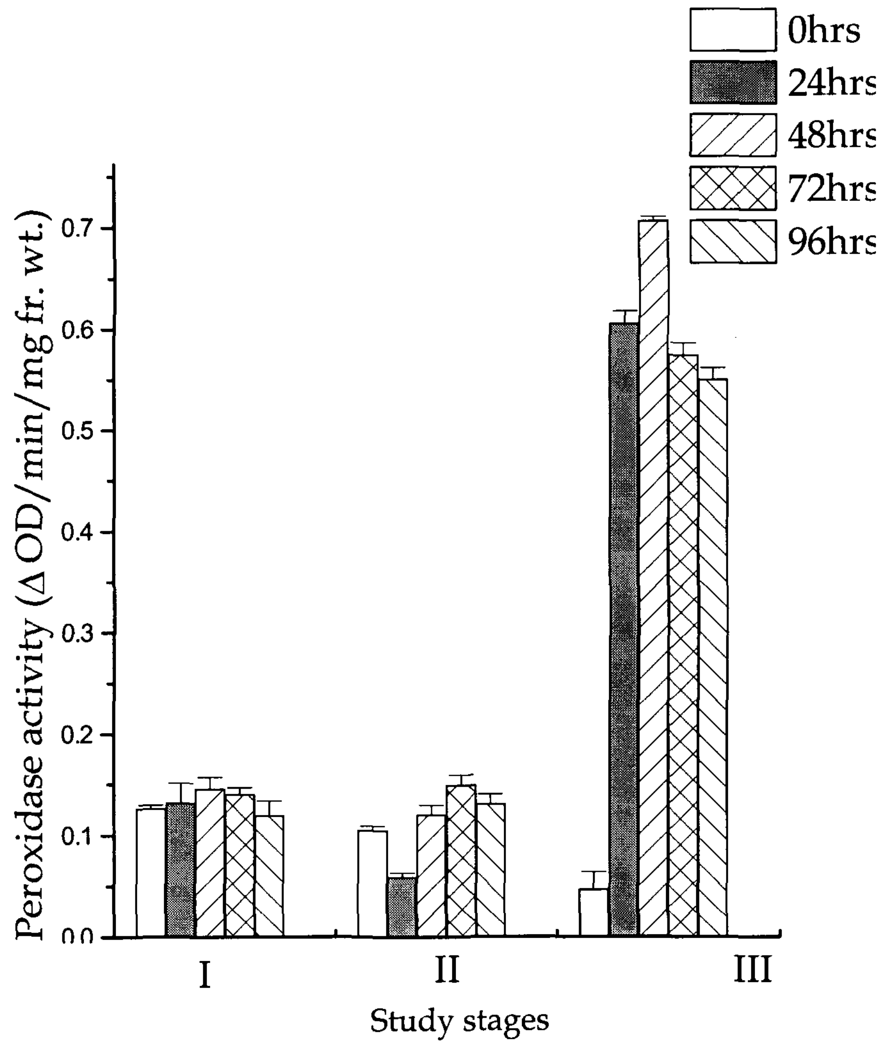

L1
L2
L3
47

Figure 48. Peroxidase activity in culture of different stages



0hrs
24hrs
48hrs
72hrs
96hrs
Peroxidase activity (Δ OD/min/mg fr. wt.)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
I
II
III
Study stages

Chapter IV

Discussion

Somatic embryogenesis in conifers was first documented from immature zygotic embryos of *Picea abies* (Hakman *et al.*, 1985; Chalupa, 1985). Since then there have been successful regeneration of conifers via somatic embryogenesis.

Polyembryogenesis being a common and natural phenomenon in conifers, most conifers undergo one of the two types of polyembryony, either simple or cleavage. In simple polyembryony, as in *Picea* and *Pinus* (Singh, 1978) proembryo is a result of fertilization of more than one egg per ovule by gametes from separate pollen grains, and therefore every proembryo is genetically different. One proembryo usually dominates and continues development while the others abort. When proembryos are cultured *in vitro* they mature into distinctive stages S1, S2 and S3. First the continued cell divisions in all planes in the head region produce club shaped S1 embryos (Fig.19). As the shoot apex starts to protrude, the head of a somatic embryo becomes cone shaped, at this intermediate stage these embryos are denoted as S2 embryos (Zhang *et al.*, 1999). With development of cotyledons, the embryos transit from early stage cotyledonary embryos to mature cotyledonary S3

embryos. These stages from I to III were obtained in the present study carried out in *Pinus kesiya* (Fig. 19).

In conifers and *Pinus,* in particular, factors limiting commercialisation include low culture survival, culture decline causing low or no embryo production and inability of somatic embryos to fully mature, resulting in low germination rates. Tautorus *et al.* (1991) indicated that 50% of the *Picea mariana* suspension cultures tested were discarded due to browning.

Gymnosperms in general show suspensor cleavage polyembryony. While many embryos develop simultaneously in an ovule by proliferation of suspensor cells it is usually the leading proembryos with suspensors which join to form the embryonal head, and which mature into early stage cotyledonary embryos.

Female gametophyte was collected 4-8 weeks after fertilization when the immature embryos were 1.0-1.11 mm in size. This stage was found to be optimum for formation of embryogenic cultures in *P. kesiya.* The developmental stage of immature zygotic embryos has proven to be a critical factor in the initiation of somatic embryogenesis in *P. sylvestris* and *P. pinaster.* The responding stage was different for both species. While initiation of embryonal masses from advanced stage of zygotic embryos have been reported in *P. strobes* (Finer *et al.,* 1989; Klimaszewska and Smith, 1997) in *P. taeda* (Becwar *et al.,* 1990) in *P. pinaster* (Bercetche and Paques, 1995) and in *P. koraiensis* from mature zygotic embryos (Bozhkov *et al.,* 1997). In pines, early

stages of development was found to respond well in *P. taeda* (Gupta and Durzan, 1987) and in *P. caribeae* (Laine and David, 1990).

For all these studies, it was observed that in pine somatic embryogenesis, the precise determination of the development stage is important for optimum response.

Stratification of seeds before culture, have been found to yield better embryogenic response. In *P. kesiya*, stratification of mature zygotic embryos at 4^0C for 12 hrs resulted in better proliferation of embryogenic callus (Table 5). Schneider and Gifford (1994) and Stous and Gifford (1997) working on loblolly pine seeds observed that stratification of seeds helped to mobilize the primary storage reserves (lipids and proteins) that are contained in the lipid bodies and protein bodies respectively. Gori (1979), and Krasowski and Owens (1993) reported that thought these reserves are present in both the embryo and female gametophyte (Ching,1966; Sasaki and Kozlowski, 1969; Kovae and Kregar, 1989; Gifford, 1998) the majority are stored in the latter. During stratification, these storage proteins get mobilized and degraded to amino acids (Durzan and Chalupa, 1968; Salmia, 1981; Lammer and Gifford, 1987; King and Gifford, 1997) and transported to the growing tissue.

Cultures in liquid medium have the added advantage of having the ability to proliferate proembryonal suspensor masses, which can be maintained for a long duration in culture. Proembryonal masses also existed in callus raised on semisolid medium; however, it was found that callus

cultures raised from mature zygotic embryos could not be maintained for long at least as observed during the present study period in *P. kesiya*. The callus raised from immature female gametophyte when eventually transferred to liquid medium, showed rapid proliferation of PEMs, and at a later stage formation of embryonal suspensor masses and eventually to embryonal heads (Fig 17).

Somatic polyembryogenesis has been described by many workers and termed the embryogenic tissues as ESMs due to their high degree of organization. The ESMs are reported to be white, transluscent in appearance when cultured on semisolid medium appear to glisten due to production of mucilage. The mucilage anatomically consists of a variable mixture of elongated cells, early stage embryos which have embryonal head and a suspensor system (Gupta and Durzan, 1987). Nonembryogenic callus appears opaque, and may not have an anatomical organization.

A possible explanation lies in the degenerating nucellar tissue present in immature female gametophyte that stimulates adjacent cells to divide and form proembryonal suspensor masses. The absence of this tissue in mature zygotic embryo cultures may possibly be the reason, why callus raised from it show lower percentage conversion to somatic embryos.

In the present study, mature zygotic embryos used as explants formed somatic embryos via embryonal suspensor mass formation following stratification for 12 hrs at $4^{0}C$ (Table 5). However, conversion to somatic

emblings was relatively less than when proliferated in cell suspensions. Immature female gametophyte raised in semisolid medium and later transferred to liquid medium formed embryogenic suspension cultures on the onset and suspension cultures showed ESM formation and embryonal heads formation eventually. Nonembryogenic suspension cultures proliferate as nonembryogenic cultures form the start if the cells lack the potential to form suspensor heads (Fig. 17 and Fig. 18).

The present study was carried out to observe the physiological and biochemical changes that were taking place in the culture right from its initiation to conversion into embryogenic masses. Cultures were grown in various media composition and culture conditions were studied in the light of to compare the different stages beginning with callus initiation till the formation of proembryonal suspensor masses and early stage somatic embryos.

The growth medium supplying the correct combination of salts is of great significance in any tissue culture medium, even more so in cultures turning embryogenic. In the present study BM medium supplemented with two synthetic auxins (2,4-D and NAA) and a cytokinin (BAP) was found most suitable for embryogenic response in culture (Tables 6, 7 and 8). While MS basal medium showed initial proliferation a low response towards proliferation and conversion to embryogenic cultures was observed (Table 8). It seems that $CaNO_3$ plays a significant role in profuse callusing and

embryogenicity. The BM medium contained $CaNO_3$ whereas it is absent in the MS medium. The presence of Ca^{2+} MS and BM media can be correlated with high proliferation of embryogenic callus (Table 7). Of all the media studied there was presence of $Mg(NO_3)_2$ in BM medium only, that supported higher percentage of embryogenic response (Table 8). Besides, the increased concentration of $CaNO_3$ in the BM medium could be related to the high percentage response in BM medium compared to other media using zygotic embryos as explant. Nitrates may also be a deciding factor in the conversion of stage I to stage II callus with conversion to early cotyledonary embryos.

Under *in vitro* conditions, the medium pH have significant influence on the potentiality of the cultures to convert to embryogenic stage. In the study carried out in *P. kesiya*, the medium pH made a significant change in the type of response of cultures to form stage III embryogenic tissue. pH 5.8 was found optimum in the present studies (Tables 9, 10 and 11). There was a low embryogenic response at lower and higher pH which is in conformity with the report of Minocha (1987) where lower pH of cell walls (about 5.5-5.8) kept the carboxyl group of the auxin less dissociated in plasma membrane than in the cytosol (where the pH is higher around 7.0). And because membranes are more permeable to noncharged solutes, undissociated auxins are believed to move from the wall into the cytosol where higher pH causes growth and differentiation of cells. Although there are many reports showing that cells in culture can significantly alter the pH of the external medium through

differential uptake of nutrients and/or through H^+ fluxes. This observation may be substantiated again with auxin mode of action and the fact that the polar transport of auxins is regulated by the pH of the cells (Minocha 1987; Smith and Krikorian, 1990). At pH 4.2, less nitrogen and phosphate were available from the MS medium than at pH 5.7. NH_4^+ was consumed preferentially to NO_3^- in all culture stages (Taber *et al.*, 1998).

The incorporation of plant growth regulators into tissue culture medium has, historically, been of special significance. Growth hormones are the natural compounds occurring in the plant systems, which in small amounts promote, inhibit or otherwise modify the physiological activities within the system. They are endogenously produced and their regulation is controlled by the physiological needs of the plant. Auxins are known to be principal agents to mediate transition from somatic to embryogenic cells (Smith and Krikorian, 1990). Auxins promote cell elongation by inducing wall acidification. In soyabean, hypocotyl sections elongation in response to a low pH only occurs for an hour or two. After this relatively short period, sections still respond to auxins for a day or two, but they respond no longer to H^+ ie., pH. Sustained growth therefore requires auxin-induced production of cell wall precursors to promote transcription of enzymes that enhance cell wall synthesis (Vanderhoef, 1980). But sustained growths of cells require much more than cell wall synthesis. It requires synthesis of plasma membrane (lipids & proteins), because that membrane grows in contact with the wall. At

the same time it requires that the turgor pressure in the cells do not decrease too much, even though the wall is loosened, because if the turgor drops excessively as the cell expands, insufficient pressure will be exerted against the walls preventing sustained growth.

It is therefore probable that this enhanced formation of plasma membrane and maintenance of turgor pressure was being kept up in the presence of two synthetic auxins, NAA and 2,4-D in *P. kesiya* (Table 26) while cultures maintained only in IAA a phytohormone, did not convert embryogenic. Turgor pressure within the cells is maintained also by enhanced solute uptake from the growth medium, and it is here that the importance of a proper concentration of mineral salts in the growth medium for auxin induced growth becomes necessary.

Application of external growth regulators is believed to effect cell polarity by interfering with the pH gradients and electrical fields around the cell (Dijak *et al.*, 1986; Smith and Krikorian, 1990). Therefore, a proper combination of auxins and cytokinins only may result in the development of somatic embryogenesis from the cultured cells, and this varies from species to species, ranging over different genus and taxa. In *P. kesiya,* the best combination was found to be 22.6 μM 2,4-D, 26.8 μM NAA and 11.1 μM BAP.

Most studies on carbohydrate utilization *in vitro* have indicated that sucrose is the best carbon source for optimal growth. However, depending on the genotype, too, explants selectively take up sucrose or maltose, i.e.,

respond differentially to carbohydrate sources (Tremblay and Tremblay, 1991). Low percentage of sucrose (1-2%) resulted in more ESMs formation (von Arnold, 1987; Becwar *et al.*, 1988a, b). In the present study in *P. kesiya*, callus from mature zygotic embryos showed better response in maltose supplemented medium (Table 17) compared to sucrose (Table 14). Maltose is reported to be broken down more slowly than sucrose, providing a metabolizable carbon source over a longer period of culture (Orshinsky *et al.*, 1990). In general, sucrose in a medium can serve as an osmotic agent and also as a carbon and energy source. Many cultures, especially embryo and some shoot cultures, require a medium with high osmotic potential (Tremblay & Tremblay 1995). Large and available carbon amounts are necessary for structural growth, differentiation and development.

The effect of temperature too can be explained in relation to auxin action and it is believed that the flux or quantity of auxin transported per unit time increases with temperature. This can be correlated with our study on incubating cultures under different temperature conditions. It was found that callusing was inhibited at lower (4-12^{0}C) and also at a higher range of 36^{0}C while cultures kept between 22-24^{0}C showed profuse callusing and conversion.

The effect of light in the induction of somatic embryogenesis is not very clear. Cell polarity and asymmetric cell division are involved in the initiation of somatic embryogenesis. In alfalfa, stimulations by auxins

promote asymmetric cell divisions to form embryogenic protoplasts (Bogae *et al.*, 1990; Dudits *et al.*, 1991), while protoplasts with nonembryogenic lines divide symmetrically. The first asymmetric cell division is generally initiated by a gradient of light, the plane of division always being perpendicular to the light axis. In zygote and female gametophyte (immature fertilized organs that are protected from light, the first division plane is predetermined. Abscisic acid production peaked at the heart stage of embryogenesis and synthesis was most pronounced in the dark (Michler and Lineberger, 1987). Since in the present study on *P. kesiya,* callus initiation and proliferation occurred in the dark, while light turned the explants green, light may not be a decisive factor in somatic embryogenesis.

Effect of light could be related to cytokinin activity from the observation in oats leaf culture (Thinmann, 1980; Thinmann *et al.*, 1982) that cytokinins delay senescence by keeping the stomata open and allowing CO_2 to enter the cell. The CO_2 is believed to inhibit competitively the strongly promotive action of ethylene on senescence. Interestingly, seeds are the highest producers of ethylene, and auxins are present in high amounts in seeds, and therefore, it is believed that auxins greatly stimulate ethylene production. In dark culture conditions and in the presence of cytokinin (BAP) the activity of the CO_2 is probably diverted towards competitively controlling ethylene levels and this could probably be related to the present aspect of study in some way.

Ethylene in many reports has been found to be associated with induction of somatic embryogenesis even in many conifers. Ethylene in the ambient microenvironment was observed to be lower in the embryogenic culture environment compared to nonembryogenic tissues of *Picea abies* (Wann *et al.*, 1987). Silver nitrate in the culture medium is known to be a potent ethylene action inhibitor. In conflicting reports, $AgNO_3$ was found to stimulate induction of somatic embryogenesis in *Pinus taeda* (Li and Huang, 1996), but inhibited somatic embryo induction and/or maturation in *P. pungens* (Afele *et al.*, 1992) and *P. pinaster* (Bercetche and Paques, 1995) suggesting that ethylene may be beneficial for somatic embryogenesis in these species. In *P. glauca* ethylene inhibited growth of embryogenic tissue (Kumar *et al.*, 1992).

In the present work, the influence and effect of ethylene on induction of somatic embryogenesis was carried out by incorporating silver nitrate into the medium. The idea was to find out if at reduced level of ethylene in the ambient microenvironment of the cultures could indeed enhance somatic embryogenic cell formation, silver nitrate being a potent ethylene inhibitor. It was found that there was inhibition of embryogenic callus initiation in lower concentration of $AgNO_3$ while in higher concentrations, there was no callus formation at all (Table 25). However, study of the effect of ethylene on embryogenic system does not end here. More work needs to be done in this aspect which could not be covered here. For now, on the basis of the study

carried out it can only be assumed that it is possible that in case of *Pinus kesiya* presence of ethylene may enhance induction and maturation of embryogenic culture in the medium. Another possibility of artificially increasing the ambient ethylene level in the culture microenvironment may speed up the physiological process.

Application of increased concentrations of Ca^{2+} in the maturation medium increased the number of embryogenic cell lines (Table 29 and 34). BM and 1/2 Litvay's medium contain, $CaNO_3$ and $CaCl_2$ respectively. An increase or decrease in their concentrations reduced the number of cell cultures that converted to ESMs. Insoluble Ca^{2+} salts may play an important role as pools of Ca^{2+} and buffering pH, callose formation or cell wall stabilization (Pedroso and Pais, 1995). Without organic nitrogen (glutamine and casein hydrolysate) i.e., with only inorganic nitrogen in the medium, the fresh weight increase of tissue masses was significantly less than with organic nitrogen in both initiation and maturation medium. No differences were found between the dry/fresh weight ratios obtained with the various nitrogen treatments (Barret *et al.*, 1997).

Macromolecule accumulation and synthesis (proteins, polysaccharides and nucleic acids) are indicators of cell growth and physiological state that have proved to be useful in characterization of embryogenic cultures in conifers. Intracellular protein content has been positively correlated with changes in growth of embryogenic cultures in white spruce *P. glauca* (Donga

and Dunstan, 1994). There are several studies on changes in protein content and protein pattern during somatic embryogenesis in conifers.

In *P. kesiya* the protein profile study revealed 98, 65, 42 and 25 kDa doublets under nonreduced conditions, and 65, 63, 35, 30, 23 and 22 kDa proteins under reduced conditions (Fig. 26). In all these studies, the vast majority of the resolved proteins remain constant between unorganised cell clusters of the stages I and II differentiated when somatic embryos (or proembryonal masses) were formed (Fig. 29). Roberts *et al.* (1989) and Flinn *et al.* (1991) reported that there was no significant changes in the protein pattern in the early stages of induction of somatic embryogenesis in *P. glauca.* Storage protein accumulation took place during the later stages of maturation following the onset of cotyledonary embryo development i.e., stage III in the present studies. A 55-57 kDa doublet was observed under nonreduced conditions while reduced extract showed 41, 35, 33, 30, 27.5, 24 and 22 kDa proteins in stage III calli. It is believed that somatic embryos closely resembling zygotic embryos not only morphologically but biochemically would display more vigorous growth.

In the present study, SDS-PAGE protein profile revealed that at stage I, the proteins were the same as compared to that in mature zygotic embryos (Fig. 27). Major proteins with apparent molecular weights of 66, 65, 63, 45, 32, 30, 28 kDa, and minor proteins at 37 and 35 kDa were observed.

Comparison of protein profile of calli at different stages of somatic embryogenesis from stage I undifferentiated calli to stage III calli having proembryonal masses showed major proteins of 45, 43-41, 32-35, 30, 28, 25, 18 and 15 kDa molecular weights. These proteins remained constant between the stages, but intensity of the bands increased with age of calli (Fig. 28).

It has been reported that there was an appearance of a 41 kDa protein during the later stages of somatic embryogenesis in interior spruce (Flinn *et al.*, 1991). In somatic embryos this 41 kDa protein showed an initial rapid accumulation that continued to accumulate during the entire cotyledon/embryo maturation and differentiation period. When compared, in zygotic embryos, this 41 kDa protein initially accumulated more rapidly over a 3 week period and thereafter the protein levels appeared to remain relatively constant or increase only slightly.

Comparison of protein patterns between early and mature somatic embryos with initial zygotic embryo explants showed differences as well as similiarities as reported in interior spruce (Flinn *et al.*, 1991) and in silver fir (Kormut *et al.*, 2003). In silver fir, compared to zygotic embryos, the protein pattern of somatic embryos involved additional protein fractions of 36, 35, 32, 31 and 19 kDa size. As reported, in contrast to zygotic embryos, a 14 kDa fraction was rather faint in somatic embryos, while the main difference was a major protein 43 kDa size distinct in cotyledonary stage of somatic embryos, which was absent in dessicated mature zygotic embryos.

In the present study, three phases in the growth cycle were observed during the study period, an initial steady phase (stage I) following the second subculture in initiation medium, followed by a rapid linear increase during the next two subcultures (stage II calli). This was followed by a declining phase (stage III) when the protein content went low. Similar pattern of changes in intracellular protein content has been shown as a positive correlation with fresh mass accumulation of total DNA content and embryo number in *P. glauca* (Dong and Dunstan, 1994). While the first two phases could be correlated with proliferation, comprising elongated suspensor vacuolated cells undergoing cleavage polyembryony. This involves asymmetric rapid cell divisions in single cells and within cell clusters (Tautorus *et al.*, 1991) resulting in repetitive somatic embryo production. Several possible explanations for this have been proposed, like nutrient mostly carbohydrate depletion, which has been found to coincide with maximum cell mass followed by browning of cultures (Fowler *et al.*, 1982; Lulsdorf *et al.*, 1992).

Several studies for the presence of proteins in the growth medium (extracellular proteins) have been carried out to reveal their function in somatic embryogenesis (Egertsdotter *et al.*, 1993; Dong and Dunstan, 1994). In the present study, carried out, the protein content in the spent medium showed a linear increase with maturation of cultures (Fig. 37). Initiation of somatic embryogenesis can be related with a marked change in the pattern of

secreted proteins (deVries *et al.*, 1988). There have been aberrant protein patterns in suspension cultures that have lost embryogenic potential and in nonembryogenic mutant cell cultures. Somatic embryogenesis could be restored in some of the cell lines by the addition of wild type extracellular proteins, thus indicating that one or several extracellular proteins are involved in somatic embryogenesis.

When mature zygotic embryo explants of *P. kesiya* were raised in semisolid medium, followed by callus initiation and proliferation in semisolid medium, then transferred to liquid medium from stage II calli onwards till the end of the study period. SDS-PAGE profile showed a gradual accumulation of some low molecular weight proteins in the spent medium of 35, 32, 20, 15, 13 kDa size. Spent medium from stage II cultures showed a faint protein band of 15 kDa size while following 2 more subcultures on the maturation medium, there were 15 and 13 kDa protein size. Stage III cultures on spent medium showed intense protein bands of 20, 15 and 13 kDa size (Fig. 39).

Secretion of proteins into the growth medium do not appear to result from leakage or lysis of cells *in vitro* as observed in *P. abies* embryogenic suspension cultures, and differed in those cultures from which cotyledonary somatic embryos could be matured and those not capable of maturation (Egertsdotter *et al.*, 1993). There is increasing evidence to indicate that this is regulated by genes reflective of normal requirements for embryo development.

A number of gaseous or volatile compounds have been found to be accumulated in the culture flasks (Thomas and Murashige, 1979; Kumar *et al.*, 1989), some of these compounds have been shown to affect growth and differentiation of cells *in vitro* including ethylene, CO_2 (Kumar *et al.*, 1989), oxygen (Kessel and Karr, 1972) and ethanol (Perrata *et al.*, 1986). Such compounds might inhibit culture growth rate, affect cell structure or accelerate degradation or lysis of living cells or tissues and the decline phase observed could be a reflection of cell lysis into the suspension medium. This was also reflected in the protein content in the nonembryogenic calli of *P. kesiya* which was comparatively much less and showed a decline as the calli matured (Fig. 36).

Peroxidases take part in processes occurring inside and outside the cell. Their activity is regulated developmentally by changes in the level of Ca^{2+} ions (Pullman *et al.*, 2003). Plant tissue necrosis and subsequent cell death are usually observed during *in vitro* regeneration in conifers, especially in plant regeneration via somatic organogenesis in pine species. Cell death is correlated with the elevated levels of peroxides. The effects of antioxidants on *in vitro* regeneration of Virginia pine (*Pinus virginiana* Mill) showed that antioxidants, PVP and DTT were found to improve callus formation. In this case higher peroxidase activity of tissue cultures during subculture from callus proliferation medium to shoot differentiation medium and to rooting

medium was observed. The addition of antioxidants reduces and inhibits browning by reducing the accumulation of peroxidase (Pullman *et al.*, 2004).

The peroxidase activity and protein content could be used as useful markers to identify the onset of embryogenesis (Jain *et al.*, 1990). Zhou *et al.*, (1992) also reported that the peroxidase activity decreased prior to visual manifestation of embryoids and also synthesized several isoperoxidases. This phenomenon was not observed in the nonembryogenic cultures. However, in the present study, an increase in peroxidase activity could be related to the development and maturation of cultures following organogenesis. This was indicated by the increase in activity of peroxidase and protein content in the intracellular tissues as well as in the spent medium in embryogenic cultures.

In the present studies, it was observed that the changes in the protein levels as well as changes in their profile could be used as parameter to recognize cultures with embryogenic potential. Peroxidase activity also indicated the embryogenic potential in the cultures.

Chapter V

Summary

Somatic embryogenesis is the process of embryo formation from somatic cells to give rise to whole plants without the fusion of gametes. Somatic embryogenesis may be *direct*, when the embryo develops directly from the somatic or vegetative cells of the explant without the intervening callus phase. It may be *indirect*, when the embryos develop from an undifferentiated mass of cells. Embryogenesis can be induced very easily in some cells like cells of mature zygotic embryos and immature zygotic embryos from female gametophyte. These are called *competent* or *pre embryonic determined* cells. There are two kinds of growth in tissue culture, *unorganised growth* (forming undifferentiated tissues) and *organised growth* (charactarised by development into embryos) are mediated by the exposure of responsive explants to cultural conditions. There are three different methods for initiation of embryogenic cultures in conifers, through the continuation of natural cleavage polyembryony of embryonal heads of explanted immature embryos, through cell division in the epidermal and subepidermal layers of hypocotyl, cotyledons or needles resulting in calli which then rapidly organise to form embryonal suspensor masses (ESMs) and through cell division of small cells within the suspensor system of explanted immature embryo.

Of the conifers, pines constitute the most divergent and economically important group of species. They provide valuable natural resources and though they are known to be pioneer species and active colonizers of degraded sites, their population in the recent years have been fast depleting. Six pine species are indigenous to India and are found scattered in the Himalayan region from Jammu to the North-east. These species are *P. roxburghii* Sarg. (syn. *P. longifolia* Roxb., *P. serenagensis* Maddeu, chirpine), *P. wallichiana* A.B. Jacks (syn. *P. excelsa*, Blue pine, Kail), *P. gerardiana* Wall. Ex Lamb. (chilgoza pine, Neoza pine), *P. insularis* Endl. (sys. *P. kesiya* Royle ex Gord., khasi pine), *P. merkusii* Jungh (Merkus pine) and *P. armandii* Franchlet (Armandi's pine). Of these *P. kesiya* is the most widespread in the North-eastern states of India. *P. kesiya* (Royle ex. Gord.) commonly known as the khasi pine is an economically important timber-yielding tree that grows mostly on the hills at an elevation of 750m to 2000m thriving best at 1350-1500m in sub-temperate and fairly moist regions. In Meghalaya, *P. kesiya* is confined to higher reaches (800m to 2000m above sea level) of the Shillong plateau in Khasi and Jaintia hills, in a narrow belt running in east-west direction. Age old practice of shifting cultivation and other anthropogenic activities such as cutting trees for timber and construction of building, collection of fuelwood during the past several decades have destroyed the climax subtropical broadleafed forests at higher elevations in Meghalaya and

elsewhere in the North-east India paving the way for invasion and successful growth of *P. kesiya.*

Cleavage polyembryony is common in pines, where the cleavage embryos result from a separation of the apical tier cells of an individual proembryo into four files of cells, each of which may develop into a separate embryo. The resulting embryos are therefore genetically identical. One of these embryos becomes dominant and the other embryos cease development. Factors such as mechanical, nutritional and growth inhibiting influences of the micro environment of the embryo play a major role in determining somatic embryogenesis in *in vitro* cultures. In culture, explants are induced to form callus that becomes embryogenic that is generally glossy, translucent, white, mucilaginous cellular mass. The callus further grows to form ESMs that anatomically consist of a variable mixture of elongated cells, early stage embryos, which have elongated densely cytoplasmic clumped cells with embryonal head and a suspensor system and sometimes later stage embryos. Somatic embryogenesis or organogenesis of tissues in *in vitro* cultures are influenced by various factors like light, temperature, relative humidity, besides composition of the culture media and pH.

Stratification of seeds of *P. kesiya* for 12 hrs at 4^0C resulted in cultures with proembryonal masses (PEMs) and ESMs (32.66%). When seeds were stratified for 12 hrs at higher temperatures, there was rapid browning of calli followed by degeneration over subsequent subcultures, while seeds stratified

for longer period 24, 48, 72 hrs at lower temperatures of 4 and 8^{0}C also showed browning and degeneration.

Stage I callus formed was soft and transluscent and was harvested following first subculture in the initiation medium. Rapidly proliferating friable callus following four to five subcultures on initiation medium contained a mixture of dense cytoplasmic cells and elongated vacuolated cells. This was termed the Stage II callus. The cultures containing more PEMs and early stage cotyledonary embryos following 2-4 subcultures in maturation medium were the stage III cultures in the present study.

Mature zygotic embryos when inoculated onto different basal media responded differently. There was early callus initiation in BM medium with rapid proliferation following the first subculture. Optimum embryogenic callus (34.16%) was recorded in BM medium which formed PEMs later in the maturation medium.

Cultures raised in the medium having pH 5.8 resulted in soft, transluscent, fast growing stage I and stage II cultures that maintained higher embryogenecity to form PEMs. Higher and lower pH of the medium was detrimental in embryogenic response.

Concentrations of sucrose at 20 and 30 gl^{-1} resulted in an early initiation of stage I callus at 34.23 and 36.1% respectively. Subsequent subcultures in initiation medium showed rapid callus proliferation to form friable and healthy stage II callus. In the absence of sucrose in the medium,

the explants turned brown with no callus formation. Stage III cultures were formed in medium supplemented with 30 gl^{-1} sucrose. These cultures could not be sustained during subsequent subcultures reducing the percentage of ESMs formation. Most of the proliferating callus in sucrose containing medium turned brown, eventually converting the small percentage of embryogenic cultures to non-embryogenic.

In the medium supplemented with maltose, initiation of stage I callus was slow compared to that in the medium containing sucrose. At 20 and 30 gl^{-1} of maltose percentage initiation of stage I calli was 19.72 and 22.10% respectively. Cultures on medium with 30 gl^{-1} maltose on subsequent subcultures showed rapid proliferation of stage II calli (32.2%) with no browning. There was formation of Stage III cultures (32.93%) having both PEMs and ESMs in the medium containing 30 gl^{-1} of maltose.

When plant growth regulators (PGR) were added to the medium in the combination of 22.6 µM 2,4-D, 26.8 µM NAA and 11.1 µM BAP, the stage I callus proliferated to stage II friable embryogenic callus with PEMs. At lower concentrations of auxins the callus was soft but turned brown while at higher concentrations of both auxin and cytokinin the callus turned hard and degenerated.

It was observed that explants formed stage I callus (24.19%) was formed in the dark while presence of varying light intensities showed lower stage I callus initiation. Light was found inhibitory to callus initiation and

proliferation. The stage I callus initiated in light at 20 µmol m^{-2} sec^{-1} resulted into nonembryogenic calli later. At 60 µmol m^{-2} sec^{-1} explants turned green. In the dark there was rapid stage II callus proliferation that converted to stage III embryogenic calli with PEMs. However, nonembryogenic cultures were also recorded.

Optimum response was obtained in cultures grown at 24^{0}C exhibiting stages I to III. While there was no response at lower temperatures of 4 and 8^{0}C, stage I callus initiated at 36^{0}C shriveled up and turned non-embryogenic.

Incorporation of $AgNO_3$ in the semi-solid medium had an inhibitory effect on callus proliferation. $AgNO_3$ in the medium induced oxidative browning of the culture medium that in turn caused browning of the cultures. Higher concentrations of $AgNO_3$ reduced the level of proliferation of stage I callus and formed clumps thus inhibiting formation of PEMs. Lower concentrations also were inhibitory for callus proliferation. Incorporation of $AgNO_3$ was not effective in proliferation and formation of PEMs. At 1/10 concentration of PGR, the cultures showed formation of PEMs. There was browning of cultures and gradual degeneration in 1/2, 1/5 and absence of PGR.

The maintanance medium contained 1/10 PGR supplemented with 30 gl^{-1} maltose. Incorporation of maltose in the medium showed less browning in the cultures compared to sucrose. 20 gl^{-1} maltose in the medium resulted

in formation of stage III callus with PEMs, while with 30 gl^{-1} a higher response of 9% was recorded. Conversion to PEMs and eventually into cultures having early stage cotyledonary embryos was higher when subcultured in medium with 30 gl^{-1} which was therefore considered the optimum concentration.

The medium with 1/10 PGR, 30 µM abscisic acid (ABA) and without polyethylene glycol (PEG) was most suitable in the maturation of stage III suspension cultures (6.73%). Presence of PEG at higher concentrations in the medium singly or in combination with ABA converted the embryogenic cultures to nonembryogenic where the dissociated cell mass clumped and turned brown and hard. Absence of ABA in the medium induced the formation of nonembryogenic cultures. Medium supplemented with full concentration of PGR without ABA and PEG resulted in reduced embryogenic response (0.87%) in the cultures.

Higher and lower concentrations of $CaNO_3$ in BM medium and $CaCl_2$ in 1/2 Litvay's medium was found to be inhibiting the number of suspension cultures that retained embryogenic cell masses during subcultures in suspension medium. The normal concentrations of Ca^{2+} of the respective media were optimum for formation of ESMs.

The cultures were established from female gametophyte on 1/2 Litvay's semi solid medium having 30 gl^{-1} sucrose and 22.6µM 2,4-D, 13.4µM NAA and 11.1µM BAP with 1 gl^{-1} mesoinositol and 0.2 gl^{-1} PVP. Stage I and

stage II calli thus formed were used for further studies. Further subcultures on the semi-solid maintenance medium, formed callus with PEMs and early stage cotyledonary embryos in suspension cultures supplemented with 1/10 PGR, 30gl^{-1} sucrose and 30 μM ABA.

There is a similarity in the developmental stages in zygotic and somatic embryogenesis. There was callus initiation from micropylar end of mature zygotic embryos within 10 days of culture forming stage I callus. From female gametophytes, there was extrusion from micropylar end within 12 days of culture in the initiation medium. The callus was transluscent to white. Proliferating callus was considered established when they were 1-1.5 cm in diameter. In *Pinus,* the proliferating callus generally comprises of suspensor cells with cells having cone shaped heads. Early stage somatic embryos (Stage III in the present study) separated out from the embryogenic callus showing embryogenic heads.

Estimation of intracellular proteins in cultures that turned embryogenic in BM medium showed an increase in the protein content from 0.3 mg g^{-1} fresh weight in stage I to 2.1 mg g^{-1} fresh weight in stage II. The protein content decreased to 0.62 mg g^{-1} fresh weight in stage III callus. There was increase in protein content from stage I to II and then a decline in all the cultures excepts the ones grown in MS, DCR and MSG medium where there was no stage III formation. In cultures that turned nonembryogenic the stage

I protein content saw a sharp increase at stage II which however could not be sustained and the cultures degenerated.

SDS-PAGE protein profile of intracellular proteins revealed 65, 63, 35, 30, 23 and 22 kDa proteins under reduced conditions (+ β-mercaptoethanol) and 98, 65, 42 and 25 kDa doublets under nonreduced (- β- mercaptoethanol) extraction conditions. Protein profile also revealed protein bands of callus obtained from mature zygotic embryo and female gametophyte were similar to mature zygotic embryos. Major proteins with apparent molecular weights of 66, 65, 63, 45, 32, 30, 28 kDa, and minor proteins at 37 and 35 kDa were observed.

In this study, the majority of the resolved proteins remained constant between unorganised cell clusters (Stage I and Stage II) but could be differentiated with zygotic embryo by the absence of two bands between 40-42 and one band between 30-32 kDa present in the zygotic embryo and stage I callus from female gametophyte but absent in stage I callus from mature zygotic embryo. Comparison of protein profile of callus at different stages of somatic embryogenesis from undifferentiated callus (stage I) to callus cultures having PEMs (stage II) showed major proteins of 45, 43-41, 32-35, 30, 28, 25, 18 and 15 kDa molecular weights. These proteins remained constant between the stages. However, intensity of the bands increased with age of callus.

When the intracellular protein patterns of initial zygotic embryo explants was compared with the later stage embryogenic calli, major difference was in the presence of 22-25, and 25-27 kDa major protein present in both zygotic embryo and cotyledonary embryos (in maturation medium, minus plant growth regulators). Besides, two very low molecular weight proteins with low intensity bands were observed in stage III callus having bands of 12-13, and 7-9 kDa size. Also a 15-17 kDa band was observed in the Stage III callus after 2 subcultures which disappeared when subcultured into medium devoid of PGR.

When gels were stained for glycoproteins some low molecular weight bands appeared in stages II and III with high molecular weight bands in stage I. There were no bands seen in extract of nonembryogenic cultures perhaps due to their browning and degeneration. Low molecular weight bands of 25, 18, 13 and 12 kDa were observed in stage III cultures after 4 subcultures in the maturation medium. Quantification of intracellular proteins at different stages of culture showed an increase in protein content from stage I to III. However, on formation of cotyledonary somatic embryos (stage III) there was a decrease in the protein content. A similar trend was observed for nonembryogenic cultures but the content of protein was markedly lower.

Three phases in the growth cycle were observed during the study period, an initial steady phase at stage I, followed by a rapid linear increase

of protein contents during the stage II. This was followed by a declining phase at stage III. Following two subcultures on PGR free medium there was an increase in the protein content which could be related to increasing embryogenesis.

The SDS-PAGE profile of extracellular proteins in the spent medium revealed a gradual accumulation of some low molecular weight proteins in the spent medium (cultures from female gametophytes), of 35, 32, 20, 15, 13 kDa size. Spent medium from stage I cultures showed a faint protein band of 15 kDa size while medium collected from cultures following 2 subcultures on maintenance medium showed 15 and 13 kDa protein size. Proteins obtained from spent medium of cultures following 4 subcultures in maintenance medium showed intense bands of 20, 15, and 13 kDa size. Staining for glycoprotein too revealed low molecular weight protein bands in the profile.

Callus raised under different treatments were tested for specific activity of peroxidase and the following general trend was observed. There was a decrease in activity with maturation, thus stage II cultures had lower activity of peroxidase compared to stage I cultures. The specific activity increased with further maturation and was higher in stage III cultures grown in BM and mMS media.

Profile study of peroxidase activity with PAGE on different stages of callus showed four significant bands that appeared during stage I and remained in stage III. The observable difference was increase in intensity of

the bands with maturation of cultures and formation of ESMs. Peroxidase content in developing embryogenic cultures in stage I and stage II cultures were generally similar at all sampling durations. However, 3-4 fold increase in the activity was recorded in stage III cultures.

These studies were significant to analyse the embryogenic potential of cultures before any morphological changes were observed. This could be used for culture assessment in regeneration studies in *P. kesiya*.

Chapter VI

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Curriculum Vitae

a)	NAME (in full)	: Sanghamitra Purkayastha
b)	DATE OF BIRTH	:15th March,1973
c)	NATIONALITY	: Indian
	CATEGORY	: General (female)
d)	FATHER'S NAME	: Sunirmal Purkayastha (Retd.CentralGovt.)
e)	MOTHER TONGUE	: Bengali
f)	HUSBAND	:Dr. Nirman Chatterjee M.B.B.S. DNB. ACLS Provider
g)	ADDRESS (for correspondence)	: C/O Mr. N.C. Chatterjee 57/1 Dr. A. K. Paul Road, Behala Kolkata, 700034 W.B. (033) 24687909
h)	Phone No. (for communication)	: (0364) 2534143, 9863097377 E.mail : s_p_c15@rediffmail.com s_p_c15@yahoo.com

g) **EDUCATIONAL QUALIFICATION :**

EXAM PASSED	YEAR OF PASSING	UNIV/ BOARD	CLASS/ %	SUBJECTS
I.C.S.E.	1989	C.I.S.C.E N.Delhi	1ST CLASS/ 72.0	Eng, Beng,Hist, Civics, Geog, Phy, Chem,Maths,Bio, H.Sc, & S.U.P.W.
PRE-UNIV	1991	NORTH EASTERN HILL UNIV. Shillong	1ST CLASS/ 71.8	Eng, Phy, Chem, Bio, & Maths,
B.Sc. (hons). in Botany)	1994	-do-	1ST CLASS/ 67.9	Eng, Bot, Zoo, Chem & GFC
M.Sc. (in Botany)	1996	-do-	1ST CLASS/ 69.83	Botany with Speciali- -zation in Pl. Biotechnology

Ph.D. Currently underway, soon to submit thesis –On "Developmental Physiology and Biochemistry of Somatic Embryogenesis of *Pinus kesiya* Royle ex Gord."

h) WORK EXPERIENCE :

NAME OF EMPLOYER	POST HELD	PERIOD OF SERVICE	NATURE OF SERVICE
1) DBT sponsored- "Development of an Embryogenic System for Genetic Improvement And Mass Propagation of *Pinus kesiya* Royle ex Gord.	**JRF**	1st Nov'97 to 30th April'98	Assisting the RA in the work referred, and preparing a work plan and working schedule.
2) NATP Sponsored,"Mission Modeproject for Increasing Food security and Life Support System in Tribal, Backward & Hilly areas."	**R.A**	Feb'2001-May'2002	Establishing Seed bank for microtuber production & propag- -ation in potato.
3) St. Anthony's College, Dept. of Botany, Shillong, as	**Lecturer**	15th May'02 -10th Dec'04	Full time lecturer- against lien post,

i) SCHOLARSHIPS/ FELLOWSHIPS AWARDED:

1. NEHU Merit Scholarship 1994- 1996.
2. Junior Research Fellowship from DBT (1st Nov'97 to 30th April'98).
3. NEC Fellowship for the period April 1998 to March 2000.

j) **College Award** (Lady Keane College) for 2nd position in B.Sc. Degree Hons.(Botany), for the year 1994.

k) RESEARCH EXPERIENCE :

- Seven years of research experience in the field of Plant Biotechnology for PhD work.
- For two and half years, as a Lecturer of Botany in St. Anthony's College, Shillong.

CONFERENCE / WORKSHOPS ATTENDED :

a. Participated/Attended National Symposium on ' **Role of Plant Biochemistry and Biotechnology in Improving Crop Productivity**' held from 18th to 20th March'1997 at ICAR Complex for N.E. Hill Region, Barapani 793 103, India.

b. Participated/Attended workshop on ' **Agriculture, Biodiversity and Climate Change**' sponsored by the National Committee of INSA for IGBP and Dept, of Botany, N.E. Hill University, held from 9th to 10th March'1998 at NEHU., Shillong-22. India.

c. Participated in the National Seminar on '**Role of Microbes in Environmental Protection and Rural Development**' organized by Dept. of Botany, NEHU., Shillong and International Society for Conservation of Natural Resources, Benaras Hindu University, held from 23rd to 25th October' 1998 at NEHU, Shillong 22, India.

d. Attended & Participated in ' **Workshop on Protein Structures : Analysis, Modeling and Evolutionary Aspects**', organized by the Bioinformatics Center Bose Institute, Calcutta, funded by Dept of Biotechnology, New Delhi, during 9th to 10th March' 2000

e. Attended & Participated in a **Training Course on Basic Computer Concepts & Application**, Bioinformatics Centre, N.E. Hill University, Shillong, sponored by the Dept. of Biotechnology, Ministry of Science & Technology, Govt of India (12th June to 14th June '2000).

f. Attended & Participated by presenting a paper in a '**National Conference on Plant Biotechnology**' organized by Dept. of Botany, Kakatiya University, Warangal, during 2nd to 3rd March'2001. ' ***Initiation of embryogenic cultures from mature zygotic embryos of Pinus kesiya Royle ex Gord. using various carbohydrate sources and changes in the protein content associated with it.*** '

g. Participated in the **Seventy Second Annual Session of the National Academy of Sciences, India**, held at the North-Eastern Hill University, Shillong, held 25th to 27th October, 2002.

h. Attended & Participated in the **National Roving Seminar on Patenting in Biotechnology**, organized by the Department of Biotechnology, Ministry of Science & Technology, New Delhi, held at the Bioinformatics Centre, North-Eastern Hill University, Shillong, held 26th October, 2002.

i. Attended, participated and presented two papers in the **National Seminar on Intellectual Property Rights**, organized and held at the St Anthony's College, Shillong, held on **6-8 October 2004** sponsored by the Department of Human Resource Development, Govt. of India, New Delhi and North Eastern Council, Govt. of India, Shillong

(a) " ***Ethnobotany at its threshold*** ".Dey, B.P., C.S.Rao, J.Sharma, A.K.Das and S. Purkayastha Department of Botany, St. Anthony's College, Shillong, 793001, Meghalaya

(b) "***Stem Cells - the Final Frontier - a review***". Purkayastha, S., A. Chatterjee and N. Chatterjee .

l) OTHER ADDITIONAL RELEVANT INFORMATION :

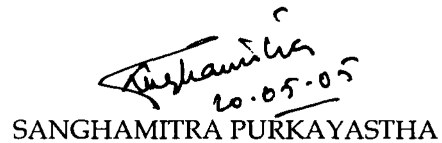
a. Participated in NCC (Sr. Cadet) during 1991- 1992.

b. A regular participant in the youth programmes of All India Radio Station (Shillong) and North eastern Service, Shillong, occasional participant in DDK, Shillong.

c) **Proficiency working in Computer Operating System (DOS, Windows 95, Window 98, Window 2000, Window ExP), programs like MS Word, MS Excel, MS PowerPoint, MS Access, Microsoft Origin and Internet.**

m) **PUBLICATIONS :**

- ***'Use of Plastics in vegetable production'*** V.K.Gupta, B.K. Srivastava, M. Bezbaruah , S. Purkayastha 2003. Journal of North Eastern Council, Vol 23 (1); 17-23.



SANGHAMITRA PURKAYASTHA